STUDIES IN FLUORESCENCE MICROSCOPY

by

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A C K N O W L E D G M E N T S

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ABSTRACT

Studies in fluorescence microscopy

Histochemical techniques suitable for fluorescence microscopy have been developed for the detection of the prinicpal chemical groupings and substances likely to be present in tissue sections. The mechanisms and specificities of the chosen reactions were confirmed wherever possible.

The following methods were found to be the most satisfactory for the detection of:

1. Amines.

(a) An extremely intense green fluorescent product was produced in sites of proteins in tissue sections treated with methanolic solution of pyridine containing a few drops of aqueous cyanogen bromide solution (the König-Sassi reaction).

(b) Treatment of sections with an alkaline solution of salicylaldehyde gave a thermolabile, green fluorescent conjugate.

(c) Tryptophane residues gave a characteristic purple fluorescence after reaction with a solution of dimethylaminobenzaldehyde in hydrochloric acid.

(d) The complex formed by mixing solutions of Solochrome dyes and alum fluoresced red after adsorption onto acidophilic primary and polymethyl amino groups.

(e) Chloramine T oxidised the primary amine groups of α-amino-acid residues to aldehydes which were subsequently demonstrated as their blue fluorescent salicyloylhydrazones.

(f) The specificity of the preceding reactions were confirmed by the following deamination experiments.

The diazotisation of amine groups with a cold solution of sodium nitrite and sulphuric acid, followed by deazotisation with warm ethanol was usually effective, but took some time to accomplish.

Oxidative deamination with a dilute solution of sodium hypochlorite within the pH range 5.5 - 7.0 worked very efficiently and rapidly. Stable aldehydes were produced predominantly in nuclear sites provided the excess hypochlorite in the sections was destroyed by washing them in a neutral solution of ammonia containing a trace of copper sulphate. Cytoplasmic proteins were probably converted to ketoacids. If sodium thiosulphate was used for destroying the excess hypochlorite, the induced aldehydes were destroyed. Instead strongly basophilic groupings, probably derivatives of sulphonic acids, were formed in both protoplasmic and nuclear proteins.

(g) Many of the reagents suggested by Danielli (1950) for blocking amine groups were found to be ineffective.

2. Thiols

(a) Thiols condensed with N- ethyl maleimide (NEM) selectively to give a product containing an

unconjugated ketone group. The zinc complex of the non-fluorescent hydrazone of the NEM-thiol conjugate derived from salicyloyl hydrazide fluoresced bluish-green.

(b) When all the basophilic substances and compounds containing hydroxyl groups were extracted from tissue sections by treating them with methanolic hydrochloric acid at 60°- 90° for several hours (drastic methylation), it was found that the remaining thiol groups could be converted to basophilic thiosulphonic acids by exposing dry sections afterwards to sulphuryl chloride vapour. These acid groups exhibited an anomalous bright blue fluorescence after the treated section had been stained in dilute solutions of auramine O or acridine yellow.

3. Disulphides

Oxidation with peracids yielded basophilic sulphinic acids which were unique in resisting methylation. Subsequent staining with coriphosphine gave an intense red fluorescence in the oxidised sites. Previous blocking of other basophilic materials with methanolic thionyl chloride and of thiols with iodoacetate was essential.

4. Phenols

(a) Indirect method. Acidified solutions of 1-nitroso-2-naphthol containing a trace of sodium nitrite coupled with tyrosine-rich sites to form an unstable green fluorescent conjugate. This test relied on the activiating influence of the phenolic group on the

ortho- and para- positions in the aromatic nucleus. The specificity of the reaction was confirmed by iodinating these positions.

(b) Direct method. Dinitrofluorobenzene reacted only with phenols and thiols (but not amines) at a pH below 5.5. By blocking the latter with iodoacetate, only the former reacted. The nitro groups of the conjugate were reduced to amines, but unfortunately attempts to demonstrate these groups by fluorescent methods (1) were unsuccessful.

5. Carboxylic acids

(a) C-terminal carboxyl groups have been converted to methyl ketones by treating them with a mixture of acetic anhydride and pyridine at 60°. The ketones thus formed were demonstrated as the Blue fluorescent zinc complexes of their salicyloyl hydrazones. This, and other experimental evidence, vitiated the hypothesis put forward by Karnovsky and Farmer (1960) that the principal reaction here was the conversion of the side-chain carboxyl groups to mixed acid anhydrides.

(b) Sites containing side-chain carboxyl groups were detected by the changes in the colour of their fluorescence from blue to green in tissue sections stained with 0.01% solutions of coriphosphine at pH 2 - 3 and at pH 5. The interference of the C- terminal carboxyl groups was eliminated by previously converting them to their

methyl ketones (5a). RNA was also extracted beforehand with hot perchloric acid.

6. Nucleic acids

(a) Zirconium ions had an affinity for the phosphate groups of both types of nucleic acid which were subsequently demonstrated as their greenish-yellow fluorescent complex with morin.

(b)DNA yielded an aldehyde after brief (Feulgen) hydrolysis which was subsequently detectable as its blue fluorescent salicyloyl hydrazone.

7. Sulphates

No direct chemical methods have been found but the presence of this group (in acid mucopolysaccharides) was inferred from the following tests:

(a) their basophilia (towards coriphosphine) when counterstained with the acid dye, thiazol yellow. Acid mucopolysaccharides, containing only acid groups, exhibited the brown or red fluorescence of coriphosphine, while the nucleic acids and proteins fluoresced a light yellow or blue as a result of the very strong interaction between their basic amino groups and the acid dye.

(b) Their basophilia (towards coriphosphine) after selective extraction of nucleic acids.

Methylation and reduction of tissue sections with lithium aluminium hydride in hot dioxane removed phosphate groups selectively. Uronic acid groups and

some protein carboxyl groups were reduced to primary alcohols. Subsequent saponification and staining showed a reddish-brown fluorescence in sites containing acid mucopolysaccharides which contrasted with the weak green fluorescence of the remainder of the section.

Hot perchloric acid has been used for the selectrive extraction of RNA.

Other mineral acids and nucleases were found to be unsatisfactory for the selective extraction of nucleic acids because acid mucopolysaccharides were removed at the same time.

(c) Mild methods of methylation (using methanolic thionyl chloride or diazomethane) have been succesfully used for both the temporary and permanent blocking of sulphate and other basophilic groups.

The normal technique for methylating tissues, using hot methanolic solutions of hydrochloric acid (Fisher and Lillie, 1954) were found to be unsatisfactory; much of the reactive material was extracted from tissue sections instead of being methylated. Sulphated acid mucopolysaccharides were desulphated to a limited extent by this reagent.

(d) Using published methods based upon iron mordants (Hicks and Mathaei, 1958; Hale, 1946), it was found that acid mucopolysaccharides did not always take up ferric ions whereas nucleic acids did. Experimental evidence

has been collected to show that the results published previously were fortuitously successful and were not strictly specific.

8. Uronic acids

(a) Sections were reduced with lithium aluminium hydride (7b) to show that the non-fluorescent dye alcian blue had a selective affinity for this group. When sections were stained with coriphosphine, they were first stained with alcian blue so as to quench the potential fluorescence of coriphosphine adsorbed onto acid mucopolysaccharides which otherwise would have been difficult to distinguish from that of nuclei.

(b) An intense blue fluorescence and a visible purple colour was observed in sites known to contain uronic acid groups after immersing sections in concentrated sulphuric acid at 60 - 75°.

9. vic-Glycols

1,2-glycols were cleaved to "dialdehydes" by periodic acid. Those in neutral mucopolysaccharides and glycogen were oxidised completely within 10 minutes. Those in acid mucopolysaccharide required 24 hours or longer before they were oxidised significantly.

The engendered dialdehydes have been conjugated with:

(a) solutions of homo- and heterocyclic amines (particularly aminoacridine dyes) containing dissolved sulphur dioxide (pseudo-Schiff reagents). Preliminary

blocking with methanolic thionyl chloride of the basophilic substance initially present in tissue sections was essential for the observation of specific staining of the engendered dialdehydes. Protein thiol groups nevertheless still interfered with the specificity of the reaction for glycols.

Evidence has been accumulated in favour of the following mechanism for this type of reaction which differs from that suggested by Kasten (1958); the engendered dialdehydes take up sulphur dioxide from the dye solution to form sulphonic acids which subsequently combine with the basic dye in a salt-like linkage. Apart from p-aminosalicylic acid, no potentially fluorescent amine has been found to condense with engendered aldehydes *in situ*, contrary to what Kasten(1958) conjectured and to the numerous reports of fluorescent anil formation of aldehydes in solution.

(b) Recent chemical work (Guthrie and Honeyman 1959) has indicated that hydrazines and hydrazides always react with engendered dialdehydes whereas Schiff-type reagents may not, particularly if the dialdehydes are converted to hemiacetal or hemialdal forms. This fact has been confirmed histochemically: salicyloyl hydrazide formed intensely blue fluorescent complexes reliably and more extensively with engendered dialdehydes than do pseudo-Schiff reagents. Moreover, salicyloyl hydrazides

did not form any fluorescent artefacts with protein thiol groups after oxidation in periodic acid (Cf.9a).

The phenylhydrazones of the dialdehydes formed *in situ*, unlike those *in vitro*, did not form formazans except with diazotised aniline in pyridine.

10. Hydroxyl groups

The basophilic properties (towards azure A or coriphosphine) of this group after it had been sulphated were studied. The sulphating efficiencies of various Lewis base complexes of sulphur trioxide were investigated in both acid and basic media. The dioxan-sulphur trioxide dissolved in the weak base dimethylformamide (DMF) or sulphur trioxide dissolved in DMF were very efficient sulphating reagents, even for glycogen which could not be sulphated by acidic sulphating reagents. The amine groups of proteins were sulphated simultaneously by the DMF-sulphur trioxide complex. However, the N-sulphate groups, unlike O- sulphate groups, were easily hydrolysed off by methanolic hydrochloric acid at room temperature. If the amine groups were protected beforehand with suitable blocking reagents, the access of the sulphating complex to hydroxyl groups was completely hindered.

Although glycogen in tissue sections can be sulphated normally by sulphuryl chloride vapour, the hydroxyl groups of neutral mucopolysaccharides were not sulphated in the same way: it is believed that in the

latter, non-basophilic cyclic sulphate groups were formed.

11. Primary hydroxyl groups

The selective sulphation and subsequent basophilia (toward azure A or coriphosphine) of this group by the pyridine-sulphur trioxide complex in the DMF was investigated.

12. Lipids

(a) Unsaturated lipids were oxidised by peracetic acid to aldehydes and subsequently condensed with salicyloyl hydrazide to form a blue fluorescent hydrazone.

(b) Lipids emitted a reddish-pink fluorescence when sections were stained with an acidified solution of protoporphyrin IX.

(c) Ketosteroids formed blue fluorescent salicyloyl hydrazones, most of whom, unlike aldehydo-salicyloylhydrazones, were stable towards alkalis. The interfering aldehydes originating from the auto-oxidation of lipids were blocked with sulphanilic acid.

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Studies in Fluorescence Microscopy

Section 1. Introduction

1. The historical background

Fluorescence microscopy has only come into prominent use during the past few years, although the principles upon which it is based have been known since its original development from the ultra-violet absorption microscopy studies of Köhler (1904). Its recent growth is mainly due to the impetus it has received from the immunofluorescent protein tracing techniques devised originally by Coons and his collaborators (1941) and their application to many medical and biological problems (Nairn, 1962). Apart from this application, fluorescence microscopy has been used for studying such things as the effects of fixation on the natural auto- or primary fluorescence of tissues, the observation of fluorescent cytoplasmic inclusions such as pigment granules, and the intracellular localisation of fluorescent drugs or foreign substances. The earlier work on these applications has been reviewed by Ellinger (1940) and later work by Price and Schwartz (1956).

When biological tissues are stained in dilute solutions of fluorescent dyes (sometimes called fluorochromes), they emit under suitable conditions of illumination an intense, so-called secondary fluorescence often in many colours which

present an aesthetically pleasing appearance. The classical studies on secondary fluorescence were carried out by Haitinger and culminated in his monograph published in 1938. Since then, the phenomena of secondary fluorescence have been applied empirically to various problems connected principally with clinical diagnosis. For example, smears of exudates have been stained with auramine O or acridine orange for the rapid diagnosis of pathological conditions such as cancer (von Bertalanaffy, 1961) or tuberculosis. It is claimed that malignant cells contain a comparatively large amount of an acidic substance (possibly RNA) which combines with the basic dye to form an unusually intense fluorescent complex. Normal cells form the same complex, but to a much smaller extent. The difference in total fluorescence output per cell is sufficiently large for semi-automatic scanning methods to be used for the routine screening and approximate counting of stained smears.

2. The aim of this investigation

Despite the many papers which have been published on the practical realisation of fluorescence microscopy and on observations of primary and secondary fluorescence, very few studies have been reported on the development of fluorescence microscopy as a systematic histochemical technique or on fundamental investigations into the mechanism of the polychromatic fluorescence of tissue sections stained with a

single, basic aminoacridine dye such as acridine orange. This thesis is concerned with these problems: because it is not possible to formulate any theories for explaining polychromatic secondary fluorescence until it is knownexactly what chemical groups in the tissue itself are responsible for the uptake of fluorescent dyes, it was decided that the first step should be the development of a systematic scheme of fluorescence histochemistry. The higher sensitivity of fluorochemical methods would make such a scheme, if it could be devised, an important contribution to histological research itself.

Histochemistry is essentially the detection and demonstration *in situ* of specific chemical groups or substances in tissues. However biologists who practise histochemistry sometimes *presume* the presence of a particular compound in the tissue which they are studying, and proceed to identify it with one or two histochemical reactions which are known to give positive results for that compound *in vitro*. This approach is fundamentally unsound. The complete identification of one complex compound by one or two tests on what is essentially an even more complex mixture of materials would not satisfy chemists familiar with more rigorous schemes of organic analysis. In this investigation, the emphasis has been shifted to the identification of functional chemical groups. Only from the results of these tests, have inferences

be made as to the compounds most likely to have that particular combination of chemical groups.

It is hoped that this approach will lead to the development of histochemistry as a discipline in its own right rather than, as at the moment, lagging behind developments in chemistry and biochemistry. In furtherance of this ideal, the mechanisms of the various histochemical reactions used or developed in this investigation have been studied wherever possible. These included reactions which were known to give positive results *in vitro*, but which failed when tried on tissue sections; instead of attributing the failure of these reactions to the vagaries of biological materials, explanations have been sought in chemical terms so as to avoid wasted effort in developing comparable histochemical reactions in the future.

In later sections the results and conclusions obtained in this investigation are discussed in detail. Such results can be distinguished from the results and conclusions of previous investigators by the appropriate reference which is always cited for the latter. It is hoped that this format for discussing the results obtained in this investigation will lead to a unified account of histochemistry. In the experimental sections at the end of each discussion, references to authors are only cited when their experimental techniques have been used in this investigation. Enumerated techniques, without author references, are new techniques evolved during

this investigation. The symbols v and/or f after each technique indicates that it can be used for white transmitted light and/or fluorescence microscopy respectively.

3. Fundamental principles

Before considering the realisation of fluorescence microscopy and its various applications to histochemistry, it is useful to appreciate the origins of fluorescence. When a molecule absorbs light, the extra energy so acquired may be dissipated in one of four ways:

(1) it can be transferred to another molecule, usually by collision or "radiationless" transfer;

(2) the kinetic energy of the molecule may be increased, which is manifested in the form of heat;

(3) the absorbed energy may be large enough to break one or more chemical bonds within the molecule, giving rise to the phenomenon known as photolysis;

(4) the absorbed energy may be re-emitted as radiation called fluorescence.

Some of these processes may occur at the same time, so that the energy emitted as fluorescence is usually less than that absorbed originally. As the energy of a light quantum is directly proportional to its frequency (or inversely to its wavelength), the wavelengths of the fluorescence radiation are longer and are shifted towards the red end of the spectrum with respect to the light absorbed originally.

This forms the theoretical basis for fluorescence microscopy. If a specimen is illuminated or "excited" with light of short wavelength in the ultra-violet or blue regions (assuming, of course, that the object will absorb radiation appreciably in this region), the ultimate image will consist of visible fluorescent light emanating from the object itself and excess transmitted light. However, if the latter is absorbed by a suitable filter transmitting only the fluorescent light, the final image will be that of a self-luminous object.

The advantages of this system over conventional light microscopy are improved contrast and sensitivity. The enhanced contrast can be illustrated by a simple example. Suppose that a microscopic specimen absorbs 1% of the light falling on it. The comparative light intensities of the object and its background will be 99:100, which means that the contrast will be low. But if the excess incident light passing through the object and its background is removed before reaching the observer, and if the light absorbed by the object is re-emitted as fluorescence, the relative intensities will be 1:0 and the contrast would be theoretically infinite. The relative contrast in the second system appears much larger to the human eye than the first; the eye can distinguish large differences of low light levels much more easily than small differences at high light levels.

Fluorescence phenomena have the additional advantage of

being specific: only a few substances exhibit a distinctive fluorescence and only then under certain conditions. Not only must potentially fluorescent materials have certain intrinsic properties, but their physical state and surroundings must be favourable. Most fluorescent dyes hardly fluoresce in the solid state, but emit an intense fluorescence in solution The effect of impurities may be important; most solid samples of anthracene, for example, fluoresce an intense green colour, although the fluorescence of highly purified anthracene is blue (Weigert, 1940). The green fluorescence is due to the presence of traces of naphthacene. Not only does the naphthacene emit fluorescence per se, but it also "absorbs" the fluorescence emitted by the anthracene which it re-emits again as its own characteristic fluorescence. However, impure anthracene in solution emits only the typical anthracene fluorescence. For these reasons, small quantities of impurities may alter or even quench completely the specific fluorescence of biological materials in unpredictable ways.

4. The scope of histochemistry

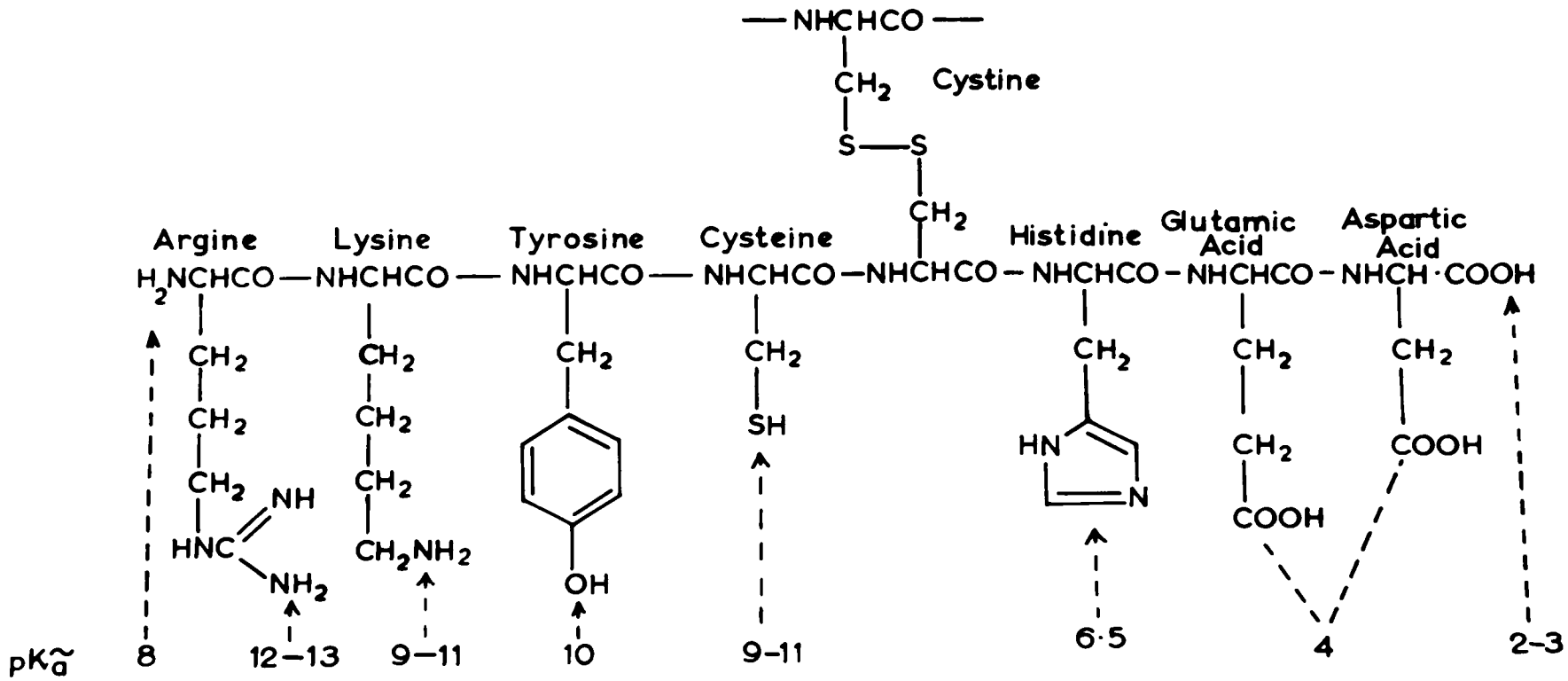
Before formulating any scheme of histochemical analysis, it is useful to consider what functional chemical groups can be present in biological tissues. Biological materials are made up from roughly four classes of organic compounds; these are proteins, nucleic acids, carbohydrates (including mucopolysaccharides) and lipids, which are usually in some form of

combination with each other. Of course, any of the more elementary components (metabolites) of these four major classes may also be present in biological tissues, particularly in fresh, unfixed materials.

Proteins constitute the major proportion of the organic matrix of biological materials. They are built up from various amino-acid units condensed in unique sequences through peptide (-CONH-) linkages. Each amino-acid in the polypeptide chain may have free chemical groups which play no essential part in the structure of the protein itself, but which can affect the overall chemical properties of the complete protein. Some of the chemical groups which are most likely to occur in common proteins are illustrated in the hypothetical peptide shown in Fig. 1. They are amines, thiols, disulphides, phenols, side-chain and C-terminal carboxyls. The fluorescence histochemistry of each of these groups is discussed in separate sections later in this thesis.

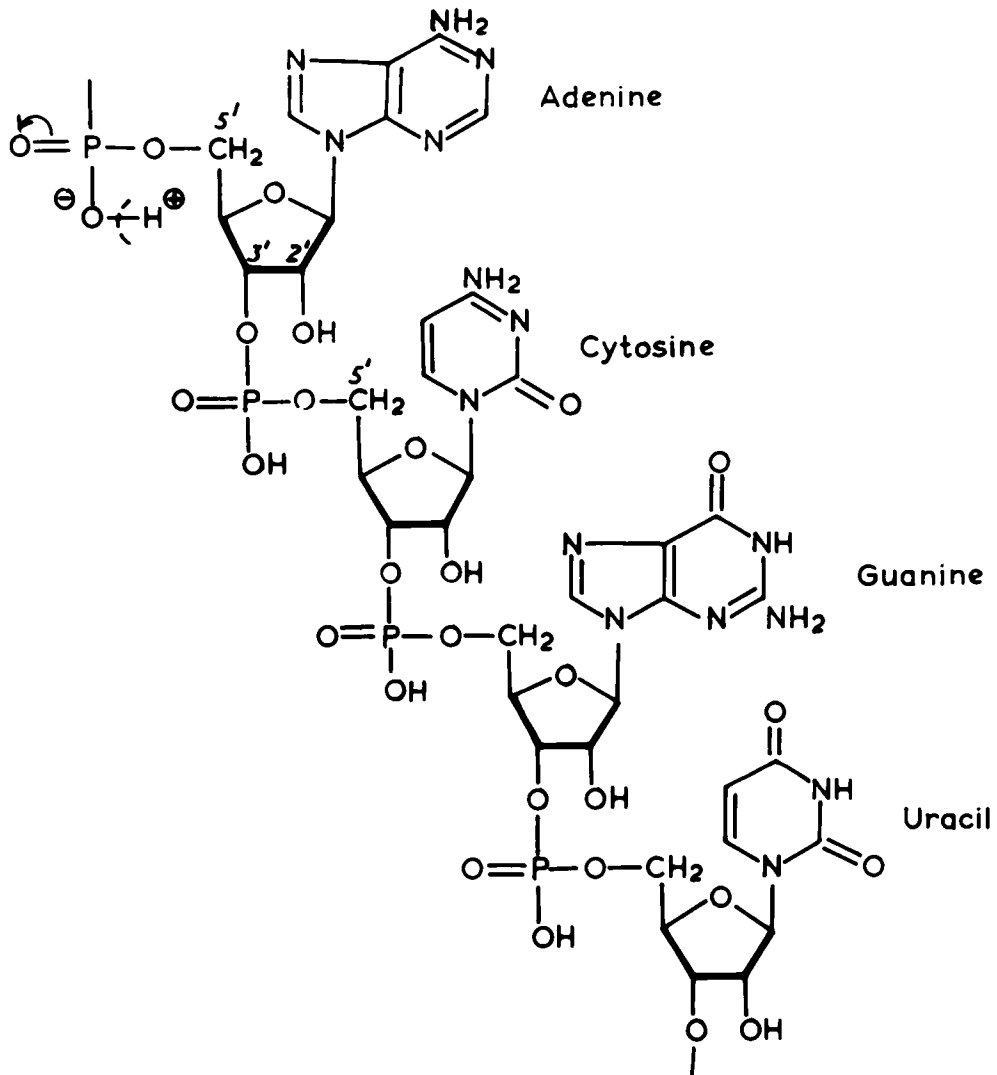
There are two types of nucleic acid in biological tissues, ribosenucleic acid (RNA) and deoxyribosenucleic acid (DNA). Without digressing into the important work which has been done on the stereochemistry of these acids, in particular on DNA (Watson and Crick, 1953), the only features which need be considered in this investigation are their chemically active centres. The structural formulae of the two nucleic acids, which are shown in Fig. 2 (after Fieser and Fieser, 1961),

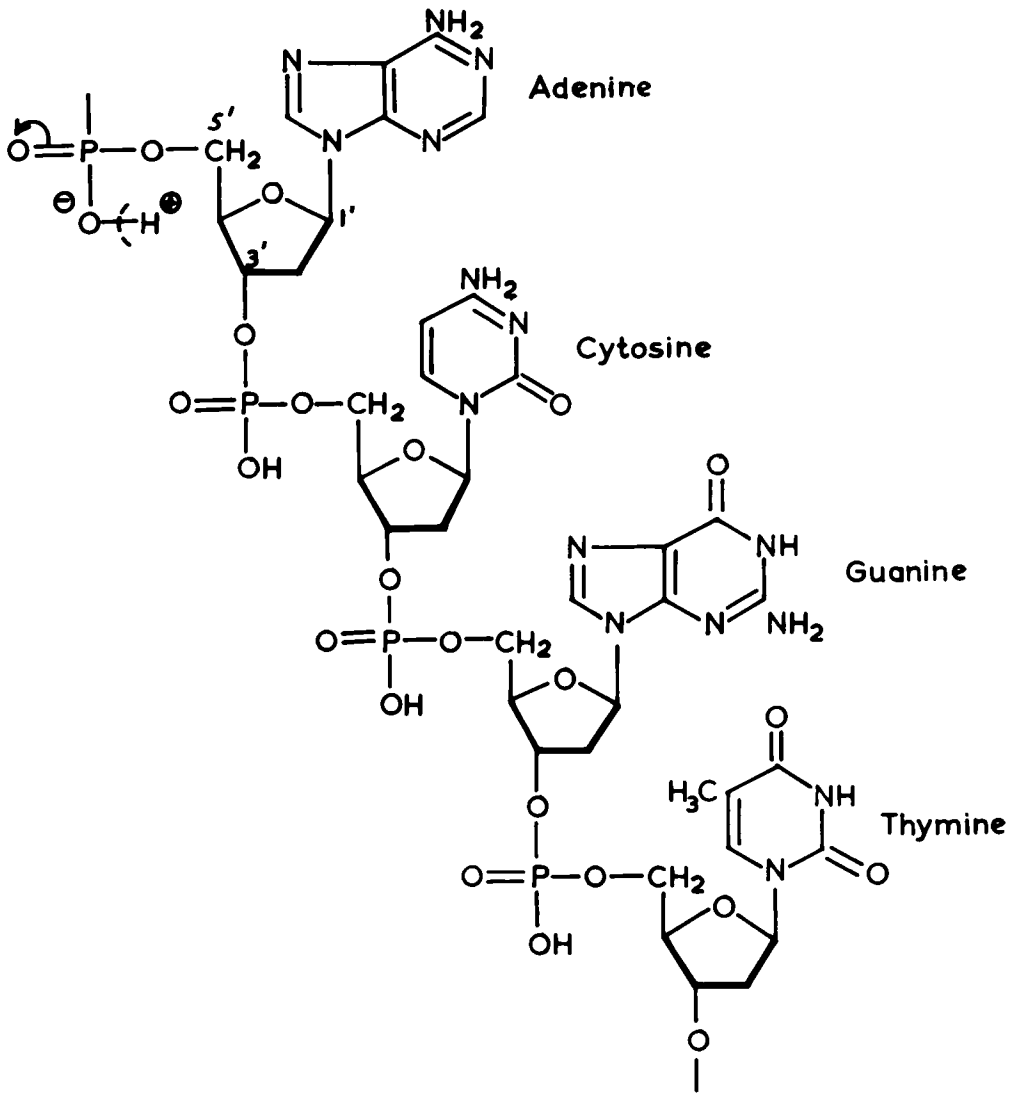
Fig. 1.



A hypothetical peptide sequence.

Fig. 2


Adenine
Cytosine
Guanine
Uracil
Ribonucleic acid (RNA)

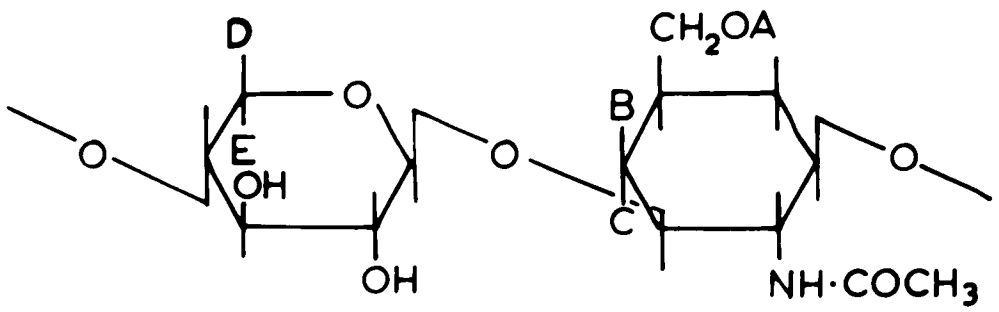

Adenine
Cytosine
Guanine
Thymine
Desoxyribonucleic acid (DNA)

indicate three such centres: anionic phosphates, acidophilic amines, and heterocyclic bases. DNA differs from RNA in two respects. First, the base thymine is only present in the former. Secondly, the acid hydrolysis of DNA reveals an aldehyde grouping at the C_3 position of the sugar moiety. RNA, which has a hydroxyl group at the C_2 position of its ribose moiety, is not hydrolysed similarly. The histochemical reactions of both nucleic acids are discussed in sec. 8.

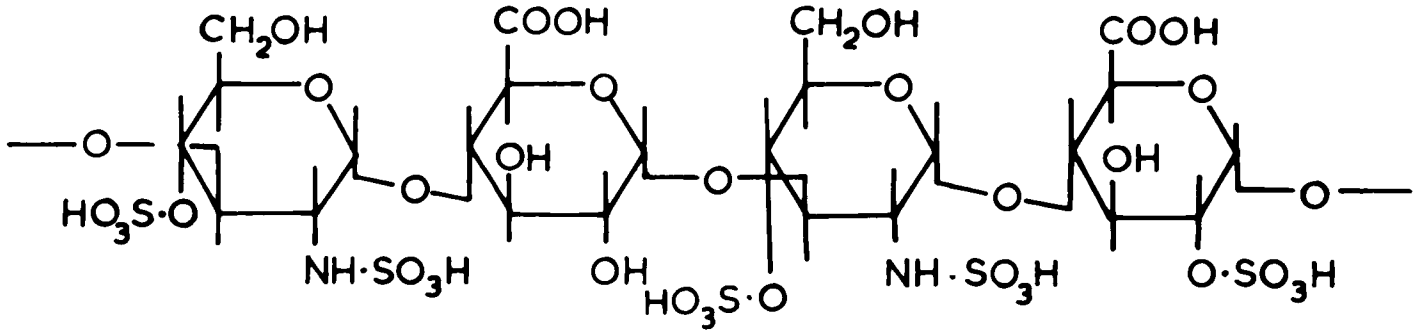
Mucopolysaccharides are closely associated with the intercellular ground substance and are of particular importance in the growth and repair of tissues. They contain hexosamine in combination with other polysaccharides units and can be found in tissues as free entities or as constituents of protein molecules. Mucopolysaccharides are classified in the chemical literature as being either "acid" or neutral, but unfortunately the terminology used by various authors is not uniform. Stary's (1959) classification will be used in this thesis.

"Acid mucopolysaccharides" are defined as mucinscontaining hexosamine together with either uronic acid or sulphate residues or both. The structural formulae of the six commonest acid mucopolysaccharides are shown in Fig. 3 (after Stacey and Barker, 1962). A partially desulphated form of heparin which is not shown in Fig. 3 is sometimes found in tissues. It is now called heparitin sulphate (formerly heparin mono-

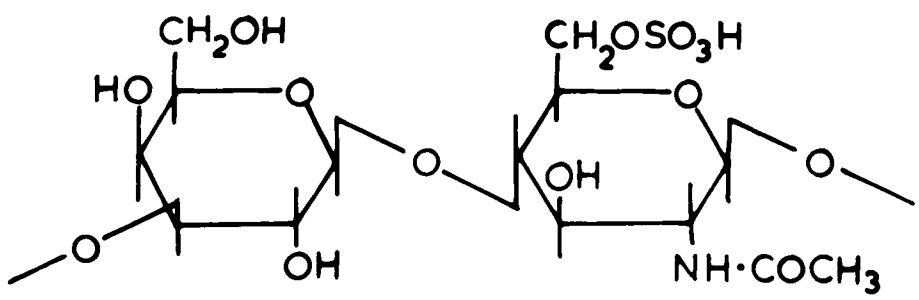
Repeating structural units of acid mucopolysaccarides



		A	B	C	D	E
Hyaluronic Acid		H	H	OH	COOH	H
Chondroitin Sulphate	A	H	O·SO$_3$H	H	COOH	H
"	B	H	O·SO$_3$H	H	H	COOH
"	C	SO$_3$H	H	H	COOH	H



Heparin



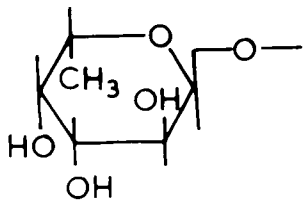
Keratosulphate

Fig. 3

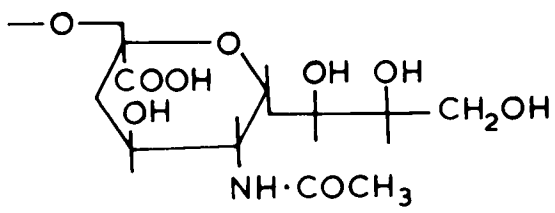
sulphuric acid; Meyer, 1956) and probably differs from heparin histochemically by having a strong reactivity towards periodic acid (sec. 12.4/4). Keratosulphate is not often found in tissues; so far biochemists have only extracted it from the cornea and cartilage (Stacey and Barker, 1962). The histochemistry of the three principal groupings which are found in acid mucopolysaccharides is discussed in the sections dealing with 1,2-glycols (sec. 12), sulphates(sec. 9), and uronic acids (sec. 10).

"Neutral" mucins are protein bound mucopolysaccharides consisting of hexosamine and hexose residues. Many substances of this group contain methyl pentose (fucose) or sialic acid residues, and hence are not always strictly 'neutral'. Glycogen will also be included in this category in this thesis for the sake of convenience, although its structure does not fit in with the definition laid down for neutral mucins. Glycogen also differs from neutral mucins in being catobolised rapidly, its turnover being about 10^3 times that of the latter (Stary, 1959). The structural formulae of some of the residues found in members of this group are shown in Fig. 4. Their most important chemical groups are primary (1^o) and secondary (2^o) hydroxyls, whose histochemistry is discussed in secs. 14 and 13 respectively. In addition, the 2^o hydroxyl groups usually occur in pairs as 1,2-glycols and have a unique reactivity towards periodic acid which is discussed separately

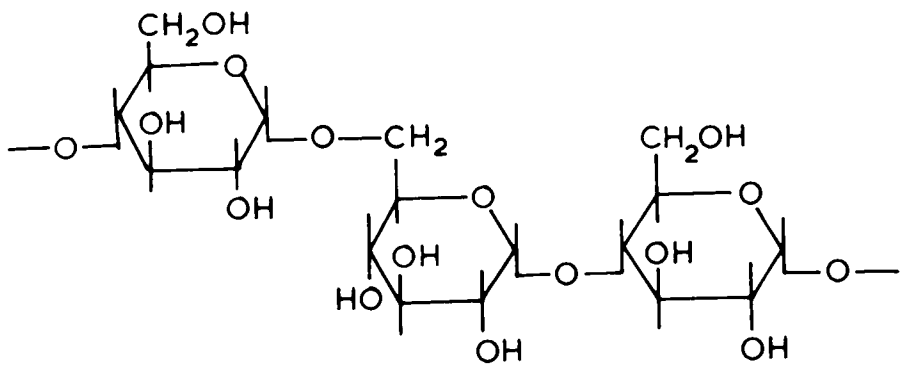
RESIDUES PRESENT IN NEUTRAL MUCOPOLYSACCARIDES



L-Fucose
(Pyranose form)



O-Sialic acid
(N-Acetyl Neuraminic Acid)



A typical repeating unit in Glycogen

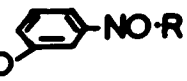
Fig. 4

in sec. 12.

Lipids have been studied perfunctorily in this investigation. They are not likely to be found in the fixed and paraffin-embedded tissues sections normally used in histological research. However, when they are present, they can be characterised by the affinity of their long hydrocarbon chains for certain dyes (e.g. protoporphorin), or by the cleavage of their olefinic groups to reactive aldehydes, or, in the case of certain steroids, by the reactions of their ketone groups.

Fig. 5 contains a summary of the chemical groups which might be present in any given biological tissue together with a diagrammatic key to the fluorochemical reactions which can be used for their histochemical identification. This key forms the preliminary basis of a possible systematic scheme of histochemical analysis and summarises the most important reactions discussed in the following individual sections.

AN OUTLINE OF THE FLUORESCENCE HISTOCHEMICAL REACTIONS USED FOR THE DETECTION OF THE CHEMICAL GROUPS FOUND IN BIOLOGICAL TISSUES

* Reactions with:		$D^{\ominus}$	$D^{\oplus}$ Dir.	$D^{\oplus}$ Indir.	$\phi{\cdot}NH_2$ Dir.[2]	$\phi{\cdot}NH_2$ Indir.	$\Psi{\cdot}CHO$	Special tests
PROTEINS								
Amines	$-NH_2$	+					+	
Thiols	$-SH$			$\rightarrow S{\cdot}SO_3^{\ominus}H$		$\rightarrow S{\cdot}CH{\cdot}C(=O)-$	+?	
Disulphides	$-SS-$			$\rightarrow SO_3^{\ominus}H$				
Phenols	$Ar{\cdot}OH$							$\rightarrow$  DNFB ?
Histidines	$\gg NH$							
C-terminal carboxyl	C-COOH		+			$\rightarrow CO{\cdot}CH_3$		
Side-chain carboxyl	$-COOH$		+					$D^{\oplus}$ pH $\gg 2$
NUCLEIC ACIDS								
Phosphates	$-PO_3H$		+					{Zr + Morin LAH
Amines	$-NH_2$	+					+	
(DNA)	$-CHO$					+		
Het. bases								
ACID MUCINS								
Sulphates	$-O{\cdot}SO_3H$		+					
Uronic acids	$+COOH$		+					conc. H_2SO_4
2° Hydroxyl	$>OH$			$\rightarrow O{\cdot}SO_3^{\ominus}H$?				
1,2-glycols	$>(OH)_2$					$\rightarrow CHO$		
N-acetyl								$\rightarrow CHO$?
NEUTRAL MUCINS								
1° Hydroxyl	$-CH_2OH$			$\rightarrow CH_2O\,SO_3^{\ominus}H$				
2° Hydroxyl	$>OH$			$\rightarrow O{\cdot}SO_3^{\ominus}H$		$\rightarrow CHO$		
1,2-glycols	$>(OH)_2$							
LIPIDS								
Hydrocarbon	$-(CH_2)_n-$		(+)					
Olefin	$>CH=CH<$				(+)	$\rightarrow CHO$		
Ketone	$>C=O$				+			

* Key to symbolism of test reagents

$D^{\ominus}$ acid dyes; $D^{\oplus}$ basic dyes; $\phi{\cdot}NH_2$ hydrazine fluorochromes; ΨCHO aldehyde fluorochromes.

Fig. 5

Section 2. Methods and Materials

This section is concerned with the practical realisation of fluorescence microscopy, photomicrography, and the fixation of the test materials used in this investigation.

The three main features of the fluorescence microscope system, the light source, the isolating and absorbing filters, and the microscope optics are illustrated diagrammatically in Fig. 6. Each feature will be discussed separately.

1. Light sources

The prime requirement for obtaining the maximum fluorescence emission from a specimen is to illuminate it with the brightest possible light at wavelengths near its absorption maxima. The total ultra-violet and blue content of the tungsten lamps used in conventional microscopy is insufficient to induce much fluorescence from most objects. The best results are obtained by using the intense spectral lines emitted by high-pressure mercury discharge lamps. The choice of the most suitable lamp for fluorescence microscopy from those now available commercially is a matter of compromising between stability, intrinsic brilliance and physical convenience. The compact source A.E.I. type ME/D lamp rated at 250 watts has been found fairly satisfactory in this investigation, although it is not perfect. A particularly convenient form of this lamp, known as the "box lamp", was used because it is built into a

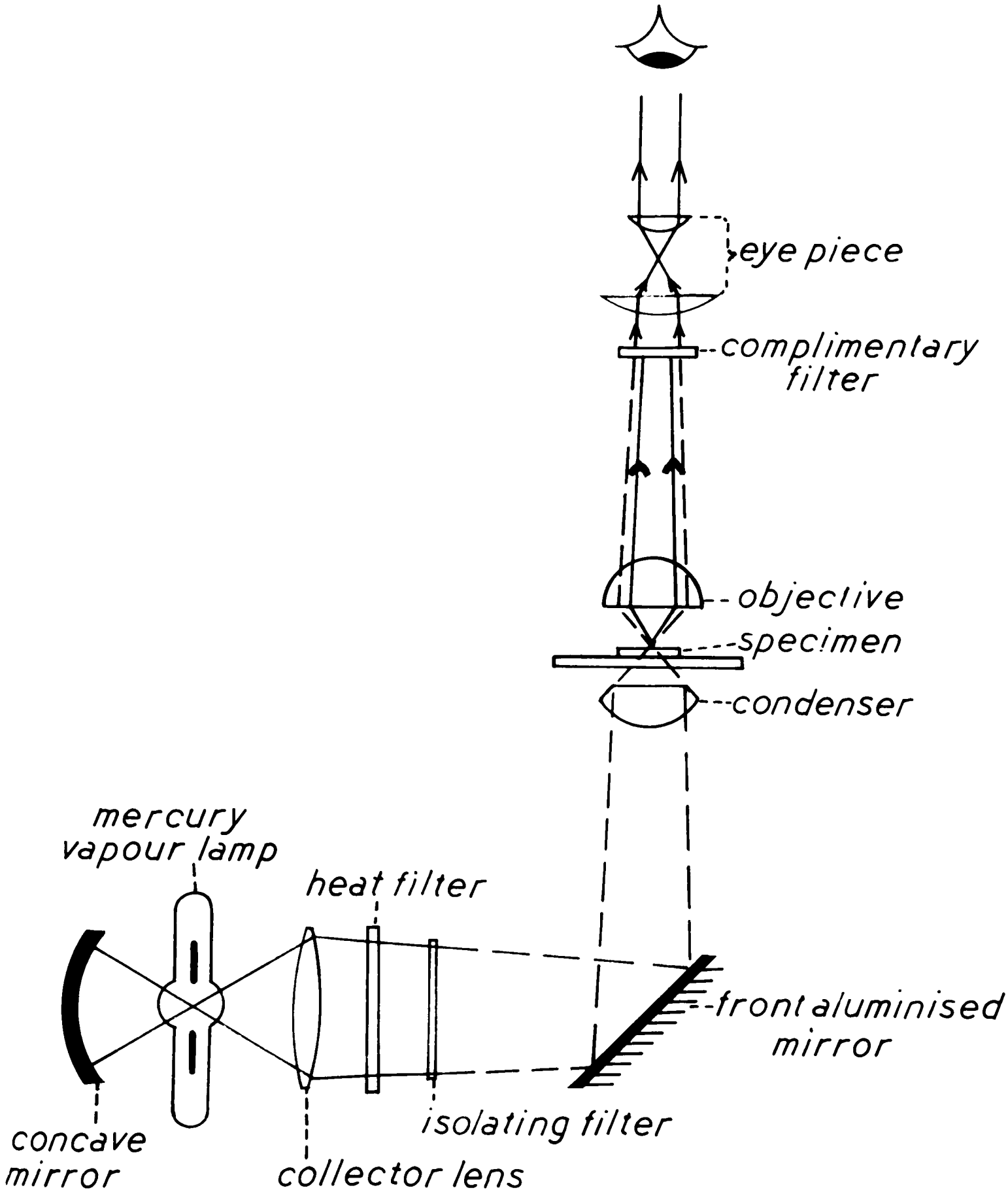

eye piece
complimentary filter
objective
specimen
condenser
mercury vapour lamp
heat filter
front aluminised mirror
isolating filter
concave mirror
collector lens

Fig. 6

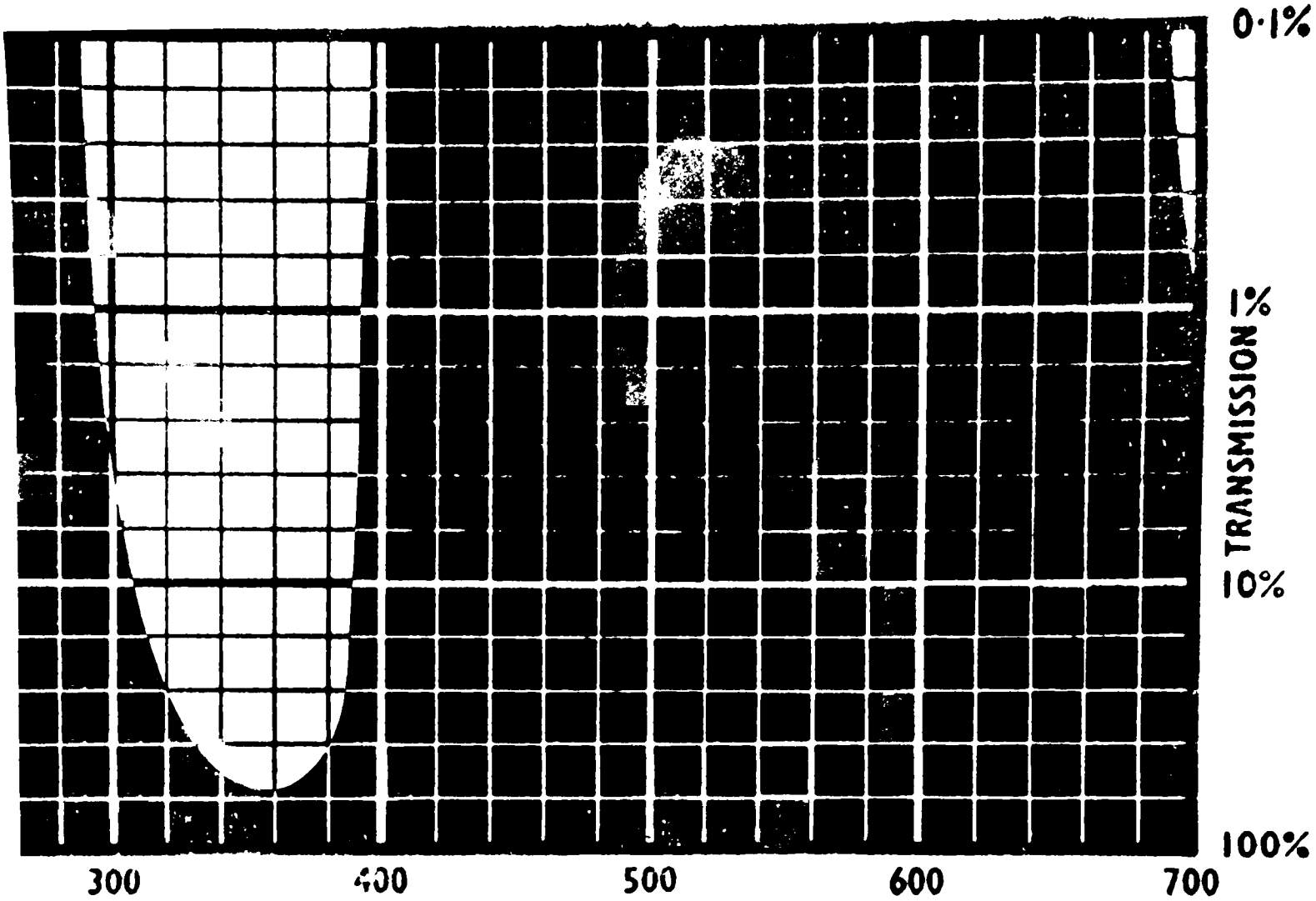
small metal housing provided with a three-pin base, which fits into a standard electrical socket. The arc of the lamp was struck and operated from a special choke (on alternating current) for this type of lamp. The length of arc was only 3.75 mm. and its width 1.5 mm. The intrinsic brilliance exceeds that of any mercury lamp except the air or water-cooled types, being about 20,000 candles per sq. cm. at the centre of the arc.

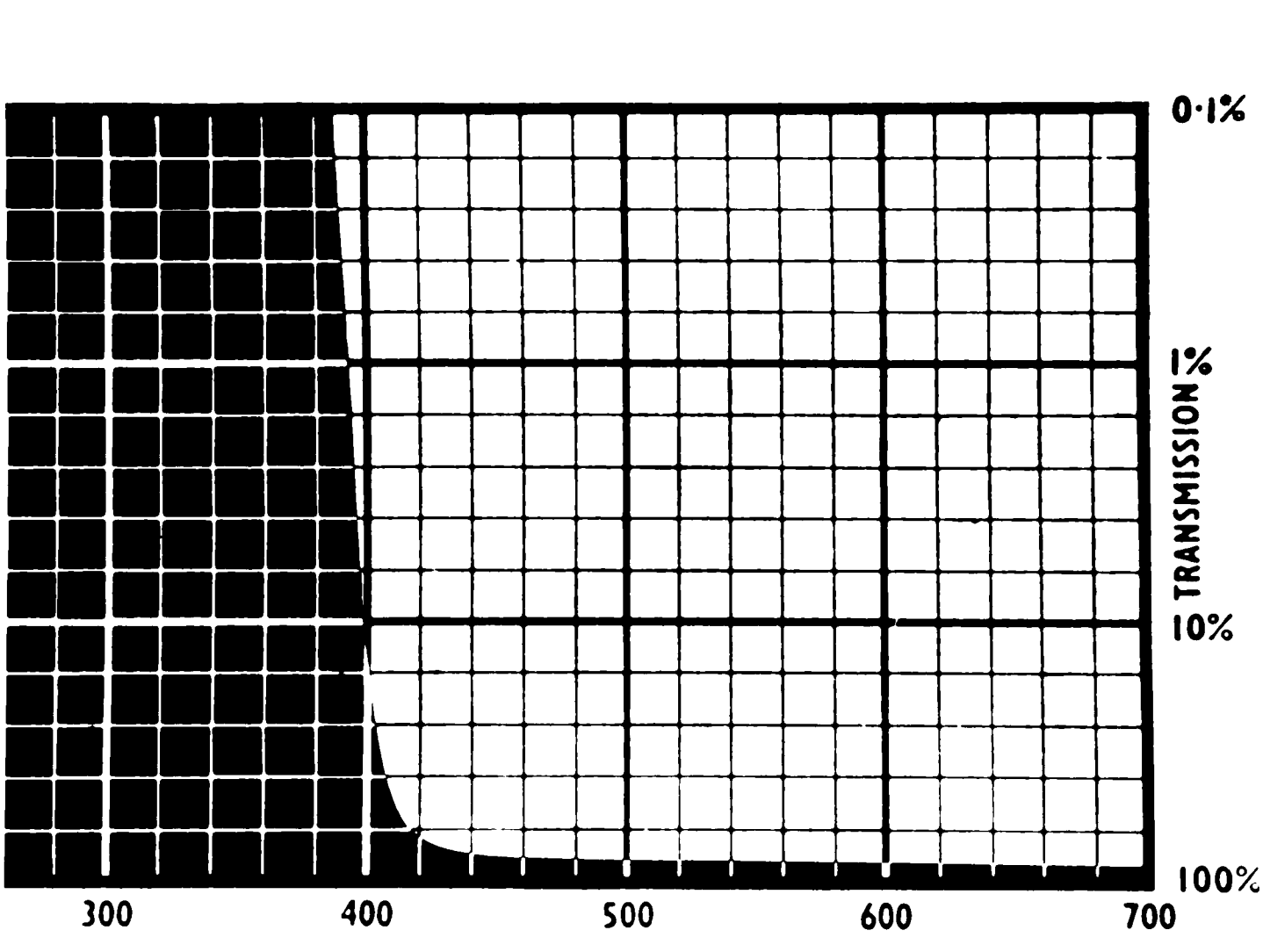
The apertures of the lens systems which are available commercially for collecting the light emitted from the source are usually too small; only a fraction of the available light ultimately enters the microscope substage condenser. For this reason, Professor R. Barer commissioned Dr. W. T. Welford of the Technical Optics Department, Imperial College, to design a dry 3-lens system for collecting the maximum amount of light from the boxed A.E.I. ME/D lamp. This collector lens system has been used throughout this investigation; the fluorescence emitted from test sections using this system was much greater than that obtainable from commercial systems such as the one incorporated into Reichert's "Binolux" microscope. The working distance from this lamp to the substage condenser was 70 cm. The N.A. on the lamp side was 0.6. A field of diameter 0.5 mm. was illuminated in the substage. In practice, the field was increased by slightly defocussing the condenser. This, of course, led to a corresponding decrease

in intensity of illumination. The form of illumination is not important in fluorescence microscopy as it is in conventional transmitted light microscopy. In fluorescence microscopy, the final image in the field of the eyepiece is that of a self-luminous object at the focal point of the objective and is not derived in any way from the lamp source as it is in conventional microscopy, so that defocussing of the substage condenser has no effect on the optical formation of the final image and there is no loss of resolving power. The collector lenses were made from fused silicia instead of ordinary glass which would crack in the intense heat of the mercury arc. The transmission of U.V. light through fused silicia is also better than that through glass.

2. Filters

The intense group of lines around 366 mμ has been used almost exclusively for the excitation of fluorescence in this investigation. An empirically adjusted solution of copper sulphate to which a little ammonia had been added was used for partially isolating the ultra-violet and blue mercury lines. It had the added advantage of acting as a heat filter and absorbing the red light emitted by the source. The Schott UG1, UG4 or the Wratten 18A glass filters were used for effectively isolating the 366 mμ group of lines after the copper sulphate solution filter. The spectral characteristics of the Wratten 18A filter are shown in Fig. 7.





Figs. 7 & 8. The spectral characteristics of the Wratten 18A and 2B filters.

The complementary filter for absorbing the excess exciting radiation was placed in the body of the microscope below the eyepiece. Two circular discs of the light yellow, gelatine Wratten 2B filter were used for this purpose. Its spectral absorbance curve (Fig. 8) shows that it completely absorbs all the 366 mμ radiation but transmits practically all the visible region, above about 400 mμ.

In practice a perfectly black background was not always obtained, particularly when some types of apochromat objectives were used. This was due to the exciting light causing the lens cement to fluoresce. The only cure for this was to place a filter between the specimen and the objective. The yellow coverslips made from "Euphos" glass were very efficient for absorbing the unwanted ultra-violet light. As Euphos cover glasses are very expensive, the cost of making permanent biological mounts would have been prohibitive. Fortunately many mounts could be examined in the dry state so that one cover slip was used repeatedly. It was later found that the lens cement of certain Zeiss Planapochromats did not fluoresce. In spite of their smaller numerical aperture (N.A.) and the consequent lower light gathering power (see below), they were used in preference to more complex apochromats of higher N.A. Normal white-glass cover slips were then used in place of the Euphos glasses and enabled permanent mounts to be made.

3. Optics

Special optics are unnecessary in fluorescence microscopy, despite statements that are sometimes made to the contrary.

As it is desirable to excite a specimen with the maximum amount of illumination, a condenser with the largest possible N.A. was used. However complex condensers were avoided as their lens cements absorb ultra-violet radiation heavily and fluoresce appreciably. A simple 3-lens quartz condenser of the Abbé type was finally chosen, as it had the added advantage of transmitting the strong mercury lines around 313 mμ.

The most important criterion underlying the choice of objectives was their light gathering power; this is related to the quantity $(\text{N.A./magnification})^2$, so that objectives of the highest possible N.A. compatible with the lowest convenient magnification were employed. However the final choice was influenced by two other factors. The self-luminous objects studied in fluorescence microscopy are often in diverse colours. It is therefore preferable to use a chromatically-corrected objective. The best objectives for this purpose are apochromats. Unfortunately they contain numerous components in their lens systems which may lead to the difficulties mentioned earlier. Even when a suitable non-fluorescent objective has been found, the curvature of its image field may be too large for taking a reasonable photomicrograph suitable for publication. This is a recurrent difficulty in fluorescence microscopy at the

present time. Corrected flat-field objectives are now being produced commercially, but it still remains to be seen whether the complexity of their lens systems outweigh their potential advantages. Objectives whose image field has been partly flattened have been found to be the best compromise so far: these included a Zeiss 10X Planapochromat (N.A. 0.32) and a Baker X20 achromat (N.A. 0.5). A Cooke 20X apochromat of higher N.A. (0.65) collected more light and was used for the observation of comparatively weak fluorescent tissue sections. Unfortunately its image field was noticeably curved and its lens cement also fluoresced appreciably.

A conventional monocular Zeiss microscope stand was used. A front-aluminised mirror was clipped onto the substage silvered mirror: the latter has a low coefficient of reflectivity in the ultra-violet region.

4. Photomicrography

Fluorescence photomicrographs were always taken on colour film, since variations in fluorescence colour were usually more informative than differences in light intensity recorded on a black and white film. The essential problem in fluorescence colour photomicrography is one involving high contrasts and low intensities. This has been partially solved by using fast colour film (High Speed Kodak Ektachrome, ASA 160) and comparatively long exposure times. Initial trials to establish the approximate exposure times were carried out

on black and white film (Ilford Pan F). The fluorescence image was focussed onto a clear glass screen with cross wires in the plane of the film carrier with the help of a magnifying lens. The screen was then replaced by the film carrier, and an appropriate exposure carried out. A second series of trial exposure experiments were carried out with the colour film in a Leitz 35 mm. camera body fitted with a focal-plane shutter. It was subsequently found that the colour film required approximately double the exposure times intimated by the black and white film experiments for the best reproduction of colour balance.

Because of the tediousness and eye fatigue associated with the individual focussing of each specimen with the glass screen device, focussing was carried out with a prism lateral eyepiece which was adjusted such that its image was in focus both in the eyepiece and in the focal plane of the camera. The prism was swung out of the optical path during the actual photographic exposure.

The initial exposure-time experiments were also correlated with the reading of a prototype photomultiplier exposure meter (Barer and Underwood, 1956, 1961) which is now available commercially (Evans Electroselenium Ltd.). The meter readings were used to gauge the optimal exposure times in later experiments.

The positive colour transparencies obtained by this process

hardly ever matched the original images observed in the microscope either in quality or in exactness of colour balance. Moreover these defects were greatly exaggerated in the positive prints processed (by Haagman's Colour Laboratories, London) from such transparencies. The quality of positive prints could, of course, be improved by special processing techniques but the cost of doing this would be prohibitive. Therefore the positive prints used as illustrations in this thesis are poor reproductions of the original colour transparencies.

Other difficulties encountered in fluorescence microscopy were the reductions of fluorescence intensity or changes in colour in some tissue sections during the long exposure times that were sometimes required. These effects were usually due to photolytic decomposition, or in some cases, to the slow diffusion of a fluorescent dye initially adsorbed onto a definite cytological feature into surrounding sites or into the mountant medium itself. The "secondary" effects were usually apparent in the final photomicrograph.

Tissues which were examined in the transmitted white-light microscope were photographed on Kodachrome I (ASA 10). The intensity of light in the image field was adjusted by means of a rheostat in series with the tungsten microscope-lamp source until the current generated in a photocell placed on top of the eyepiece had reached an arbitrary, predetermined level. Constant exposure times (0.5 sec.) were used.

5. Test materials: their fixation and mounting

The following tissues have been used as test materials in this investigation: the stomach, embryonic umbilical cord, and epiphysial plate cartilage of the rabbit; the stomach, duodenum, intestine, gut, liver and kidney of the guinea pig; the pancreas, trachea, salivary glands, and the skin and attached hairs, of the mouse; the adrenal, ovary and testis of the rat; artery from the ovine umbilical cord; cock's comb; the gullet and adrenals of the frog.

Small pieces of these tissues were placed in "fixative" solutions which co-agulated rather than reacted with the tissue constituents. Fixatives containing heavy metals such as mercury or dichromate were deliberately avoided; it is generally accepted that if these metals were absorbed into the tissue during the fixation process, the fluorescence of the histochemical reactions carried out later would be quenched. In the early stages of this investigation, Carnoy's fluid (an alcohol-chloroform-acetic acid mixture) and absolute alcohol were used for fixation, particularly for mucopolysaccharides. Unfortunately both these fixatives harden the tissue blocks considerably so that they were difficult to cut. Later it was found that ethylene glycol monoethyl ether acetate (abbreviated to 'glycol ether' in the rest of this thesis) preserved proteins, nucleic acids and mucopolysaccharides equally well whilst leaving the tissue blocks in a suitable condition for

cutting sections easily down to about 3-5 μ. The washing out of the fixative was unnecessary before taking the tissue blocks through the alcohols prior to embedding in paraffin wax. Tissues were usually fixed in glycol ether overnight (12-18 hours) at 0-5°. This fixative and similar ethers and esters were introduced into histology by Shelley and Florence (1961). However glycol ether induced the polarization or streaming artefacts of the glycogen content in liver sections which are usually associated with alcohol fixation.

Fixatives containing formalin were also found to be satisfactory for carrying out histochemical reactions on most tissue constituents <u>except</u> for the demonstration of neutral mucopolysaccharides and glycogen with pseudo-Schiff reagents after the periodic acid reaction (sec. 12.7). A <u>diffuse</u>, intense fluorescence was always obtained in tissues fixed and stained in this way. Vassing and Culling (1959) similarly reported that the fluorescence of tissue sections which had been stained in dilute solutions of basic fluorescent dyes, was usually indifferent after formalin fixation. The interference of formalin is not surprising in view of the fact that it combines with many chemical groups in the tissue, particularly with amines, thiols and hydroxyls (French and Edsall, 1945). However strong fluorescence histochemical reactions for groups other than <u>vic</u>-glycols could usually be obtained by washing tissue <u>sections</u> in running water for about

5 mins. after removing the paraffin wax and taking them down to water.

The mixed cetylpyridinium chloride-formalin fixative of Williams and Jackson (1956) led to strong fluorescence histochemical reactions for acid mucopolysaccharides in thick (50μ) ~~un~~decalcified sections of cartilage (in the tibia of the rabbit; Fig. 54).

Thin, white (iron-free) glass slides were used for mounting tissue sections. The cheaper, "green" slides sometimes fluoresced and were unsatisfactory for fluorescence microscopy. Albumin was usually used as the tissue adfixative; its possible interference in fluorochemical reactions was always checked against dry sections fixed to the slide with heat.

In the early stages of this investigation, sections which had been stained in solutions of basic fluorescence dyes were "dehydrated" in 30% alcohol, allowed to dry on the hot plate (40°) and examined for fluorescence as dry mounts as recommended by Reichert and Haitinger in Reichert's (1952) manual. In view of the fact that fluorochromes are extracted from tissue sections by alcohols, it appeared worthwhile following Haitinger's advice instead of trying to mount the stained sections in synthetic media, which of course, would have necessitated dehydration through the alcohols. However, the optical resolution of the dry fluorescent sections was

poor. Moreover, the extent, intensity and colour of the fluorescence in any cytological feature was variable and not always distinct. It has now been found that a satisfactory, reproducable fluorescence appearance could usually be obtained if fluorochromed sections were mounted in water. The "dehydration" stage in 30% alcohol was omitted in this procedure.

The use of aqueous phosphate buffers as a mounting medium (von Bertalanaffy and Bickis, 1956) was not investigated for the theoretical reasons put forward in sec. 12.

Sections on which fluorochemical condensation-type reactions have been carried out (e.g. the periodic acid-salicyloyl hydrazide technique for vic-glycols; sec. 12.19) could usually be dehydrated through the alcohols and mounted in the non-fluorescent "Fluormount" (E. Gurr Ltd., London) or the DPX medium made up as follows (Lillie, 1953): 5 ml. dibutyl phthalate, 25 ml. polystyrene and 70 ml. xylene.

Section 3. Amines

1. Introduction

Amines are particularly amenable to detection by fluorochemical reactions which can be classified roughly under three headings; reactions with aldehydes; oxidative deamination reactions; and affinity for acidic dyes. The efficacy and specificity of each reaction on tissue sections has been checked by deamination experiments.

2. Reactions with aldehydes

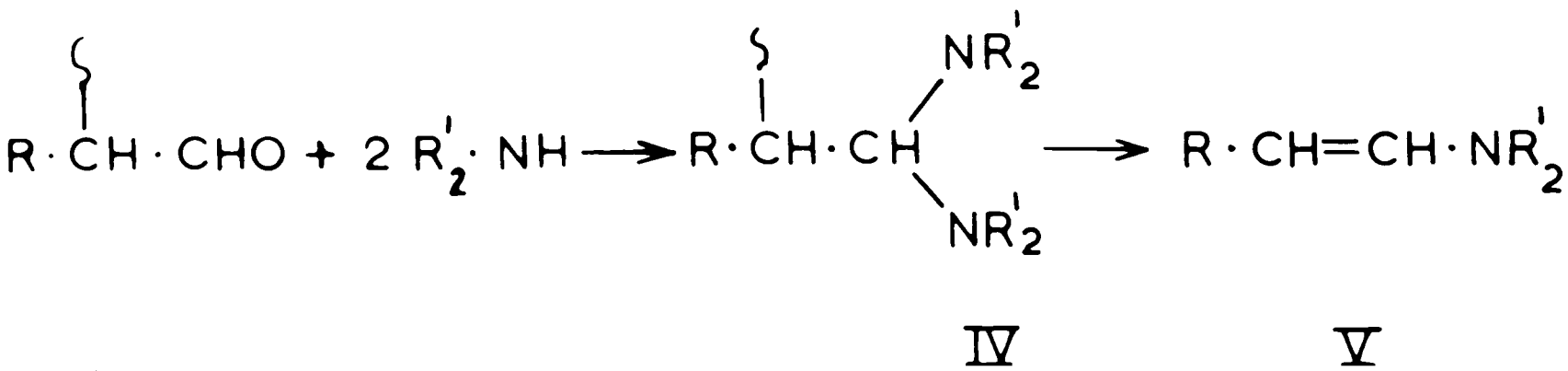
Aldehydes can condense with most groups containing an active hydrogen atom. Therefore they do not necessarily react specifically with amines. Thiol groups, for example, can react *in vitro* to form half mercaptals, $R.CH(OH)SR'$, or mecaptals, $R.CH.(SR')_2$ (Obata and Yamanishi, 1951). Fortunately, these derivatives are unstable and are readily dissociated in water (Ryzheva, 1946). This means that thiol groups in tissues are unlikely to interfere with the aldehyde - amine reaction. One exception might be the reaction between glutathione and O-phthaldialdehyde in which an exceptionally intense fluorescent product is said to be formed (Cohn and Shore, 1962). Glutathione is widely distributed in high concentrations in tissues (Stern and Timonen, 1954) but so far, no intense fluorescent reaction has been observed in various tissue sections treated with solutions of O-phthaldi-

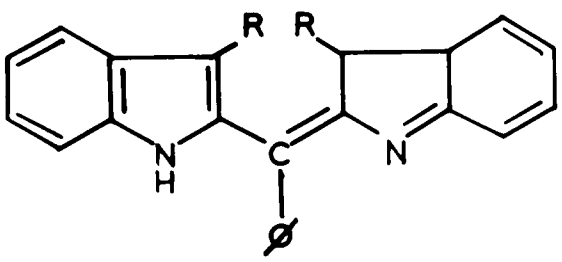
aldehyde. The failure to detect glutathione in fixed tissues is probably due to its diffusion from the tissue during the embedding process.

The activated hydrogen atom in the 2-position of indole residues of proteins can be displaced by aldehydes *in vitro*; coloured derivatives of the type I or the leuco form II are produced (Malowan, 1951; Dobeneck and Maresch, 1952). However such derivatives are not likely to be formed in tissues; the stereochemistry of proteins is probably unfavourable for the condensation of a second indole molecule with the mono-adduct III that is undoubtedly formed initially in tissues (Glenner, 1957). The derivatives of 3- substituted indoles can undergo further internal condensation and oxidation to yield fluorescent β-carbolines.

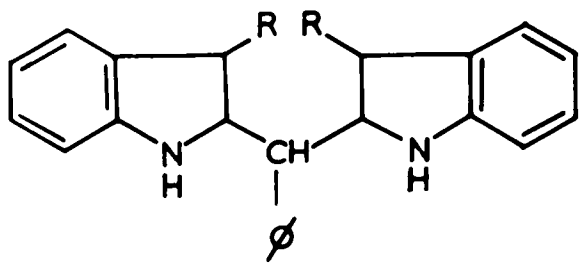
Phenolic residues of proteins (e.g. tyrosine) should not react with aldehydes under the reaction normally used in histochemistry (cf. Akabori and Ohno, 1950).

Both primary and secondary amines can react with aldehydes, and therefore such reactions are not specific for the former. Secondary amines *in vitro* react more easily than ketones or aromatic aldehydes to form diimines, IV, and enamines, V (DeBenneville and Macartney, 1950).

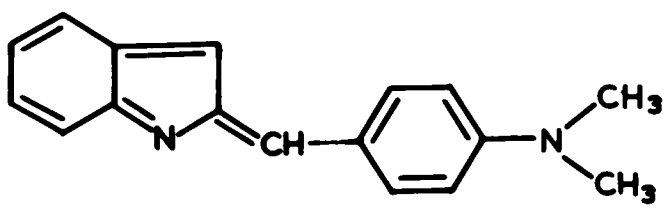




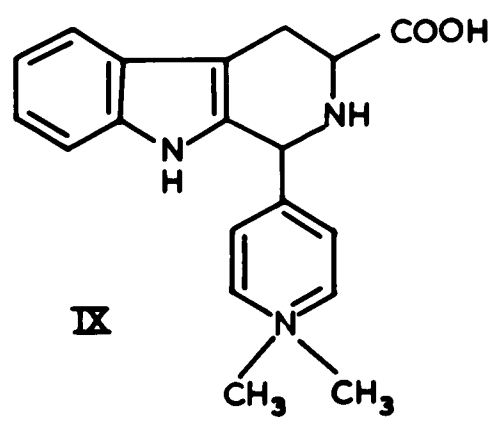
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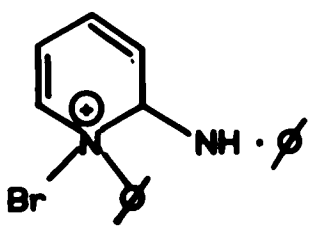
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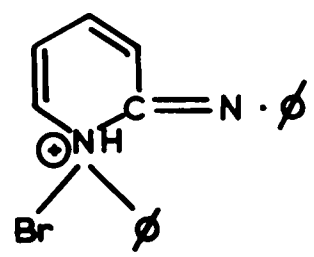
III



IX



VI



VII



VIII

Of the simple fluorescent aldehydes which have been tried in this work as histochemical reagents, only salicyl-aldehyde gave intensely fluorescent anils which were, unfortunately, labile to heat. Very stable, highly fluorescent anils were obtained with the aldehyde intermediates produced by the action of cyanogen bromide on pyridine. These two reactions are discussed in detail below.

3. The König-Sassi reaction

When tissues were treated with a methanolic solution of pyridine containing a few drops of aqueous cyanogen bromide solution, a very intense green fluorescence was developed in the protein sites containing amine groups. The intensity of fluorescence was many times greater than that produced by any other type of fluorochemical reaction investigated so far. The green fluorescence of the anil could be converted to a lighter green (Fig. 30) or sometimes to a blue colour by washing the sections afterwards in methanolic hydrochloric acid. The fluorescent complex was fast towards alcohols and was stable towards heat.

The reaction was discovered by König (1904a,b) and has been applied by Sassi (1956) for detecting amino-acids on chromatograms as intense yellow or red fluorescent complexes. In view of its high sensitivity, it is surprising that the reaction has not been used by other chemists. Nicotcnamide in solution has been assayed fluorometrically by a technique

Fig. 29. Guinea pig gut. Fixed in glycol ether.

0.1% coriphosphine, pH 5 : 0.001% thiazol yellow. Water mount, X 180.

The acid mucopolysaccharides emit a bright orange fluorescence. The protoplasmic and muscle proteins fluoresce blue. The fluoresence colour of the nuclei is light yellow. Some nuclei have an orange tint.

Fig. 30. Guinea pig gut. Fixed in glycol ether.

Mönig-Bossi reaction: 0.1% coriphosphine, pH 5. Water mount, X 180.

An intense green fluorescence is emit ed by the nuclear and protoplasmic proteins.

The special processing required for this prit is responsible for the apparent diminution of the brown-red fluorescence of coriphosphine adsorbed onto acid mucopolysaccharides.

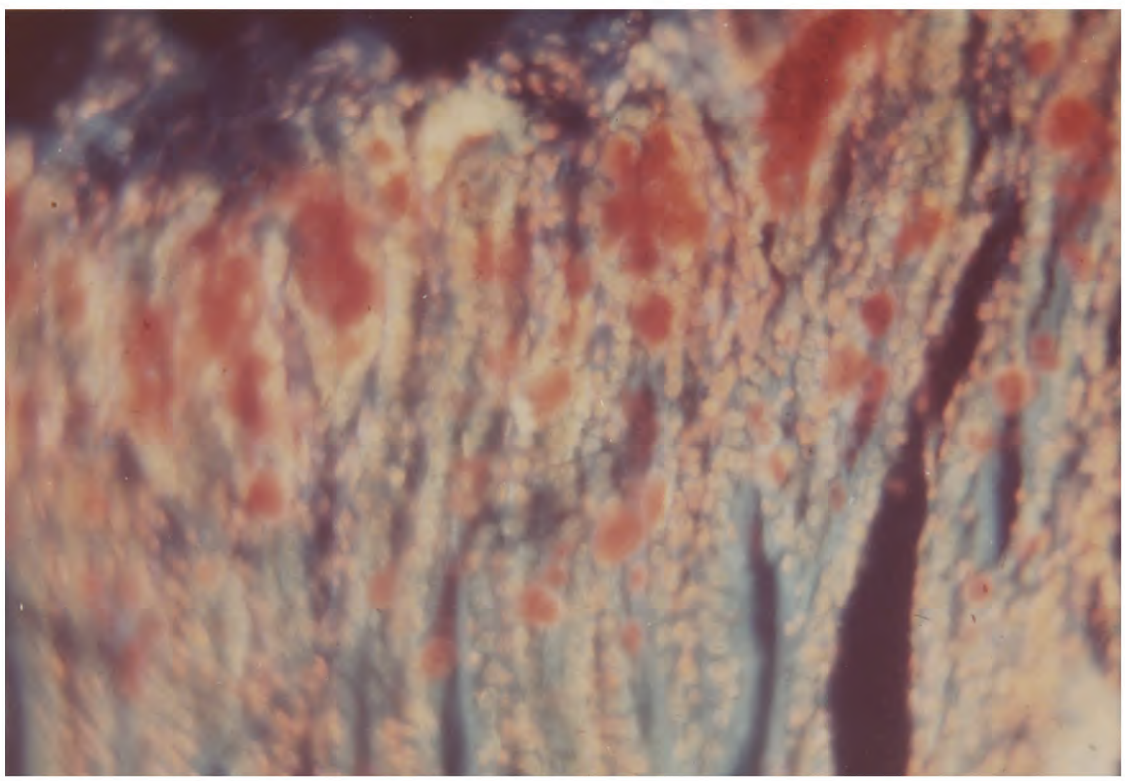


Fig. 29

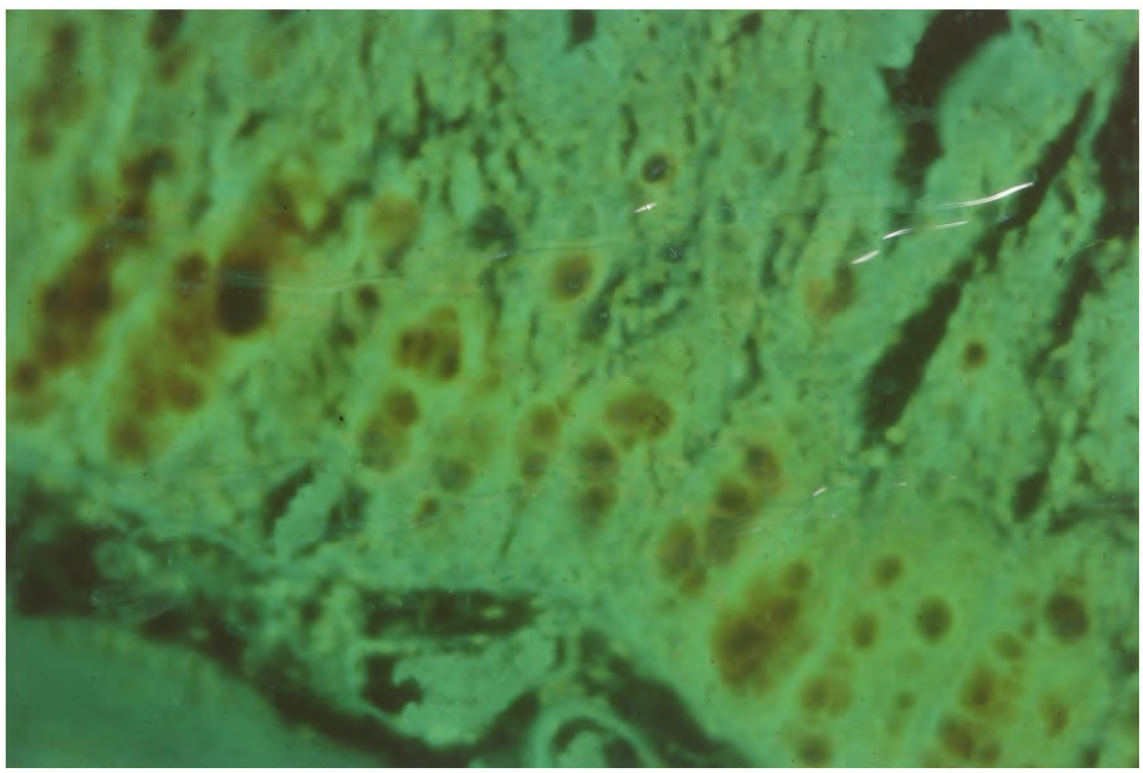


Fig. 30

which depends essentially on the same reaction (Donzou and Le Clerc, 1955).

König (1904) thought that polymethine dyes were formed with structures which would now be written as VI and VII. Creyf and Roosens (1956) found that cyanogen bromide and nicotonamide in the presence of water does not form the N-Cyano-3-carbimido-2-pyridone as reported by Donzou and Le Clerc (1955). Instead the pyridine ring opened to form the intermediate VIII. Presumably, in the König - Cassi reaction, pyridine is cleaved similarly to an aldehyde intermediate which then condenses with the amine.

The tissue proteins in both unmethylated and methylated sections gave a positive reaction, from which it can be inferred that both primary and secondary amines can react.

The details of the devised histochemical technique are given in Experimental Section 3.1.

4. The dimethylaminobenzaldehyde (DMAB) method

The condensation of indoles with dimethylaminobenzaldehyde (Ehrlich's reagent) is well known as a biochemical test. Goessner (1947) and Glenner (1957) believe that the condensation products formed in tissue sections with this reagent have the initial structure III, which is in equilibrium with a leuco form. 3-indoyl derivatives, such as tryptophane (3-indoyl-aminopropionic acid), tryptamine (3-indoyl ethylamine), serotonin (5-hydroxy-3-indoylethylamine) and indoylacetic

acid, undergo further internal condensation to form β-carbolines, IX (Snyder et al., 1948; Portner and Högl, 1953) which are usually fluorescent (Jepson and Stevens, 1953; Davis and de Ropp, 1960). These products can be oxidised in situ by sodium nitrite to blue pigments (carboline blue) of unknown structure (Adams, 1957) which absorb little ultra-violet radiation at 366 mμ (Spies and Chambers, 1948) and are non-fluorescent.

In tissue sections, the tryptophane - DMAB product exhibited a weak, purple fluorescence which was destroyed on oxidation by chemical means or by long exposure to ultra-violet light. Boyd (1929) similarly found that the initial tryptophane - DMAB product was slowly converted in vitro to the visible blue complex by sunlight, which he attributed to ultra-violet radiation. Other amino-acids could also form condensation products with DMAB, but in tissues, they would not be fluorescent nor would they be oxidizable by sodium nitrite (Adams, 1957). Only hydrazines or free aromatic amines in solution can form fluorescent anils with DMAB (Feigl and Mannheimer, 1952).

5. Salicylaldehyde

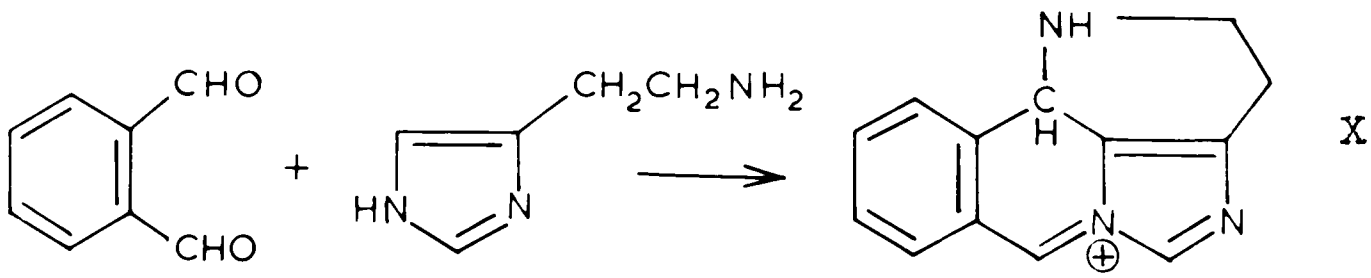
Feigl (1938) used a 1% solution of salicylaldehyde in 2% acetic acid for the fluorochemical detection of hydrazine in solution.

A green fluorescence (similar to that shown in Fig. 30)

was produced in tissues treated with acidified salicylaldehyde. The intensity of the fluorescence in tissues was increased considerably by using alkaline solutions of the aldehyde dissolved in alcohol. The anil was fast towards alcohols but the salicylaldehyde moiety was completely removed from tissues sections by warming them to about 40° or by leaving them exposed to the air for about one week. The amine groups were regenerated in this process.

6. O-phthaldialdehyde

Alkaline methanolic solutions of O-phthaldialdehyde have been used for the fluorometric detection and assay of histidine and histamine (Shore et al., 1959), arginine, cysteine and glutathione (Cohn and Shore, 1962) and agmatine (Cohn and Shore, 1961) both in solution and on paper chromatograms. Shore et al. (1959) suggest that the reactions shown below take place. Subsequent rearrangement and oxidation may yield a fluorophor X of somewhat different structure than the initial condensation product.



Patton and Foreman (1949) found that intense yellow fluorescent products could be formed with histidine and tryptophane on chromatograms sprayed with the aldehyde dissolved

in 80% alcohol. When glycine was treated similarly, it fluoresced brown, but other amino-acids including arginine, glutathione, cysteine, phenylalanine and tyrosine did not react.

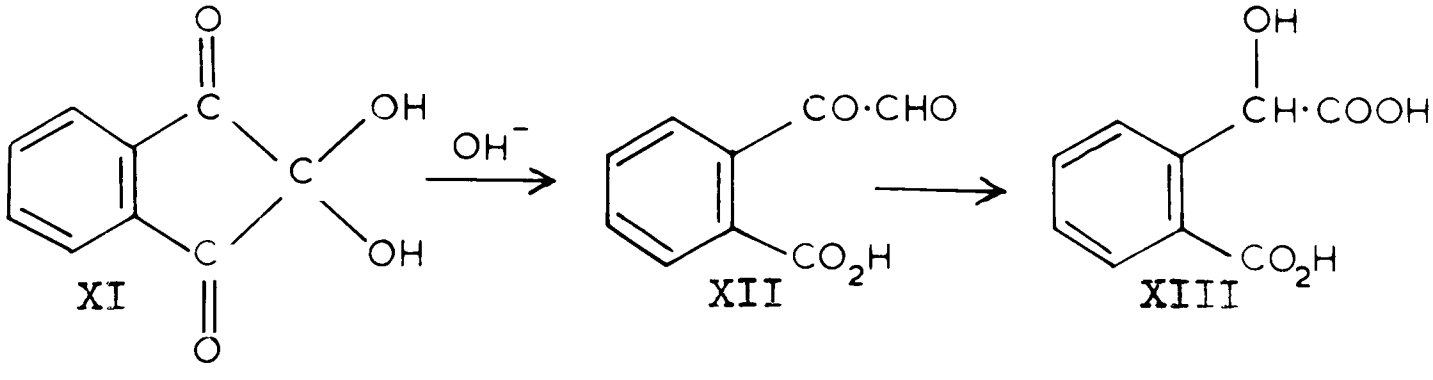
A strong blue fluorescence, comparable to autofluorescence, was obtained by using alkaline solutions (pH 8) of O-phthaldialdehyde on sections which were post-treated with dilute hydrochloric acid. Only a weak blue fluorescence was produced in tissues treated with an alcoholic solution of the aldehyde. Neither variation was therefore useful as a fluorescence histochemical technique.

7. Ninhydrin

Ninhydrin, IX (1,2,3-indanetrione hydrate) can react with the amino-acid residues of proteins in two ways: alkaline solutions of ninhydrin can condense with the guanidine group of arginines, creatines, and related compounds to form fluorescent products. On the other hand, acidic or neutral solutions of ninhydrin oxidize, deaminate and decarboxylate many amino-acid residues to form stable aldehydes. In the special case of tryptamines, highly fluorescent β-carbolines are produced.

Alkaline solutions of ninhydrin are usually used for the fluorochemical detection or assay of amino-acids on paper chromatograms (Lowe et al., 1958) or in solutions, particularly for arginine (Conn and Davis, 1959) and creatine (Cohn, 1960). A solution of ninhydrin in sodium carbonate buffer (pH 9.4),

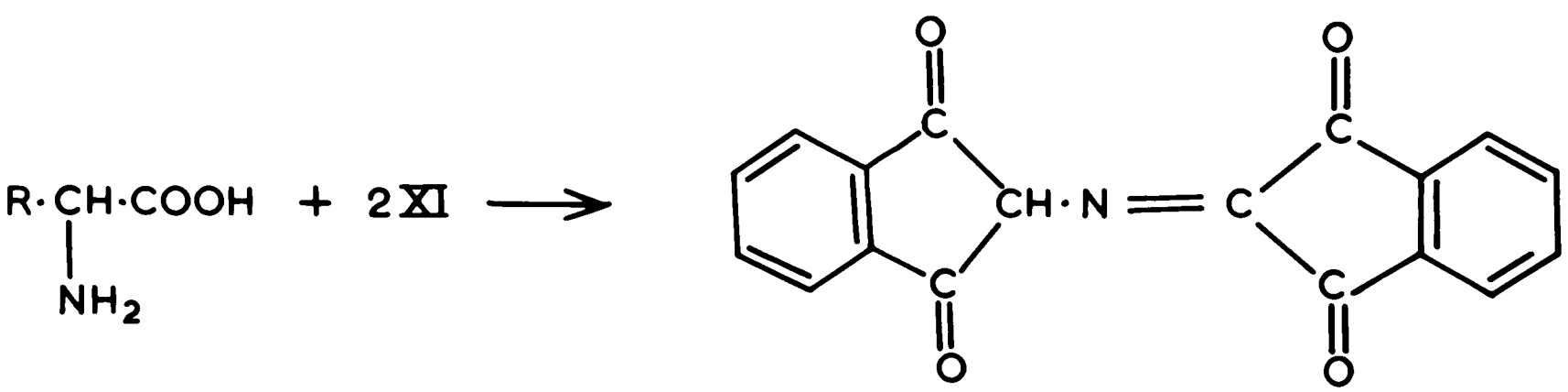
in my hands, induced a strong blue fluorescence in sections of mouse pancreas. The fluorophor in this example was not derived from arginine or creatine as neither of these amino-acids react *in vitro* (Lowe *et al.*, 1958) unless strong alkali (pH 10) is present. Of the naturally occurring amino-acids, γ-aminobutyric acid and phenylalanine yield the most intense fluorescent products (Lowe *et al.*, 1958). The first-named acid is widely distributed in nature, but in mammals, it is limited almost entirely to the brain (Awapara *et al.*, 1950; Roberts and Frankel, 1950). Therefore the reacting component in pancreas and other test tissues was probably phenylalanine. The precise mechanism for the formation of the fluorophor has not yet been formulated. It is thought that the reaction proceeds in two stages. The alkali opened up ninhydrin to form initially O-carboxyphenyl glyoxal (XII), which was eventually degraded to non-fluorescent O-carboxymandelic acid, XIII (Ruheman, 1910). The ketone groups of XII presumably condensed with phenylalanine to form the fluorophor.



XI XII XIII

Ninhydrin has been used in visible light histochemistry for the mild oxidative deamination and decarboxylation of

terminal amino-acids. Serra (1946) found that an aqueous solution of XI gave indifferent results when used on fixed materials, but gave a satisfactory blue colour with freeze-dried materials due to the formation of Ruhemann's complex, XIV. However Langeron (1949) and Pearse (1960) have pointed out that the extreme diffusibility of XIV makes the localisation of amine groups by this reaction unsatisfactory.



XIV + R·CHO + CO_2

Yasuma and Ichikawa (1953) used Schiff's reagent to demonstrate the stable aldehydes formed simultaneously. The formation of both non-fluorescent XIV and aldehydes has now been confirmed by condensing the latter with salicyloyl hydrazide (sec. 12.19). However not all the protein amine groups were oxidised even after 24 hours treatment as was shown by a positive König-Sassi reaction (sec. 3.3) after ninhydrin oxidation. Others have also found that XI is not universally satisfactory for the histochemical demonstration of amino-acid residues. A re-evaluation of the reaction is now being carried out by Glenner (1963).

8. Chloramine T- salicyloyl hydrazide

The use of chloramine T (sodium p- toluene sulpho-chloroamide) in histochemistry was first suggested by Chu et al., (1953). Burstone (1955) showed that this reagent produced stable aldehydes in tissues. The reaction was prevented by prior deamination, but after acetylation the nuclei would still react. In my hands, the structure of tissues oxidised in aqueous solutions of chloramine T at 37° were left in a poor condition, although a moderately intense blue fluorescence was produced in the protein areas after treating the oxidised tissues with salicyloyl hydrazide (sec. 12.19). In view of the structural distortion and the loss of other important constituents from the tissues (e.g. neutral muco-polysaccharides) after such treatment, this method is not recommended for general use except for demonstrating primary amine groups. If amine groups were in the secondary form, as they were in methylated tissues sections (sec. 11), no oxidation took place.

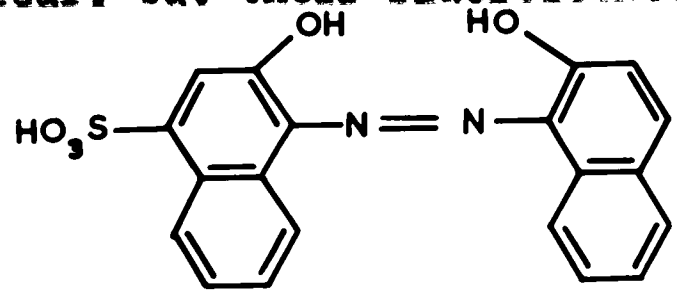
Acid dyes

9. Thiazol yellow

The use of dilute solutions of this dye (Fig. 69) for "counterstaining" the nuclear and protoplasmic proteins surrounding acid mucopolysaccharides is discussed in sec. 9.4.

10. Solochrome Black-aluminium complex

In a fruitless attempt to show that the fluorescent complex formed between Solochrome Black and alum (Radley, 1943) might be adsorbed specifically onto acid mucopolysaccharides (sec. 9.11), it was found that only the protein sites adsorbed the complex which fluoresced a brick-red colour. If alum was omitted from the dye solution, the tissues were stained a weak, visible red colour. but their fluorescence was negligible.



XV

Presumably aluminium ions formed a fluorescent chelate with the two hydroxyl groups of the dye XV whose sulphonic acid groups were the essential centres reacting with the amine acidophils.

Other Solochrome dyes (patented by I.C.I. Ltd.), such as Solochrome Violet RS (Radley, 1938) or Solochrome Cyanine RS (Pearse, 1960) can be substituted for Solochrome Black.

The fluorescence reaction was not prevented by extracting basophilic substances from tissue sections with hot aqueous hydrochloric acid (sec. 9.7/1) or by blocking the basophilia with methanolic thionyl chloride (sec. 11). In fact the intensity of the reaction after methylation was increased. This was probably due to the methylation of the protein primary amine groups to form stronger cationic polymethylamines. Prior

deamination (sec. 3.13) abolished the reaction.

The complex was especially useful for "counterstaining" the tissue background after the periodic acid-salicyloyl hydrazide reaction for neutral mucopolysaccharides. The complex adsorbed onto amine groups was fast towards alcohols and therefore stained sections could be mounted in DPX. A typical appearance of sections treated in this way is illustrated in Fig. 58. The actual intensity of the Solochrome complex is much greater than is apparent from the photomicrograph. The fluorescence emanating from the salicyloyl-hydrazones was so intense that only short photographic exposures were required. These times were insufficient to form the correct red images on the film.

11. Naphthoquinone-4-sulphonic acid

Feldman (1959) and Opienska-Blauth et al. (1960) assayed amino-acids on chromatograms as coloured fluorescent complexes after heating them with sodium 1,2-naphthoquinone-4-sulphonate. In this application, the reagent had a special advantage in that secondary and tertiary amines did not interfere. Unfortunately no fluorescence could be detected in tissue sectionstreated with 1% methanolic solutions of this reagent at 60°.

The selective destruction or blocking of amine groups as confirmatory tests.

12. Introduction

The specificity of histochemical reactions for amines can be checked by selectively destroying or blocking the amine groups in tissue sections beforehand. Such techniques are also useful for protecting amine groups which might react with reagents intended for the demonstration of other groups. One example of the second application is the investigation of the sulphation of hydroxyl groups in the presence of amines with the DMF-sulphur trioxide complex (sec. 13.8).

The diazotisation and deazotisation of amine groups is theoretically the best specific method for the removal of amine groups, but in practice, it is inconvenient and leads to the extraction of other components from tissue sections.

On the other hand, techniques based on oxidative deamination, such as with hypochlorites, are both rapid and specific, and are the methods of choice.

Danielli (1950, 1953) suggested various reagents for the selective blocking of the end groups of proteins in tissue sections without trying them out in practice. Those reagents which I have tried (e.g. phenyl isocyanate) had hardly any effect on the fluorescence histochemical reactions for amines carried out afterwards. Some reagents named by Danielli were not investigated for theoretical reasons. For example,

he stated that S-phenyl thiocarbonyl chloride ($C_6H_5.S.CO.Cl$) reacts specifically with amine groups; this compound does react with amines *in vitro* (Rivier, 1907), but it reacts with phenol and thiol groups in the same way (Ehrensvärd, 1947). The formula of another reagent stated by Danielli to be specific for amines was given as $Cl.CH_2.O.CH=CH_2$. No reference to this compound, chloromethyl vinyl ether, could be found in the chemical literature, so that it is doubtful whether it exists at all!

13. Deamination by diazotisation and deazotisation

Most histochemists (e.g. Lillie, 1954) use Van Slyke's (1929) nitrous acid reagent at room temperature for the deamination of tissue sections as originally recommended by Monné and Slautterback (1950). This reagent destroys most of the eosinophilia of blood cells, for example, and does not impair the PAS reaction. Lillie (1954) claims that mixtures of acetic acid and sodium nitrite act more rapidly on sections and smears than mixtures containing hydrochloric acid in spite of their higher pH levels. Burstone (1955) and Pearse (1960) used cold nitrous acid for deaminating tissues.

Others (Pearse, 1960; Bradbury, 1963) including myself have found that Monné and Slautterback's technique does not abolish the specific reactions of amine groups, such as the terephthaldialdehyde-Schiff (Pearse, 1960), or the König-Sassi

reactions. This failure is to be expected for the following reasons:

(1) Some terminal amino-acids in tissues have an inherent resistance to rapid deamination by nitrous acid. Most of the amine groups in wool, for example, require up to six days treatment for their removal (Speakman, 1936; Rutherford et al, 1937). Some basic groups which are probably unreacted arginine residues (Cockburn et al., 1948) may still remain after this time. This long reaction period also produces drastic changes in the tissue as the result of the oxidation of disulphide bonds, the extraction of tryptophane, neutral polysaccharides, etc.

(2) The belief that nitrous acid itself can completely deaminate tissues is fallacious. Amine groups can only be removed in two steps:- (a) diazotisation with nitrous acid at low temperatures, followed by (b) deazotisation, preferably with warm water or alcohol.

a) $R \cdot NH_3^+ Cl^- + HNO_2 \longrightarrow RN_2^+ Cl^- + 2H_2O$

b) $RN_2^+ Cl^- + R' \cdot CH_2OH \longrightarrow R \cdot H + N_2 + R' \cdot CHO + HCl$

or $RN_2^+ Cl^- + R' \cdot CH_2OH \longrightarrow R \cdot OCH_2R' + N_2 + HCl$

$RN_2^+ Cl^- + H_2O \longrightarrow R \cdot OH + N_2 + HCl$

This theory has now been translated into a histochemical technique (exp.sec.3.9). Tissues were first treated with a cold mixture of sodium nitrite and sulphuric acid for 48 hours. Sulphuric acid is preferable to other acids for

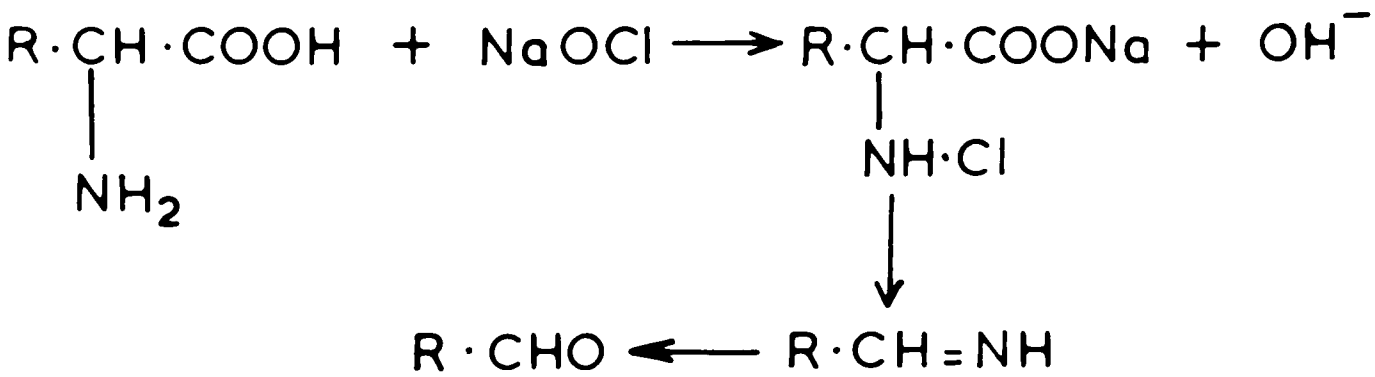
diazotisation in that it avoids rearrangement or substitution reactions of the diazonium salt (Kornblum, 1944). The diazotised tissues were then deazotised with alcohol (the Griess reaction) at 37° for 4-6 hours. Perfectly anhydrous conditions were not required, but the water content was limited to about 5-10% (Beeson, 1894). Water itself at 37° deazotated most, but not all, tissues as shown by their subsequent positive König-Sassi reactions. Soluble proteins and mucopolysaccharides were extracted at the same time.

Other reagents, such as alkaline formaldehyde (Brewster and Poje, 1939) or sodium hypophosphite (Mai, 1902), failed to deazotate diazotised tissue proteins.

14. Oxidative deamination with hypochlorites

(1) Introduction

Langheld (1909) was the first to establish that alkaline solutions of hypochlorite could rapidly oxidise, decarboxylate and deaminate primary and secondary α-amino-acids, but since then few studies of the exact course of the reaction with amino-acids incorporated into proteins have been carried out. The initial reaction with α-amino-acids is an N-chlorination which is accompanied by the liberation of free alkali. This is followed by hydrolysis, decarboxylation, and the ultimate formation of aldehydes (Langheld, 1909; Aubel and Asselineau, 1948).



In addition to the degradation of the terminal α-amino-acid residues, Baker (1947) believes that in proteins, the thiol and disulphide linkages, the tyrosine ring, the heterocyclic nucleus of histidine, tryptophane and proline, and the guanidine grouping in arginine are all liable to be attacked. The aromatic nucleus of phenylalanine and the hydroxyl groups of serine react slowly. Contrary to earlier conclusions (Goldschmidt and Steigerwald, 1929), peptide linkages can also be attacked (Herkin and Silbersiepe, 1951). Albumin used as a tissue adhesive is certainly attacked and leads to the detachment of the tissue section from its slide if it is left in the hypochlorite solution for too long, particularly if the pH is greater than 7.5.

Although it is believed that only the terminal α- amino-acid residues can be converted to aldehydes, other types of amine groups are also destroyed rapidly by neutral hypochlorite. In tissue sections, the König-Sassi reaction is completely abolished after hypochlorite oxidation; before oxidation, the whole tissue is positive. However, after oxidation, only certain features (notably nuclei) are converted to aldehydes.

The protoplasm contains neither basophilic substances, aldehydes nor ketones after deamination. The majority of amino-acids which constitute the proteins of tissues are therefore not terminal α-amino-acids (see sec. 7.2/3) and do not react in the same way. Their anomalous oxidation has been studied *in vitro* by Wieland *et al.* (1949), who showed that only glutamic acid is quantitatively oxidised (to ketoglutarate), whereas aspartic acid loses only 3% of its nitrogen as ammonia. However ketoglutarate and hypochlorite *together* can deaminate glycine, alanine, leucine, phenyl-alanine, tyrosine, serine, threonine, methionine and aspartic acid. The nature of the oxidation products was not investigated, but it seems likely that the corresponding ketoacids, and not aldehydes, are formed (Ingols *et al.*, 1953). The thiol groups in proteins are oxidised to sulphonic acids.

Polysaccharides can also be oxidised by hypochlorite, but it is believed that very little degradation takes place under the conditions of my histochemical experiments. At about pH 10-11, the primary hydroxyl groups of monosaccharides are oxidised *in vitro* to uronic acid groups (Whistler and Schweiger, 1959; Whistler and Yagi, 1961). If the pH of the reaction mixture is lowered to about 4-5, the uronic acid is degraded further to a sugar with two fewer carbon atoms. By adjusting the pH of the hypochlorite solution to about 5.5-7.0 for histochemical oxidations and keeping the oxidation

times short, little degradation of the neutral mucopolysaccharides in the section should take place. When sections of liver were incubated in diastase to remove glycogen, there was no change in the number of aldehyde-positive sites after oxidation in acidic hypochlorite (exp. sec. 3.10). There was very little change in the periodic acid reaction of glycogen in liver sections even after drastic hypochlorite oxidation. If the glycogen had been oxidised to a uronic acid or a δ'-lactone, it probably would not undergo a rapid periodic acid reaction (Cp. hyaluronic acid; sec. 12.4/4). Apparently vic-glycol groups in tissues therefore do not react with hypochlorites.

(2) Histochemical experiments

The application of hypochlorites for deamination in histochemistry was introduced by Chu et al. (1948). They treated acetone-fixed sections with unbuffered solutions of sodium hypochlorite. The cytoplasmic contents gave a positive reaction only if the methamine-silver reagent of Gomori (1946) was used for visualising the engendered aldehydes. Burstone (1955) later found that sections treated with stronger solutions of hypochlorite at pH 7.5 were subsequently coloured strongly by Schiff's reagent (sec. 12.6). The staining could be abolished by rinsing the oxidised sections in a solution of sodium thiosulphate (to destroy the excess hypochlorite) before treatment with Schiff's reagent. He concluded that hypochlorites, unlike chloramine T (sec. 3.8), do not produce stable aldehydes in

tissues and attributed the staining to loosely-bound hypochlorite which oxidised the leucofuchsin to its coloured form.

(3) Reducing rinses

The writer considers that Burstone's interpretations are erroneous, and that the experimental techniques cited above are unsatisfactory. After using a dilute solution of hypochlorite whose pH had been brought down to 7.5 on sections which were subsequently rinsed in a solution of sodium thiosulphate and stained in coriphosphine (0.01%), a stable red-orange fluorescence reminiscent of sulphate groups was observed in the protein sites of several types of tissue (Fig. 9). These sites did not fluoresce when the oxidised and thiosulphate-rinsed sections were condensed with salicyloyl hydrazide (sec. 12.19), thus showing that aldehydes are not present. This suggested that the results obtained by Burstone (1955) were due not only to spurious oxidation reactions of hypochlorites, but also to the unfortunate choice of thiosulphate as the reducing rinse. Another unusual feature of the thiosulphate rinse was that it rendered the oxidised sections translucent and often grossly distorted them. This took place even if sections were oxidised with dilute solutions of hypochlorite at pH 5.5. In this case, however, the extent of the red fluorescent basophilia in such sections as revealed by staining them with coriphosphine was very much less than that observable in sections oxidised at 7.0 (Fig. 11).

Fig. 9. Guinea pig liver. Fixed in glycol ether. Sodium hypochlorite, pH 7.7, 3 mins. : sodium Thiosulphate wash : 0.01% coriphosphine, pH 5. Water mount, X 180.

The whole section emits a stable, orange fluorescence which is difficult to focus sharply.

Fig. 11. Guinea pig intestine. Fixed in Carnoy's fluid.

Sodium hypochlorite, pH 5.2, 10 mins. : wash in 5% sodium thiosulphate : 0.01% coriphosphine, pH 5.2. Water mount, X 180.

The basophilic red fluorescent sites are distributed patchily (cp. Fig.9). The staining is not sharply confined to any particular region.

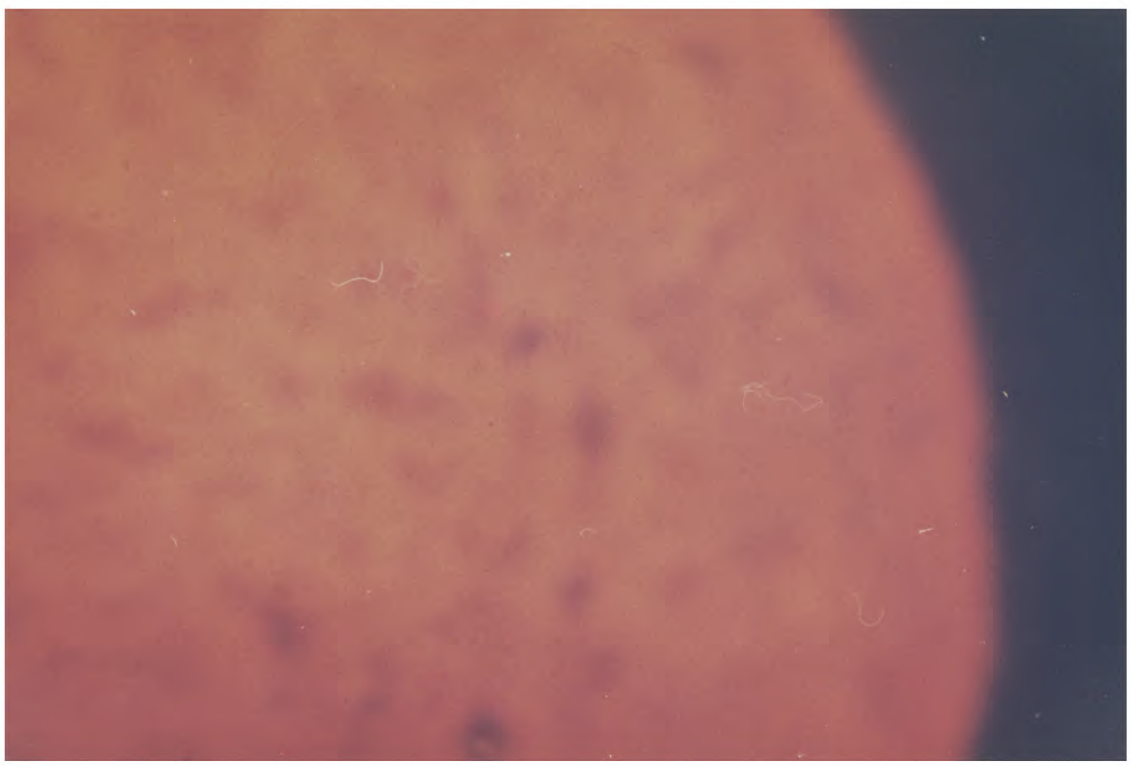


Fig. 9

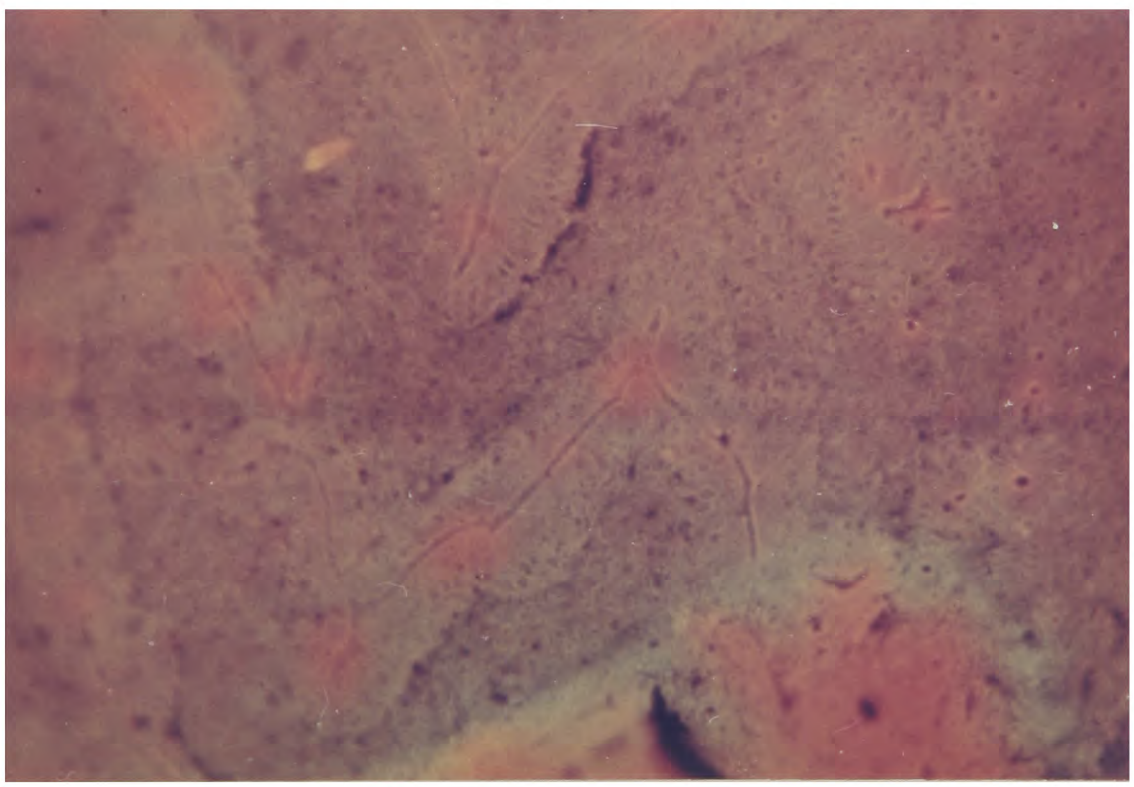


Fig.11

Although the excess hypochlorite entrapped in the tissue sections usually oxidises thiosulphate to sulphate, some hydrogen sulphide may also be formed particularly in the initial stages (Maksimyuk and Ptitsyn, 1946). The hydrogen sulphide so formed can react with the aldehydes engendered in the tissue in the presence of hydrochloric acid to form α-chlorosulphonyl chlorides (Jonas, 1952) which are then presumably hydrolysed to α-chlorosulphonic acids (Cp. sec. 13.5). If ketones are formed in the hypochlorite oxidation of ϵ-amine groups, then thioketones would probably be formed with hydrogen sulphide (Brintzinger and Zeigher, 1948) and would be susceptible to instantaneous oxidation. The formation of these basophils would explain the colour and intensity of the fluorescence of oxidised and thiosulphate-washed sections which have been stained in coriphosphine. Barrnett and Seligman (1954) noticed incidentally that ammonium sulphide reacted with engendered carbonyl groups but did not investigate the reaction any further. In order to prove that the colouration by Schiff's reagent in Burstone's sections was due to the formation of aldehydes and not to the excess hypochlorite retained in the tissue, it is necessary to wash the oxidised sections in a way such that only inert products are formed. The following solutions were therefore investigated for destroying excess hypochlorite:- (a) solutions of ammonia containing traces of copper, (b) water, and (c) allyl alcohol.

(a) Hypochlorites react rapidly with solutions of ammonia to form chloramine (equation 1), which if cupric ions are not present (Cahn and Powell, 1954), would react with more ammonia to give hydrazine (Raschig, 1907) as shown in equation (2). Hydrazine, if formed, would of course block the engendered aldehydes in the tissue to form hydrazides ($R.CO.NH.NH_2$).

$$NH_3 + OCl^- \longrightarrow NH_2Cl + OH^- \quad \text{.....} \ (1)$$

$$NH_2 \cdot Cl + NH_3 + OH^- \longrightarrow N_2H_4 + Cl^- + H_2O \quad \text{..} \ (2)$$

$$N_2H_4 + NH_2Cl + 2OH^- \xrightarrow{Cu^{++}} N_2 + 2NH_3 + 2Cl^- + 2H_2O$$

Tissues which have been oxidised in neutral hypochlorite, washed in a 70% alcoholic solution (pH9) of ammonia containing a trace of copper sulphate, and stained in a dilute solution of coriphosphine, did not exhibit the widely distributed, stable orange-red fluorescence shown by sections that had been washed with thiosulphate (Fig. 9). The nuclear areas, however, did fluoresce a brick-red colour initially, whose intensity quickly decreased after 1-2 minutes exposure to ultra-violet light: the coriphosphine which was adsorbed onto these sites dissolved into the water mountant. Because of this photolytic instability, a photomicrograph could not be taken. The fluorescence colour of the conjugate was probably due to a "pure" phosphate-coriphosphine bond. In an undeaminated section, the cationic amine groups limit the uptake and

Fig. 12. Guinea pig liver. Fixed in glycol ether.

Sodium hypochlorite, pH 7 : neutral ammonia wash : salicyloyl hydrazide. Fluormount, X 180.

The large number of blue fluorescent sites are derived from engendered aldehydes.

Fig. 13. Guinea pig liver. Fixed in glycol ether.

N Hydrochloric acid, 60°, 6 mins. : salicyloyl hydrazide. Fluormount, X 180.

The blue fluorescence emitted by the nuclei is weaker than that of the fluorescent sites shown in Figs. 12 or 27.

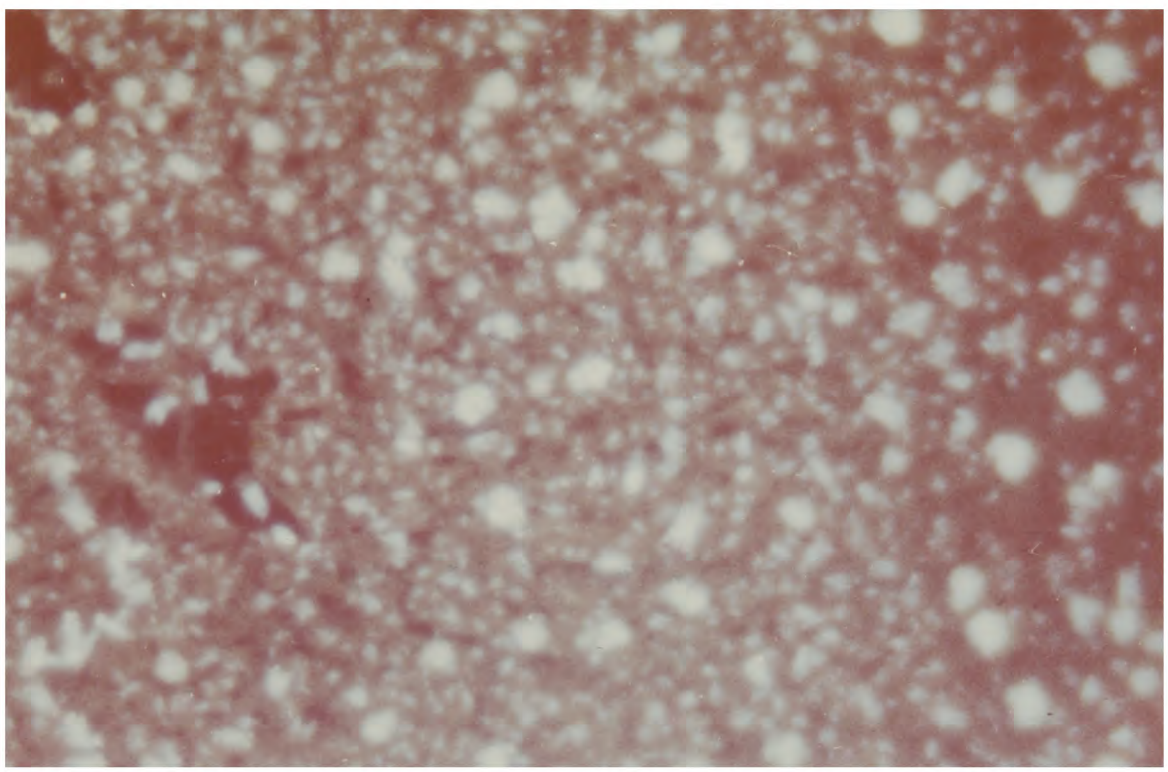


Fig.12

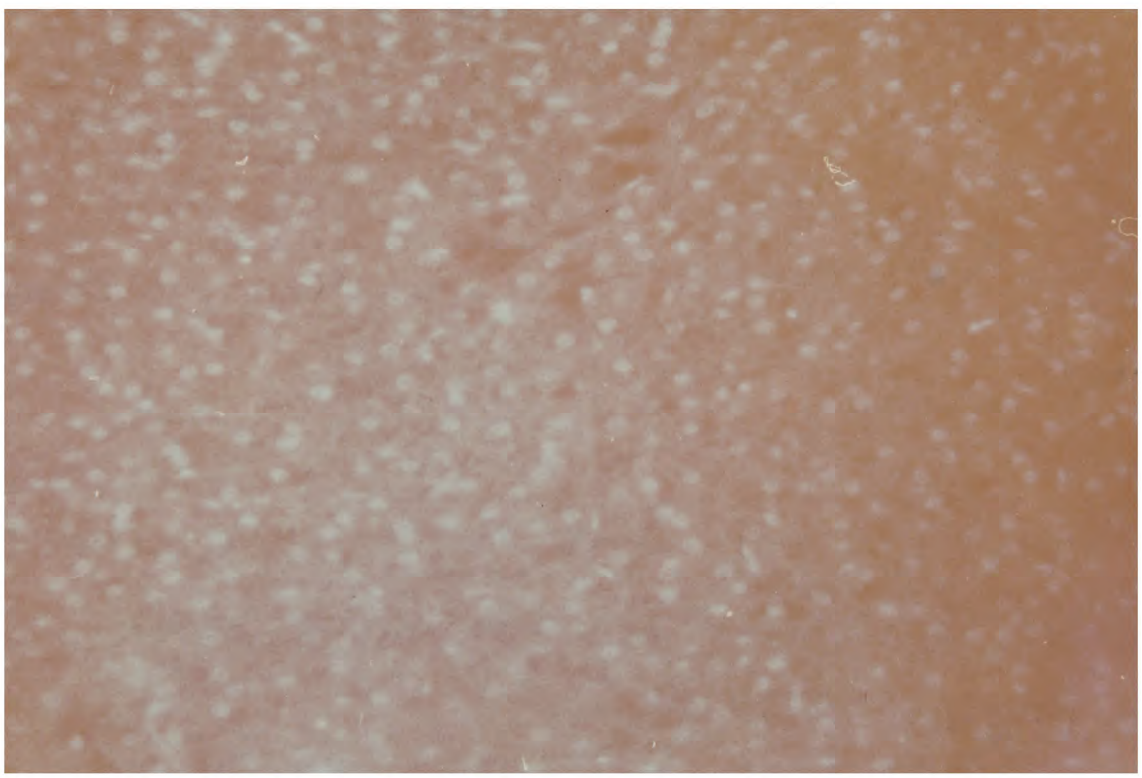
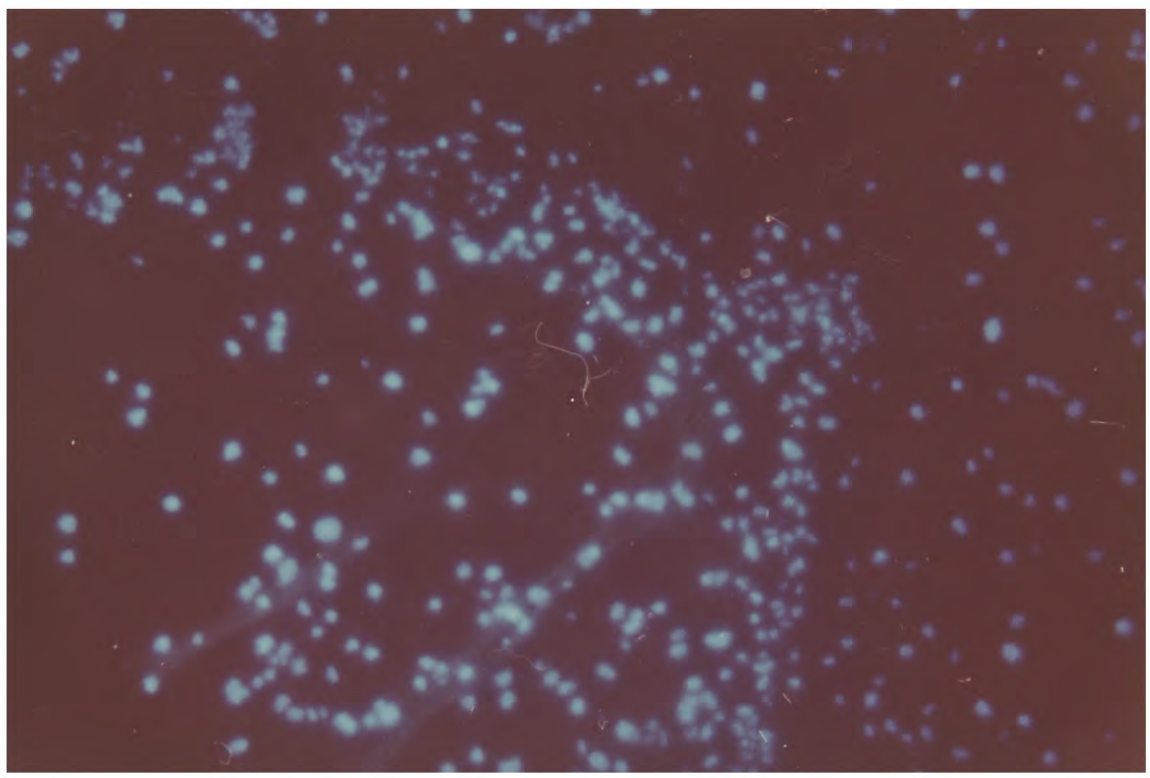


Fig. 13

Fig. 14. Frog adrenal. Fixed in formalin.

Sodium hypochlorite, pH 7.5 : ammonia wash : salicyloyl hydrazide. Fluormount, X 180.

The amino groups of proteins which are oxidised to aldehydes emit a bright blue fluorescence.



Pl .14

the "strength" of binding of the dye (and hence fluorescence) adsorbed onto anionic sites.

The König-Sassi reaction was completely abolished after the hypochlorite-ammonia wash-treatment, but only a limited number of aldehyde sites could be demonstrated. When the pH of the ammonia solution was adjusted nearer to neutrality, a very much larger number of aldehyde sites was demonstrated subsequently. Some of the engendered aldehydes are destroyed by a Cannizzaro-type reaction in the alkaline solutions of ammonia.

(b) Nearly the same number of aldehyde sites could be demonstrated when oxidised sections were washed only in water instead of neutral solutions of ammonia.

(c) Instead of being destroyed through oxidation, hypochlorites can be added across the olefinic bond of allyl alcohol to form mainly 2-chloropropane-1:3-diol (Smith, 1918). The number of aldehyde sites remaining in oxidised tissue sections after a wash in allyl alcohol is less than that observable after the ammonia or water washes.

(4) The sites of hypochlorite oxidation in tissues

The appearance of the tissue illustrated in Fig. 12 suggests that hypochlorites may not be oxidising the amine groups of proteins but are hydrolysing the DNA in nuclei to aldehydo-furanoses (Feulgen hydrolysis). The intensity and extent of the fluorescence emitted from nuclei in such sections

is very much greater than could be observed in serial sections which had been subjected to a normal Feulgen hydrolysis and condensation in salicyloyl hydrolysis (Cp. Fig. 13). Nevertheless, it might be thought that hypochlorites at room temperature are more efficient for a Feulgen hydrolysis than hot hydrochloric acid; the latter is well-known to extract the DNA aldehydes as soon as they are exposed. The time adopted for the acid hydrolysis is usually a compromise between optimal hydrolysis and minimum extraction. The possibility of a reaction between DNA and hypochlorites was investigated in two ways:

(i) Nuclear aldehydes were exposed in sections by a normal Feulgen hydrolysis and reduced to primary alcohols with a 0.1% aqueous solution of sodium borohydride buffered to pH 7.7 in boric acid as used by Jobst and Horvath (1961) for the reduction of ketolipids in tissues. The unbuffered 1% solutions of sodium borohydride (pH 9) recommended by Geyer (1963) for the reduction of aldehydes in tissues also abolished the reactivity of suitably pre-treated sections towards aldehyde-reactive reagents. The abolition with Geyer's reagent was, however, not due to the reduction of the aldehydes, but was due to a Cannizzaro-type destruction of the aldehydes by the alkali present in the borohydride mixture:

$$2\ R\cdot CHO + OH^- \rightarrow R\cdot CH_2OH + R\cdot CO_2^-$$

Control sections containing free aldehyde groups lost their reactivity towards aldehyde-reactive reagents after being immersed in dilute alkali of the same pH as the reducing mixture. Geyer's reagent therefore gives misleading results and should not be used. The nuclear aldehydes were reduced rather than "blocked" with aliphatic or aromatic amines because blocking groups can sometimes interfere with histochemical reactions carried out later for neighbouring groups (e.g. Primary hydroxyl and amine groups cannot be sulphated with the DMF-sulphurtrioxide complex on sections in which the 1,2-secondary hydroxyl groups of glycogen have been "blocked" by oxidation with periodic acid and condensation with aniline; sec. 13.8).

The reduced sections were then treated with hypochlorite and salicyloyl hydrazide in the normal way. A few aldehyde-positive sites were produced. Therefore there is some component in nuclei other than the deoxysugar of DNA which reacts with hypochlorite. The reduction in the number of positive sites might be due to two causes:

(a) the hot hydrochloric acid extracts most of the histones (Stedman and Stedman, 1950) and purine bases (Di Stefano, 1948) which are probable centres of attack for the hypochlorite

(b) the pH of the reducing medium may still be too high to prevent the destruction and/or extraction of the "protein aldehydes".

(ii) Nucleic acids were extracted before hypochlorite oxidation by incubating sections in 0.4% deoxyribose-nuclease for 100 mins. at 37° (exp. sec. 9. 10a). Despite the general objections to the use of DNase in histochemistry (sec. 9.8/2), it should be efficacious in this experiment as it abolishes the Feulgen-salicyloyl hydrazide reaction and avoids the two drawbacks cited for the borohydride experiment. A <u>few</u> aldehyde-positive sites could be demonstrated subsequently in sections after the DNase digestion and oxidation in hypochlorite. As in (i), the conclusion is that it is not the deoxysugar which is responsible for the positive hypochlorite-salicyloyl hydrazide reactions in the nuclei. An associated aminoprotein appears to be the reactive constituent.

One unusual feature of the salicyloylhydrazones of the "protein aldehydes" is the partial destruction of their fluorescence by solutions of zinc salts. Apart from one particular hydrogenated steroid (Camber, 1957), the fluorescence of the salicyloylhydrazones of all the other aldehydes which have been investigated so far in solution or <u>in situ</u> is intensified or, at least, is unaffected by post-treatment with zinc salts. The absence of fluorescence in the non-nuclear protein sites shows that ketones are not formed by hypochlorite oxidation, but it does not disprove the hypothesis that ketoacids may exist in these sites. Ketoacids (e.g. pyruvic acid) are exceptional in not forming fluorescent

salicyloylhydrazones in solution (Camber, 1957).

(5) The effect of pH on the hypochlorite oxidation of tissues

Any solution of hypochlorite whose pH is greater than 7.5 rapidly oxidises tissue sections and eventually dissolves them completely. It is therefore difficult to understand how Chu et al. (1953) obtained any positive results at all with their unbuffered solutions whose pH must have been between 10 and 11. In my experience, the 10% solutions of hypochlorite at pH 7.5, which Burstone (1955) recommended, destroyed the morphological structure of the sections used in this work after the times he used for complete deamination.

If the pH of a 1% solution is adjusted to the range 5.5-7.0, a large number of aldehyde sites are produced in tissue sections. The oxidation proceeds smoothly without any signs of over-oxidation. Aldehydic sites can be produced with acidic solutions of hypochlorites of pH down to 2.1 but the number is variable and is less than that produced by the nearly neutral solutions of hypochlorite. A similar optimal range of pH has been reported for the oxidation of hydroxy acids in vitro (Shilou and Yasnikov, 1952).

(6) Conclusion

Dilute solutions of hypochlorites can be used for the complete and rapid deamination of tissue sections provided their pH is adjusted to the range 5.5-7.0. Basophilic aldehydes are produced in nuclear sites. The proteins of other

cytological features are probably converted to ketoacids.

15. Oxidation to aldoximes

Primary amines can be oxidised by hydrogen peroxide in the presence of a catalytic amount of sodium tungstate to aldoximes through intermediate hydroxylamines (Kahr and Berther, 1960). Oxygen is liberated.

$$R \cdot CH_2 \cdot NH_2 \rightarrow R \cdot CH_2 \cdot NH \cdot OH \rightarrow RCH=N \cdot OH$$

An immediate reaction took place in tissue sections treated with 1.5% solutions of hydrogen peroxide containing 0.1% sodium tungstate. When the evolution of oxygen had finished, the König-Sassi and alkaline salicylaldehyde reactions were reduced to about half their original intensity. On the other hand, methylated sections did not react at all. This indicates that the reactive groups are primary amines. This reaction can therefore be used as a rapid diagnostic test for such groups by observing the evolution of oxygen. No means of visualising the primary amine groups cytochemically by this reaction could be devised.

Blocking techniques

16. Phenylation

Dinitrofluorobenzene (DNFB) was introduced into biochemistry by Sanger (1945, 1950) for blocking the phenol, thiol and amine end groups in proteins. The imidazole group of histidine also reacts (Porter, 1950). For histochemical

purposes, Danielli (1947, 1950) recommended the blocking of any two of these groups by other methods before treating the third group with DNFB. The function of the two nitro groups in the phenylating reagent is to activate the nuclear halogen atom sufficiently for it to be displaced by the acidic hydrogen atoms of other groups (see sec. 3.20).

Although the amine groups in tissues can be blocked very efficiently with DNFB, the presence of the resulting conjugate is not conducive for further fluorescence histochemical experiments; the nitro groups of the conjugate would quench the potential fluorescence of neighbouring groups in the tissue after they had been stained with fluorochromic reagents. Experiments were therefore carried out to remove the nitro groups from the amine-DNFB conjugate by reducing (with sodium dithionate), diazotising and deazotising them with alkaline formaldehyde. The removal of the diazonium groups was followed by treating the deazotised sections with a fresh solution of H-acid: a positive reaction indicated an incomplete deazotisation. 5% solutions of sodium hypophosphite (Mai, 1902) were ineffective as deazotising agents.

The alkaline formaldehyde reagent of Brewster and Poje (1939) completely abolished the H-acid coupling reaction and the visible yellow colouration of the original amine conjugates in the tissue, but subsequent fluorochemical reactions were nevertheless completely quenched. For example, there was no

apparent fluorescent basophilic staining with coriphosphine. Moreover, the amine groups of the DNFB conjugate could not be detected by the König-Sassi reaction at the end of the dithionate reduction stage. Whether the quenching and/or the abolition of these fluorochemical reactions was due to stereochemical effects or to the presence of unreduced nitro groups is unknown.

17. Benzaldehyde

Neither acid nor alkaline buffered solutions of benzaldehyde in alcohol or acetone and water significantly reduced the intensity of the subsequent fluorochemical reactions for amine groups in tissue sections. Even piperidine failed to catalyse the formation of Schiff bases.

18. Phenyl isocyanate

The thiol and the primary and secondary amine end groups of proteins form substituted ureas with aryl isocyanates in high yields in vitro (Hopkins and Wormall, 1933a,b; 1934a,b; Gaunt and Wormall, 1936). Of all the variants that have been tried on tissue sections, only a cold, phosphate-buffered solution of phenyl isocyanate at pH 7.9 altered the colour of the König-Sassi reaction carried out subsequently. The light green fluorescence which was produced showed that the blocking was incomplete.

The isocyanate group can be "activated" by a nitro group

para to it in an aromatic nucleus. The amine groups in tissues should therefore show a stronger reaction with p-nitrophenylisocyanate than with phenylisocyanate itself. Burstone (1955) tried this reagent on tissues. Sections were treated with the isocyanate dissolved in a pyridine-benzene mixture for 20 hours at room temperature. The tissue conjugate was visualised by reducing, diazotising and coupling with H-acid. The intensity of the final colour was extremely low, indicating that little isocyanate combined with the tissue proteins and confirmed my histochemical blocking experiments.

The reaction of proteins with isocyanates has been used for the synthesis of fluorescent labelled proteins in Coon's tracing techniques (Nairn, 1962). However the success of this technique depends on the fact that only a small proportion of the total number of protein amine groups combine with the fluorescent isocyanate coupling reagent as otherwise the specific antigenicity of the protein would be destroyed. The essential criterion of Coon's technique would have been vitiated if isocyanates were efficient blocking compounds.

19. Chloroacetone

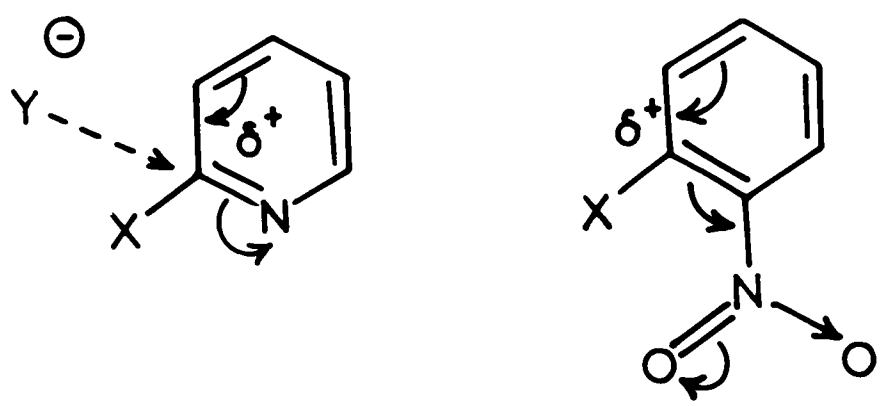
The carbonyl group in chloroacetone (chloro-2-propanone) activates the halogen atom sufficiently for it to be replaced by the conjugate base of compounds containing an acidic hydrogen atom. This explains why chloroacetone reacts *in vitro*

with the thiol groups of proteins (Bacq and Fisher, 1946) and with primary and secondary amines (Janetzky et al., 1946). The reactions with amines are sometimes anomalous; with aniline, a product of unknown structure with the empirical formula $(C_9H_{11}NO_2)_2$ is formed and not $C_6H_5.NH.CH_2.COCH_3$ as expected. Phenols require a catalyst, such as concentrated sulphuric acid or aluminium chloride, before they will react (Zaheer et al., 1954). Danielli (1950) states, without proof, that chloroacetone reacts only with the carboxyl groups in tissue sections.

In practice, neither a solution in alcohol nor pure chloroacetone itself had any effect on the subsequent fluoro-chemical reactions for amines in tissue sections.

20. Halopyridines

The heterocyclic atom in pyridine, pyrimidine or quinoline can activate α- or γ-substituted nuclear halogen atoms in the same way as the nitro groups activate the halogen in DNFB. Erlenmeyer et al. (1946, 1947) deduced the similarity between pyridine and nitrobenzene on biochemical evidence.



This activating mechanism is responsible for the reactions *in vitro* of 2- and 4-chloro and bromopyridines with primary and secondary amines (Wibaut and Tilman, 1933; Petrov, 1945; Wibaut and Brockman, 1961). The α-substituted chlorine is more reactive in pyrimidine and quinoline than in pyridine (Young and Amstutz, 1951). The halogens can also be displaced by thiols (Backer and Buisman, 1945). Phenols do not react with halopyridines in the same way as amines: complexes are formed instead between the phenol and the basic nitrogen atom (Labes and Breitenstein, 1934; Labes *et al.*, 1941). Therefore halopyridines appear to be more advantageous than DNFB as phenylating-type blocking reagents in histochemistry. They would avoid the tedious removal of nitro groups with its attendant complications. The alkaline salicylaldehyde reaction for the amine groups in tissues was only partly reduced by 24 hours treatment with 2-chloropyridine dissolved in alcohol. The incomplete blocking may be due in part to an energy barrier effect. Just as dinitro*chloro*benzene fails to react with amino groups in tissues (Abderhalden and Stix, 1923) because the heat required to make the reaction go to completion would result in the hydrolysis of the protein, so may fluoropyridines be more successful for the blocking of protein end groups. Their greater reactivity *in vitro* supports this premise (Bradlow and Vanderwerf, 1949).

21. Benzoylation

The usual histochemical method for blocking amine groups is to benzoylate them in a Schotten-Baumann reaction. Pyridine is used as the catalyst and vehicle for the benzoylating agent and for neutralising the hydrochloric acid produced during the reaction (Danielli, 1947). This reaction is efficient on tissues, but in my experience, the 10% solutions recommended by Danielli (1947) and Pearse (1960) lose their potency rapidly. A white solid, presumably benzoic acid, is precipitated. Lillie (1954) has reported similar difficulties. Moreover pyridine is an unpleasant reagent to use.

A solution of benzoyl chloride in dimethylformamide (DMF) was therefore tried. Hall (1956) suggests that the active species is an electrolyte formed through the O-acylation of DMF by benzoyl chloride. However a 50:50 mixture did not benzoylate either the hydroxyl groups of carbohydrates or the amine groups of proteins in tissues: both the periodic acid - salicyloyl hydrazide and the alkaline salicylaldehyde reactions in tissue sections were subsequently positive.

22. Acetylation

Primary and secondary amines and hydroxy compounds are usually acetylated *in vitro* in the presence of bases by the Schotten-Baumann reaction. This reaction is not often used in histochemistry for blocking purposes as it entails immersing sections in pyridine boiling under reflux (Pearse, 1960).

Lillie (1954), however, suggests that acetylation for 24 hours at room temperature is adequate for the amine groups in tissues in contrast to the hydroxyl groups of neutral mucopolysaccharides which require longer intervals and higher temperatures.

A 5% solution of sodium acetyl sulphate was tried in an alkaline phosphate buffer solution (pH 8.3) instead of pyridine. Kenner and Stedman (1952) found this compound excellent for acylating aminoacids in vitro. It had no effect on tissue sections even after 24 hours treatment.

Acid catalysed acetylation was also investigated. Such acylations in vitro are often fast and quantitative (Blumrich and Bandel, 1941; Schnek and Santiago, 1962). The usual catalyst is perchloric acid. It ruptures acetic anhydride to give an acetylium ion (CH_3CO^+), which in turn reacts with hydroxyl or amine groups as follows (Burton and Prail, 1950):

$$H^+(+ ClO_4^-) + (CH_3CO)_2O \rightleftharpoons CH_3CO^+ + CH_3COOH$$

$$R \cdot NH_2 + CH_3CO^+ \rightarrow R \cdot NH \cdot CO CH_3 + H^+$$

A fresh 10% mixture of acetic anhydride in ethyl acetate containing 0.1% perchloric acid abolished the alkaline salicylaldehyde reaction in tissue sections after 36 hours treatment.

Experimental Section 3. Amine Groups

1. König-Sassi reaction (f)

Preparation of cyanogen bromide solution (after Woolf, 1953).

4 g of potassium cyanide dissolved in 7.5 ml distilled water is added dropwise to 4 ml of liquid bromine covered with 2.5 ml water contained in a reaction flask which is immersed in an ice-salt mixture. After the potassium cyanide has been added, the reaction mixture is stirred for a further 2 hrs. The resulting orange-coloured liquid is stored in a screw-topped container at -20°C.

Method

(1) Take sections to methyl alcohol

(2) Immerse in 5% v/v pyridine-methyl alcohol mixture containing 2-3 drops of cyanogen bromide solution for 3-5 mins. The mixture is used within 24 hrs.

(3) Rinse in methyl alcohol, absolute ethyl alcohol and xylene.

(4) Mount in Fluormount and examine for fluorescence.

Result

A very intense green fluorescence is developed. This is a very rapid and sensitive technique.

2. Dimethylaminobenzaldehyde (DMAB) method for tryptophane(f)

(1) Take sections to water.

(2) Immerse in 5% solution of DMAB in concentrated HCl or a

2.5% DMAB solution in a 1:3 mixture of conc. HCl and glacial acetic acid for 5 mins.

(3) Wash with water.

Results

A strong purple fluorescence is produced. When a 5% DMAB solution in dilute (3%) hydrochloric acid is used, a blue fluorescence is produced that could be mistaken for tissue primary fluorescence.

3. Alkaline Salicylaldehyde method (f)

Method

(1) Take sections to alcohol

(2) Immerse in fresh 1% salicylaldehyde solution in 1% alcoholic potassium hydroxide for 5-20 mins.

(3) Wash with alcohol and clear in xylene

(4) Examine for fluorescence as dry mount or mount in Fluormount or DPX.

Result

An intense green fluorescence is produced, which sometimes fades on exposure to air for several days. The fluorescence is destroyed immediately on heating sections to about 50°.

Acid Salicylaldehyde (f)

(1) Take sections to water

(2) Immerse in 1% solution of salicylaldehyde in 2% acetic acid for 5-20 mins.

(3) Wash in water and take through alcohols

(4) Examine for fluorescence as dry mount.

Result

A green fluorescence is produced which is less intense than that given by the alkaline salicylaldehyde method.

4. O-phthaldialdehyde (f)

Two methods were tried:

(a) Sections taken to alcohol were treated with a 1% solution of O-phthaldialdehyde in 80% alcohol for 1 hr.

(b) Sections were immersed in a 0.05% solution of O-phthaldialdehyde in water (pH 8) for 1 hr., followed by a wash in dilute (N/10) HCl for 10 mins.

Result

When the sections were taken to alcohol and dried after treatment by either method, a blue fluorescence was produced. The intensity of the fluorescence was insufficient to commend the method for general use.

5. Ninhydrin (v,f)

Method (a)

(1) Take sections to water

(2) Immerse in 0.25% solution of ninhydrin dissolved in sodium carbonate buffer (pH 9.3) for 30 mins. at 60°.

(3) Wash in water, 30% alcohol, dry and examine for fluorescence.

Result

A bright blue fluorescence is produced at protein sites.

Method (b)

(1) Take sections to alcohol

(2) Immerse in 0.5% ninhydrin solution in absolute alcohol for 18 hours at 37°C.

(3) Wash in alcohol and take to water

(4) Condense with SH as in stages (5) - (8) of method 6.

Result

No fluorescence colours are produced by the alcoholic ninhydrin solution itself, although a visible purple coloration is observable. Ninhydrin converts the 1° amino groups to aldehydes by oxidative deamination, which are subsequently shown as their blue fluorescent hydrazones. Similarly, 0.2% solutions of ninhydrin in acetic acid-acetone mixtures give no fluorescent conjugates although a visible pale purple colour is evident after 5 mins. reaction.

6. Chloramine T - SH method for 1° amines (f)

(1) Take sections to water

(2) Immerse in 1% chloramine T solution for 90 mins. at 37°

(3) Wash in 5% sodium thiosulphate for 3 mins.

(4) Wash in water

(5) Immerse in acidified 0.5% salicyloyl hydrazide (pH 3) for 20 mins.

(6) Wash in distilled water

(7) Wash in dilute PCAF solution

(8) Wash in water and take through alcohols to xylene

(9) Mount in Fluormount and examine for fluorescence.

Result

Reactive sites are shown as an intense blue fluorescence. The structure of the section is usually distorted.

7. Thiazol yellow (f)

A 0.001% solution of thiazol yellow used for 5 mins. is used as a "counterstain" for acidophilic groups after acid mucopolysaccharides have been stained with coriphosphine (exp. sec. 9.2).

8. Solochrome Black-Alum Complex (f)

Preparation of stock solution

A 0.1% solution of Solochrome Black is diluted with twice its volume of 5% alum, and filtered. Any precipitate that appears in the filtrate on storage is removed by further filtration. The translucent pale-purple solution of the complex did not lose its staining properties after six months storage.

Method

(1) Take sections to water

(2) Immerse in Solochrome Black-Alum complex solution for 10 mins.

(3) Wash in water and take through alcohols to xylene

(4) Mount in Fluormount and examine for fluorescence.

Results

A bright brick-red fluorescence is produced in sites containing protein and nucleic acid amine groups. This method provides an excellent contrast for the bright blue fluorescence of neutral mucopolysaccharides demonstrated by the PA-SH technique (exp. sec. 12.14).

Deamination

The following methods have been used as specific confirmatory tests for amine-groups.

9. Diazotisation and deazotisation

Method

(1) Take sections to water

(2) Treat with fresh nitrous acid for 48 hrs. at 0-5°C made by dissolving 1 g sodium nitrite slowly in cold 30 ml 3% sulphuric acid

(3) Wash with water

(4) Incubate in either (a) distilled water or (b) alcohol, for 4 hrs. at 60°

(5) Test efficacy of the deamination procedure by König-Sassi reaction.

Result

This deamination method is reliable and preserves

structural details well. Unfortunately 48 hours must elapse before the results are available.

The deamination methods advocated by Lillie (1954) and Pearse (1960) which omit the deazotisation step were ineffective. Deazotisation with alkaline formalin (exp. sec. 3.12) on diazotised sections also proved inadequate.

10. Oxidative deamination with sodium hypochlorite

Method

(1) Take sections to water

(2) Immerse in 1% sodium hypochlorite whose pH has been freshly adjusted within the range 5.5-7.0 for 5-20 mins. "Milton's solution" can be substituted for the hypochlorite solution

(3) Wash in alcoholic ammonia solution for 1-2 mins. (10 ml water, 20 ml .88 ammonia solution, 70 ml alcohol) in which one crystal of cupric sulphate is dissolved. The pH of the solution is adjusted to 7 with sulphuric acid

(4) Wash twice in distilled water, one min. each

(5) Test efficacy of this deamination procedure by the König-Sassi reaction (exp. sec. 3.1) or by condensation with salicyloyl hydrazide (exp. sec. 12.14 stages 3-7).

Result

The König-Sassi reaction is completely abolished indicating complete oxidative deamination. Aldehydes are produced in nuclear sites.

The wash in 5% sodium thiosulphate which Burstone (1955) recommended for stage (3) led to the immediate destruction and detachment of the tissue section from its glass slide.

11. Oxidation of primary amines to aldoximes

Take sections down to water and immerse in 1.5% hydrogen peroxide containing 0.1% sodium tungstate for 30-60 mins. until the evolution of oxygen has finished.

The intensity of the König-Sassi reaction (exp. sec. 3.1) carried out subsequently is about half that of control, unoxidised sections.

Blocking reactions

12. Phenylation

Method

(1) Take sections to alcohol

(2) Treat with 1% DNFB in 90% alcohol saturated with sodium bicarbonate (pH approximately 9) for 16-20 hours (overnight)

(3) Wash in two changes of alcohol, 2 mins. each, and take down to water

(4) Reduce in a fresh 5% solution of sodium dithionate for 4-8 hours at 37° until sections appear colourless

(5) Wash in water

(6) Diazotise with fresh nitrous acid at 0-5° for 30-60 mins. The nitrous acid is made by slowly adding a cold solution

of fresh 5% sodium nitrite to three times its volume of cold N hydrochloric acid, keeping the temperature below 5°

(7) Wash briefly in cold water

(8) Deazotise with alkaline formaldehyde reagent for 45 mins.

(9) Test efficacy of stages 6-8 by the König-Sassi reaction and the visible H-acid coupling reaction (exp. sec. 12.12, stage 8).

Results

Both the reactions named in stage (9) are negative. The König-Sassi, the Solochrome Black-alum complex, and the periodic acid-salicyloyl hydrazide reactions are all negative after stage (5).

The phenylation blockage therefore cannot be recommended as it interferes with the histochemical reactions of other groups.

Preparation of the alkaline formaldehyde reagent (after Brewster and Poje, 1939).

5 g of sodium hydroxide are dissolved in 30 ml water. After cooling this solution, 25 g ice are added, followed by the slow addition of 4 ml of 40% formalin.

13. Benzaldehyde

1% solutions of benzaldehyde in 70% alcohol containing either a little piperidine or glacial acetic acid blocked few

amine groups after 18 hours. However, a 1% solution of benzaldehyde in M/10 barbitone buffer (pH 8) containing a little acetone blocked most amine groups of proteins but not those in nuclei which exhibited a moderately strong green or even blue fluorescence after treatment with alkaline salicylaldehyde (exp. sec. 3.3).

14. Phenyl isocyanate

A 2% solution of phenyl isocyanate in an acetone-dioxane mixture did not block protein amine groups after 3 hours as shown subsequently by a strong König-Sassi reaction.

A 5% solution of phenyl isocyanate in M/10 phosphate buffer at 5° partially blocked amine groups after 24 hours as shown by a light green fluorescence after carrying out the König-Sassi reaction.

15. Chloracetone

A 10% solution of chloracetone in alcohol did not block tissue amine groups after 24 hours treatment at room temperature or at 37°, as shown subsequently by strong König-Sassi and alkaline salicylaldehyde reactions. Immersion in chloracetone itself for 24 hours hardly reduced the intensity of the König-Sassi reaction.

16. Halopyridines

Comparable reactions to those carried out for chloracetone

using 2-chloropyridine showed that the latter reagent was ineffective in blocking tissue amine groups.

17. Benzoylation (Modification of Lillie's (1954) and Pearse's (1960) procedures)

Method

(1) Take dewaxed sections to absolute alcohol
(2) Allow to dry in air, and then in an incubator at 60° for 5-10 mins.
(3) Immerse dry sections in a 5% fresh solution of benzoyl chloride in dry pyridine (distilled and stored over barium oxide) for 10-16 hours (overnight)
(4) Rinse in acetone and alcohol, one min. each
(5) Test efficacy of benzoylation blocking with the König-Sassi technique.

Result

Amine groups are completely benzoylated as indicated by the negative König-Sassi reaction.

18. Acetylation

Method

(1) Take sections to alcohol, and dry in air
(2) Immerse in acetic anhydride for 3 mins.
(3) Treat with freshly-prepared acidic acetic anhydride mixture (4 ml acetic anhydride, 36 ml ethyl acetate, and 0.05 ml perchloric acid) for 36 hrs.

(4) Rinse in acetic anhydride for 1 min.

(5) Rinse in alcohol for 1 min.

(6) Test efficiency of acetylation procedure by the alkaline salicylaldehyde reaction (exp. sec. 3.3).

Result

The alkaline salicylaldehyde reaction is completely abolished with this acetylation mixture. Blocking was incomplete using mixtures containing 1/10th of the acetic anhydride content as recommended for the quantitative acetylation of amine groups in vitro by Schenk and Santiago (1962).

Preparation of Sodium acetyl sulphate (after van Peski, 1921)

2.5 ml concentrated sulphuric acid is added dropwise to 15 g acetic anhydride at 0-2°. 2 g anhydrous sodium acetate is added and the mixture stirred for 90 mins., followed by storage overnight at -10°. 40 ml ice-cold ether is stirred into the mixture over a period of about 2 mins. The white precipitate is collected and washed with four 20 ml portions of cold ether.

Section 4. Thiols.

1. Introduction.

The principal sulphur linkages in proteins are thiols and disulphides. It is not always clear whether the histochemical reactions now available for these groups differentiate between them. Danielli (1950) therefore suggested that the thiols already existing in tissue sections should be temporarily protected with a reversible blocking compound, such as a mercurial reagent, if a histochemical technique is ultimately intended for them alone. The interference of disulphides can be eliminated by oxidising them to sulphinic acids with peracetic acid, after which the thiol block can be reversed and a histochemical reaction carried out in the normal way.

Although some mercury compounds, such as p-mercuribenzoate, completely block thiol groups (Boyer, 1954), the resulting mercaptide linkage is not stable and is reversed in competition with other thiol reactive reagents (Pearse, 1960). In addition, the reactions with many mercurials are slow; many hours are required for their equilibration (Horowitz and Klotz, 1956).

It is therefore desirable that histochemical methods should be devised for the direct demonstration of thiol groups without disturbing the disulphide linkages. Seligman, Tsou and Barrnett (1954) have published such a method. They found that the conjugate formed between N-(4-hydroxy-1-naphthyl)-isomaleimide (HNI) and tissue thiols could be visualised by coupling it with

a stable diazonium salt. Following Price and Campbell's (1957) discovery that the HNI-thiol-diazonium salt complex was intensely fluorescent on chromatograms, Pearse (1960) suggested that Seligman et al's technique could be used for fluorescence microscopy. A comparative assessment of this technique has not been carried out as the starting materials required for the synthesis of HNI are not available in this country.

The following techniques have now been devised specifically for fluorescence histochemistry:

(1) The coupling of thiol groups with N-ethyl maleimide, followed by condensation with salicyloyl hydrazide and complexation with zinc salts.

(2) The sulphation of "drastically" methylated sections with sulphuryl chloride and staining with a basic fluorescent dye.

Each technique has been compared with the sensitive dihydroxy-dinaphthyl-disulphide (DDD) method (Barrnett and Seligman, 1952, 1954; Barrnett, 1953) used for demonstrating thiol groups in visible light histochemistry. Incidentally DDD does not react with disulphides as has been implied by Pearse (1960) because of the misleading title of Barrnett and Seligman's (1952) original paper. The specificity of each technique has been checked by previously blocking thiol groups with iodoacetamide.

2. The N-ethyl-maleimide-salicyloyl hydrazide-zinc method (NEM-SH/Zn).

(1) Introduction

N - Ethyl maleimide (NEM) reacts promptly with most thiol groups in vitro and in tissue sections (Friedman etal, 1949; Alexander, 1958) and is usually used in histochemistry as a blocking agent. The only known failure of NEM to react with thiols occurs in intact erythrocytes; Sheets, Hamilton De Gowin and King (1956) considered this to be due to the impenetrability of the cell envelope.

(2) The Specificity of the NEM reactions

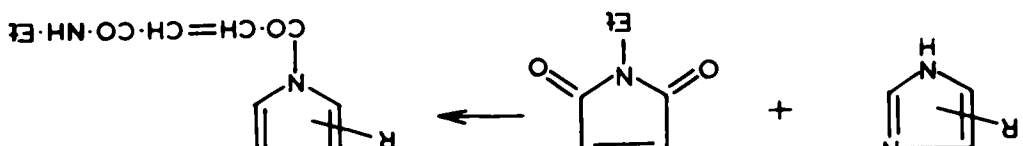
Doubts as to the specificity of the reaction have been raised by the in vitro investigations of Smyth, Nagamatsu and Fruton (1960). The following reactions are possible with the groups likely to be present in tissues:

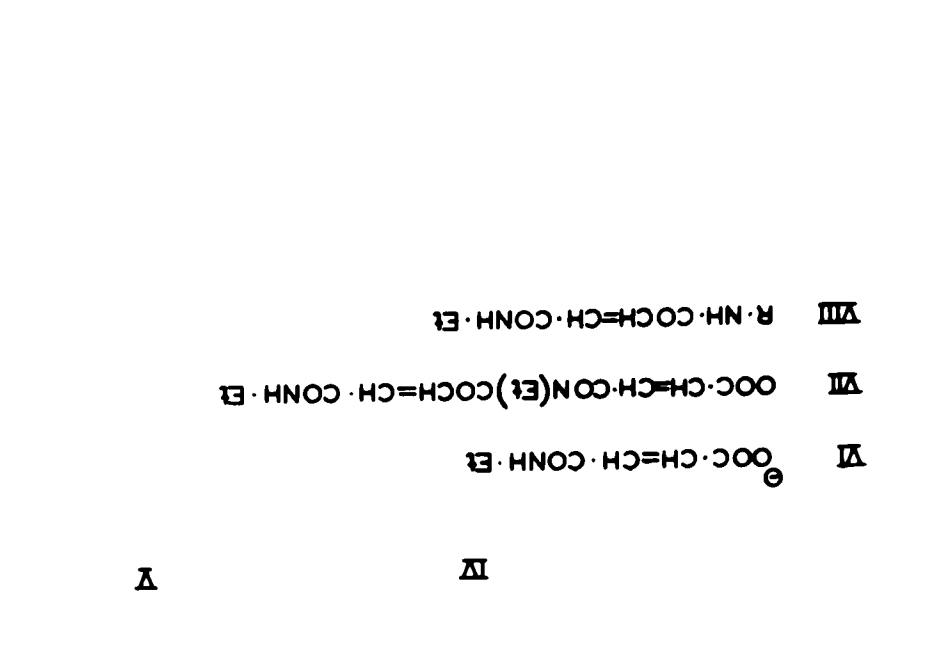
(a) with thiols

The usual adducts which is formed at about pH 7 has the structure I (Marian, 1949). A solution of the cysteine complex (II) undergoes a quantitative conversion after 36 hours at pH 9 to a cyclic compound III, 2 - (N - ethylacetomido) - 3 - keto - 1,4 - thiazine - 5 - carboxylic acid.

(b) with imidazoles and histidines.

An imidazole reacts with one carbonyl group of NEM (IV) forming a acylimadqzole (V) which may be expected to undergo rapid hydrolysis to N - ethyl maleamic acid (VI). The latter will also be produced by the hydrolysis of NEM itself (Gregory, 1955). Since acylimadazoles are reactive acylating agents (Stadtman, 1953) V may acylate VI with the formation of





of the dimer VII and the regeneration of imidazole. VII is susceptible to further acylation by V and the chain length of the resulting polymer would thus increase and it may eventually be deposited in the tissue section.

At pH 7.4, histidine derivatives give a pink colour in vitro and an insoluble NEM polymer.

(c) with amines

Stable, acylamino compounds (VIII) are formed. However, thiols react preferentially in mixtures containing an excess of aminoacids (Alexander, 1958) or histidines (Rigg's, 1960).

(3) The condensation of the NEM adducts with salicyloyl hydrazide

The difference between the thiol adduct (I) and the others is that I contains two ketone groups that are not part of conjugated amidic or carboxyl systems. They should therefore form non-fluorescent salicyloyl hydrazones which can be rendered strongly fluorescent by post-treating them with a solution of a zinc salt (cp. Sec. 7.2/3ii). This is what happens in practice. A green fluorescence has been produced in sections in which the DDD reaction gave a weak result. An application of this reaction is described in sec. 12.7/7.

There is no evidence that salicyloyl hydrazide or other hydrazides and hydrazines condense with the carbonyl group of carboxylic acids, amides, peptides, acid anhydrides or lactones. On the contrary, the few reactions that have been recorded between aromatic hydrazines and compound containing

such groupings have led to a product still containing the carbonyl group, Hippuric acid for example reacts with phenyl - hydrazine to form $C_6 H_5.NH. CH_2 CO. NH. NH.C_6H_5$ (Ohta, 1946). Methylesters of carboxylic acids form hydrazides (sec.10.4/2). Therefore salicyloyl hydrazide should not react with the amine or histidine complexes with NEM if they are formed in tissue sections.

However, amine groups in tissue sections gave a <u>weak</u> green fluorescent NEM -zinc salicylhydrazone complex, whose fluorescence quickly disappeared soon after exposing dry, unmounted sections to ultra-violet light, or after dehydrating and mounting sections in DPX.

Solutions of NEM (pH 7.4) lose their histochemical reactivity after about 2 days, which is probably due to their decomposition to VI (Gregory, 1955). NEM is stable at a lower pH but then the reaction with thiol does not go to completion and its rate is considerably reduced (Alexander, 1958; Roberts and Rouser, 1958). The pH of 7.4 used in histochemical experiments is a good compromise; at this pH the rate of combination with thiol groups is much faster than the decomposition of N (Gregory, 1955).

When tissue sections were treated with NEM dissolved in distilled water, the salicyloyl hydrazide - zinc reaction was negative. It is thought that in the absence of the phosphate buffer salts, only the precursors of the adducts obtained in the buffer solutions are formed (Friedman, 1952).

It is therefore essential to use fresh phosphate buffered solutions of NEM at pH 7.4 for successful histochemical experiments.

Benesch etal, (1956) found that potassium hydroxide in anhydrous isopropanol gives a red visible and fluorescent colour with NEM - thiol adducts on chromatograms provided they are less than 2 days old. No fluorescent colouration could be observed in freshly prepared tissue sections treated with NEM. Apparently this reaction only works for compounds in which thiols are the only functional groups (Benesch etal, 1956).

4. The sulphuryl chloride method

Sections which have been/"drastically" methylated with hot methanolic hydrochloric acid can be sulphated with sulphuryl chloride vapour. The resulting basophilia towards auramine O or acridine yellow is due to O - sulphate groups. The colour of the fluorescence is often ~~=~~ anomalous. The theory underlining this method is discussed in sec. 13.5.

An application of thismethod is described in sec. 12.7/7.

5. Confirmation of thiol groups

The validity and specificity of histochemical reactions for thiol groups can be confirmed by blocking them with iodoacetate or iodoacetamide at alkaline pH levels. The following irrevesible reaction takes place (Kapkine, 1933; Dickens, 1933).

Tissue sections were treated with fresh 0.1M solutions of iodoacetic acid, whose pH had been adjusted to 8.0 with sodium hydroxide for 20 - 24 hours at 37° as recommended by Barrnett and Seligman (1954).

The efficiency of the blocking reaction was tested with the DNFB reagent at pH 5.2 (sec. 6.3) for thiols on sections of skin and hair which had a neglible nitrosonaphthol reaction for phenols (sec. 6.2). There was a reaction before, but not after, the iodoacetate treatment thus showing that the blocking was complete and irreversible.

Experimental Sec. 4. Thiol Groups

1. N - ethyl maleimide - salicyloyl hydrazide - zinc method (f) Method

1. Take dewaxed sections down to water. A perspex ring surrounding the section is fixed to the slide with paraffin wax.

2. Cover sections with fresh 1.25% solution of N - ethyl maleimide in M/10 phosphate buffer of pH 7.4. After covering the perspex cell with a cover-slip, the sections are incubated for 4 hours at 37°.

3. Wash briefly in water, then in 1% acetic acid for 2 mins., and finally in running water for 2 mins.

4 - 7. As stages (4) to (7) in exp. sec. 12.14.

8. Immerse in 1% zinc acetate for 5 - 10 mins.

9. Rinse in water, take through the alcohols, clear in xylene and mounted in Fluormount.

Result

Areas containing thiol groups exhibit a green or greenish blue fluorescence. If stage (8) is omitted, no fluorescence is produced.

2. The sulphuryl chloride method

Method

(1) Take sections to methanol.

(2) Treat with 1% hydrochloric acid in methanol for 18 hours at 90°.

(3) Confirm that the fluorescence basophilia towards acridine yellow or auramin O (stage 6) and PA - SH reactions (exp. sec. 12.14) are abolished.

(4) Rinse sections in methanol and ethanol and dry at 60° for 5 - 10 mins.

(5) Expose dry section to sulphuryl chloride vapour at 25° - 37° for 20 mins.

(6) Stain in 0.01% acridine yellow or auramine O dissolved in 30% alcohol for 20 mins.

(7) Rinse in 30% alcohol, dry in air, and examine section for fluorescence as a dry amount.

Result

Sites exhibiting a blue fluorescence are considered to contain thiol groups.

Section 5. Disulphides

1. Introduction

There are two types of histochemical techniques available for demonstrating disulphide bonds. In both methods tissue thiol groups are blocked with iodoacetate (sec. 4.4) beforehand. The disulphide link can then either be oxidised to strongly basophilic sulphinic acids, or they can be reduced to thiols demonstrable by one of the methods described in the previous section. The first technique is particularly suitable for fluorescence histochemistry, but the second has so far given indeterminate results.

2. The peracetic acid - methylation - coriphosphine method.

(1) Introduction.

This method depends on the selective oxidation of cystine (and cysteine) to cysteic acids by performic or peracetic acids. The oxidation product has been demonstrated in visible light histochemistry with a hydrazide or with Schiff's reagent (Pearse, 1951) or with alcian blue (Adams and Sloper, 1955, 1956).

For fluorescence histochemistry, use has been made of the fact that the oxidation product, unlike all other tissue basophils, completely resists methylation and gives a stable, intense red fluorescence after staining with dilute solutions of coriphosphine. Fig. 15 shows the appearance of the skin and hair shafts after the oxidation-methylation - stain-

ing sequence.

(2) <u>The specificity of the oxidation reaction with peracetic acid</u>.

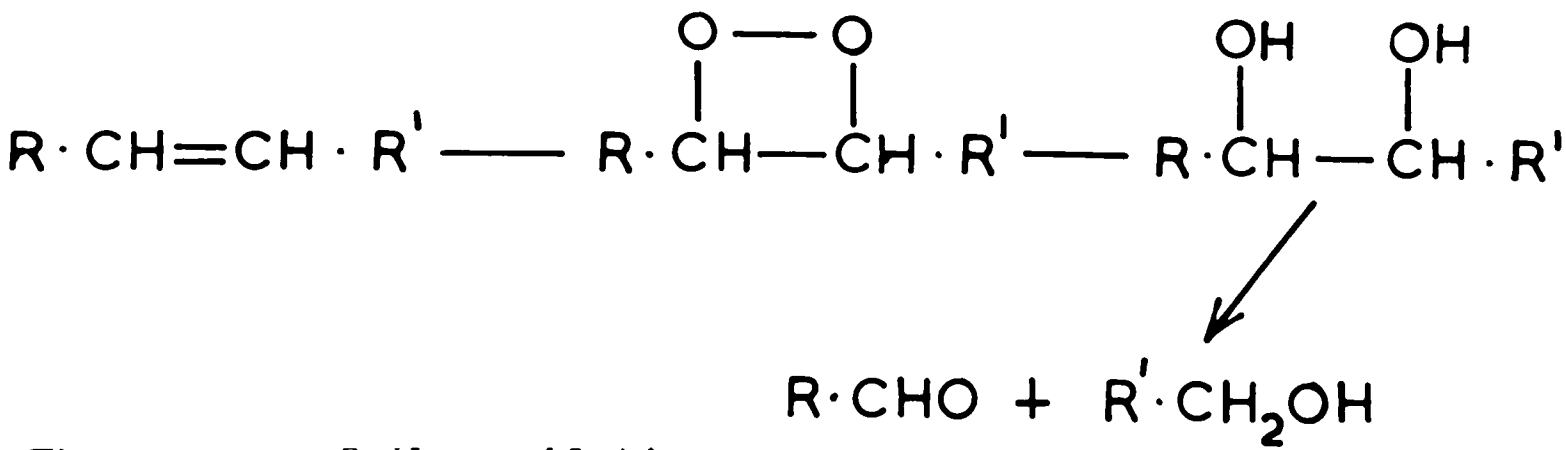
Performic and peracetic acids can oxidise the compounds and groups enumerated below. It is necessary to consider their ultimate fate before a specific technique can be devised for visualising the oxidation product originating from disulphide bonds in tissue section.

i. <u>Indoles</u>

Indoles, including tryptophane, are oxidised to numerous complex products. Hydroxytryptophane and β - oxyindolyalanine are formed from tryptophane itself (Witkop, 1946).

ii. <u>Unsaturated lipids and compounds containing olefinic bonds</u>.

Unsaturated lipids are believed to be attacked in two stages. In the first reaction, peroxides are formed across the olefinic bond. The second phase is a cleavage of the peroxide to a hydroxyketo - (Deuel, 1951) or a dihydroxy compound (Boeseken, 1926; Boeseken and Belifante, 1926) which subsequently break down to an aldehyde and an ω- hydroxy compound.



The course of the oxidation reactionof lipids in tissues

is still a matter of debate (Pearse, 1960), but the one important fact which does emerge is that their final reaction products are not basophilic.

iii. Hydroxyl and phenolic groups

The phenolic and hydroxyaminoacid constituents of proteins are partly attacked (Toennies and Homiller, 1942). Phenols can themselves be converted to muconic acids (Wacek, 1950) in high yeilds in the presence of metal salts, but this is unlikely to take place in the oxidation of tissue sections. Mucopolysaccharides may be oxidised similarly to complex products including uronic acids (Skause and Sunblad, 1942). If any of these acid products are formed during the histochemical oxidation, they would be methylated during the second stage of the devised experimental technique and would not interfere with the specificity of the oxidation for disulphide bonds.

iv. Thio compounds

Methionine is oxidised to a sulphone (Hirs, 1956) but cysteine is capable of oxidation right through to the sulphonic acid (cysteic acid) stage (Shinihara, 1932). Cystine in wool is oxidised chiefly to cysteic acid (Alexander etal, 1950).

The reaction could also go through a thiosulphonic acid ester which would decompose into a sulphinic acid (Lavine, 1936).

$$R \cdot SH \longrightarrow R \cdot SOH \longrightarrow R \cdot SO_2H \longrightarrow R \cdot SO_3H$$

Pearse (1951) carried out in vitro tests by a modified

Coujard (1943) technique to establish the nature of the final reaction products in oxidised tissue section. The formation of sulphinic acids seem to fit the observed facts best and would agree with Cecil's (1950) conclusion that sulphinic acids are the most stable intermediate oxidation products from cysteine and cystine. However, sulphonic acids are certainly formed in the oxidation of wool fractions (Weston, 1955). Lillie and Bangle (1954) disagreed with the cystine oxidation theory and suggested that an unsaturated complex lipid was responsible for the reactions of the hair cortex. Lillie (1952) has pointed out that the lipid pigment ceriod reacts with Schiff's reagent after oxidation with performic acid even in tissues which have been extracted in boiling chloroform and methanol.

The sulphinic acid theory is supported by the high intensity of the red fluorescence emitted by the keratin sites in oxidised tissue sections after they had been stained in dilute solutions of coriphosphine. This suggests that the engendered basophil is not a sulphonic acid of high electron density of the type found in acid mucopolysaccharides. Such groups tend to <u>quench</u> the fluorescence ofcoriphosphine. Sulphinic acids contain one less S = O group and therefore their overall net electron density should be less. Presumably the electron density is sufficiently high for it to attract the cationic centres of dyestufts, but is low enough for it not to distort the π- electron symmetry and hence potential fluorescence

of the absorbed dye. The structural and chemical similarity between sulphinic acids and carboxylic acids supports this hypothesis (Balfe etal, 1943).

Although solid derivatives of the type $R.SO_2H.H_2N.NH.Ph$ have been prepared by mixing solutions of sulphinic acids and phenylhydrazine at room temperature (Panizzi and Nicholaus, 1950; Bredereck and Bäder, 1954), naphthoic acid hydrazide gave only a weak reaction with sulphinic acids in a Coujard-type experiment (Pearse, 1951) in contrast to its strong reaction with oxidised tissue sections (Pearse, 1951; Bonomi, 1951). On theother hand, salicyloyl hydrazide did not give a fluorescent complex with oxidised tissue sections either before or after post-treatment with solutions of a zinc salt. This last experiment only proves that the engendered basophils contain no aldehyde groups and concurs with Findlay's (1955) experiments in vitro. However, it does not exclude the possibility that a non-fluorescent sulphinic acid - hydrazide complex (vide supra) is formed.

The unique property of the engendered basophils is their resistance to methylation (Lillie etal, 1954) which is difficult to reconcile with the formation of sulphinic acids. The fact that the methyl esters of sulphinic acids can be prepared in high yields in vitro by the action of lead tetra - acetate on the appropriate thiols or disulphides dissolved in methanol and chloroform indicates that sulphinic acids are readily methylated (Field etal, 1961).

$$R \cdot SS \cdot R + 3Pb(OAc)_4 + 4\,CH_3OH \longrightarrow 2\,R \cdot SO \cdot OCH_3 + 3\,Pb(OAc)_2 + 4\,AcOH + 2\,AcOCH_3$$

The correct explanation of the mechanism of the oxidation of sites containing sulphur compounds in tissue sections is therefore still unsettled.

(3) Reduction of disulphide groups

Danielli(1950) suggested that disulphide bonds could be demonstrated in visible light histochemistry by the following sequence:

(a) Block the preexisting thiol groups in tissue sections permanently and irreversibly (with iodoacetate).

(b) Reduce the disulphide bonds to thiols.

(c) Demonstrate the newly engendered thiols by one of the methods available for that group.

Barrnett and Seligman (1954) tested a large number of reagents for reducing disulphide bonds for this purpose, of which sodium thioglycollate was the best.

In my experience, even thioglycollates do not cleave all the disulphide bonds. Fig. 16 shows a section of mouse skin and hair (fixed in neutral formalin) which has been covered with celloidin, reduced with thioglycollate for 4 hours at 37°, incubated with alkaline iodoacetate for 40 hours at 37°, oxidised with peracetic acid, methylated for 20 hours (sec.11.4) and stained in dilute coriphosphine. This procedure should

Fig. 15. Mouse skin & hair. Fixed in formalin.

Methanolic thionyl chloride, 4 hrs. : peracetic acid, 4 hrs. : 0.01% coriphosphine, pH 5. Water mount, X 180.

The red fluorescence of the oxidised disulphide (and thiol) groups is much brighter than it appears in this photomicrograph.

Fig. 16. Mouse skin and hairs. Fixed in formalin.

Thioglycollate reduction : iodoacetic acid, pH 8.0, : Peracetic acid, 4 hrs. : methanolic thionyl chloride, 20 hrs. : 0.01% coriphosphine. Water mount, X 180.

Some of the hair shafts remaining in the skin emit a light green or yellow fluorescence. Most of the detached hair fragments fluoresce a bright red colour unchanged from that shown in Fig. 15.

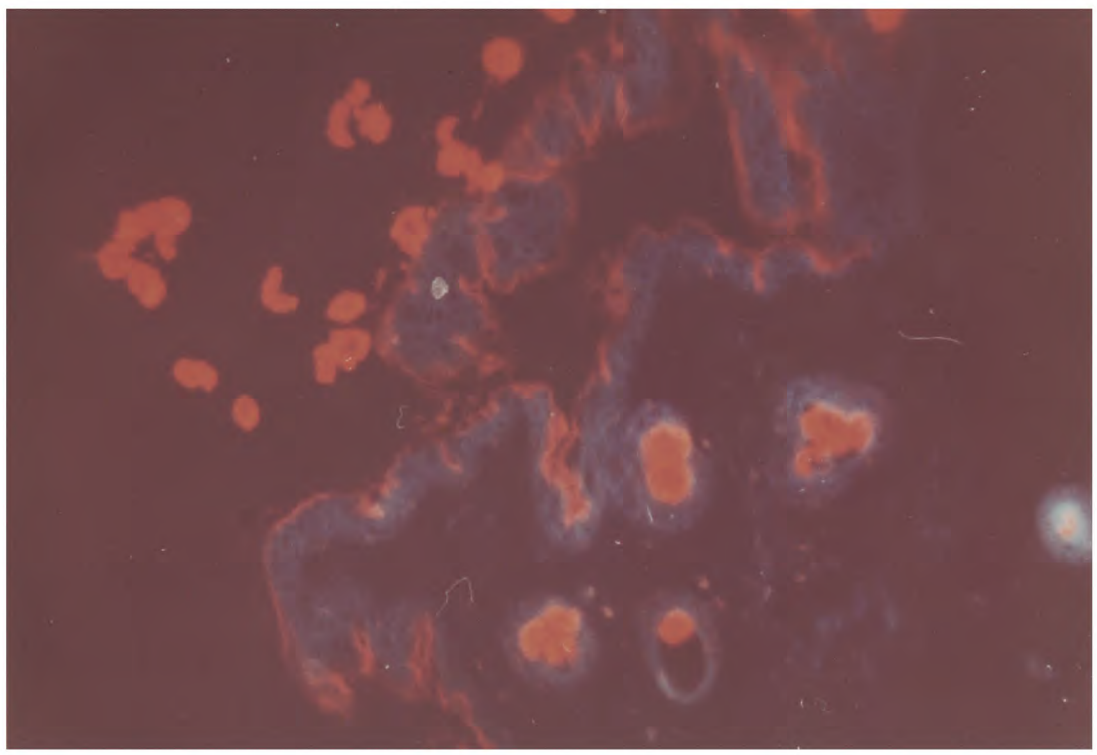


Fig. 15

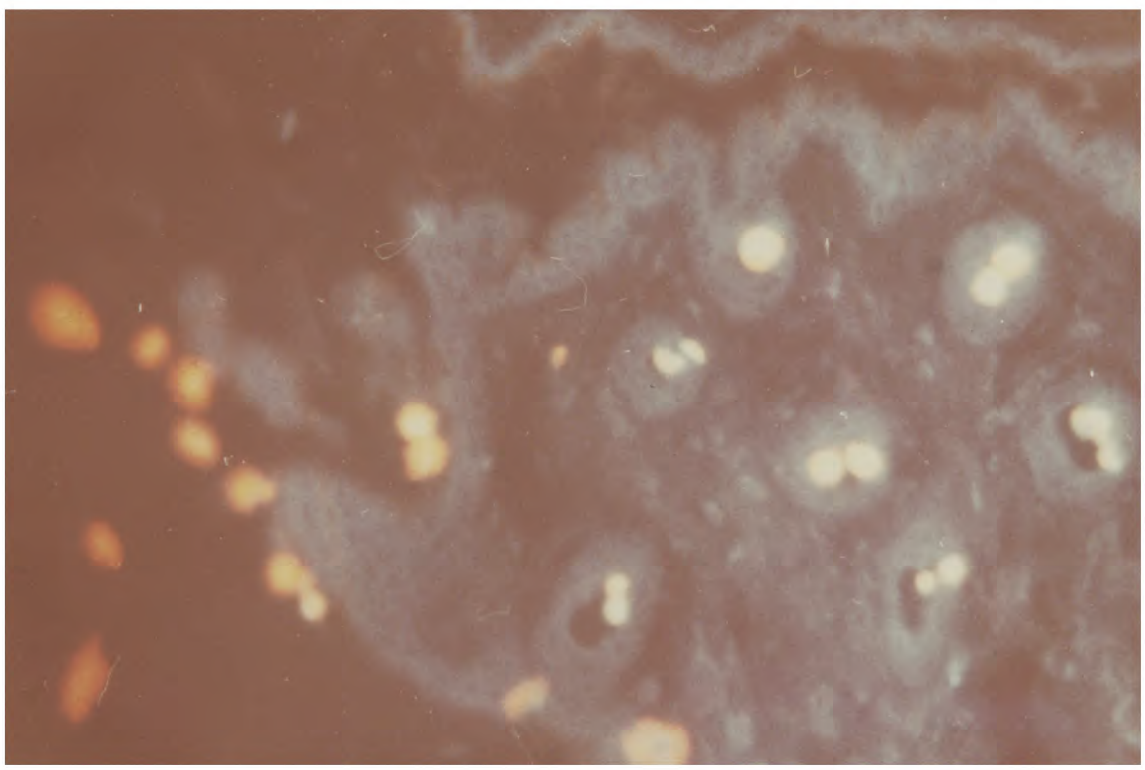


Fig.16

lead to the complete blocking of both the thiol and disulphide groups and consequently there should be no fluorescent sites in the section. In practice, the keratinised hairs still exhibit an intense red fluorescence, but there are small changes in the intensity and colour (to green or yellow) of the fluorescence in the hair shafts and epidermal keratin. As the fluorescence reaction is far from being abolished, it can only be attributed to the imperfect reduction of the disulphide groups. The iodoacetate blocking of thiol groups is less likely to be at fault (sec. 4.4).

Until better methods for reducing disulphide bonds have been found, techniques based upon them cannot be used satisfactorily in histochemistry. This also means that there are no equivocal confirmatory tests for disulphide groups at the present time.

Experimental Section 5. Disulphides

1. The peracetic - methylation - coriphosphone method (f)

Preparation of peracetic acid (after Greenspan, 1946).

9.5 ml. glacial acetic acid and 0.25 ml concentrated sulphuric acid are added to 30 ml 30% hydrogen peroxide. The mixture is "aged" for 1 - 3 days before use, and stored at 0 - 5°.

Method

(1) Take dewaxed sections down to water.

(2) Block thiols with iodoacetate (sec. 4.4).

(3) Wash in 20% acetic acid for 2 mins.

(4) Oxidise in peracetic acid reagent for 30 - 120 mins.

(5) Wash in 20% acetic acid.

(6) Wash in two changes of water, 2 mins. each.

(7) Dehydrate through the alcohols.

(8) Wash in methyl alcohol for 2 mins.

(9) Methylate in 2% thionyl chloride in methanol for 10 - 16 hours (overnight).

(10) Wash in methyl alcohol, and take down through the alcohols to water.

(11) Stain in 0.01 coriphosphine (pH 4 - 7) for 10 - 20 mins

(12) Wash briefly in two changes of water.

(13) Examine for fluorescence as a water mount.

Result

Sites containing disulphide groups exhibit an intense red fluorescence.

2. Reduction of disulphide groups to thiols (after Barrnett and Seligman, 1954).

Dewaxed, dehydrated and dried sections are dipped into a 0.5% solution of celloidin in 50:50 ether - alcohol and allowed to dry in air. The covered sections are incubated for 4 hours at 37° in 4.5% thioglycollic acid (freshly made) whose pH has been adjusted to 8.0 with dilute sodium hydroxide. The sections are washed afterwards in tap-water, rinsed briefly in 1% acetic acid and washed again in water.

Disulphide bonds are cleaved imperfectly by this method; the peracetic - coriphosphine method gives positive results afterwards.

Section 6 Phenols

1. Introduction

Phenolic groups in tissues, in particular those of tyrosine and tyramine residues, can be detected either indirectly or directly. The usual techniques used in histochemistry for this group are indirect and depend on the activating effect of the phenol group on the ortho - and meta - positions of its accompanying aromatic nucleus. The hydrogen atoms at these nuclear centres are particularly sensitive to displacement and substition by nitroso groups which, if attached to a naphthol molecule, results in a fluorescent product. The characteristic feature of this type of reaction is that it can be prevented by iodinating the aromatic nucleus beforehand.

This serves as a confirmatory reaction.

There are no well established direct methods for the histochemical demonstration of phenolic groups. Danielli (1950) suggested that their reaction with alkaline solutions of dinitrofluorobenzene (DNFB) could be used as a basis, provided the similarly reactive amines and thiols were suitably blocked. No unequivocal methods for doing this have been published. It is now suggested that if the DNFB reaction is carried out at a pH below 5.5, only the thiol and phenol groups will react. If the former are blocked with iodoacetate, only the latter reacts. The resulting conjugate can be visualised for visible light histochemistry by reducing, diazotising and suitably coupling the nitro groups. Although the reduced nitro

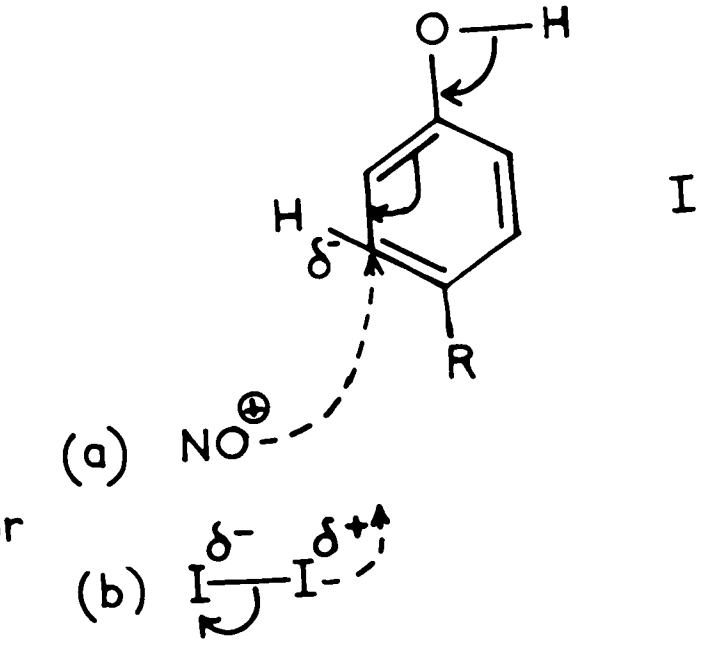
groups should, in principle, be demonstrable by one of the fluorochemical methods devised for amines, no fluorescence reaction ever took place in practice.

2. The Nitrosonaphthol reaction

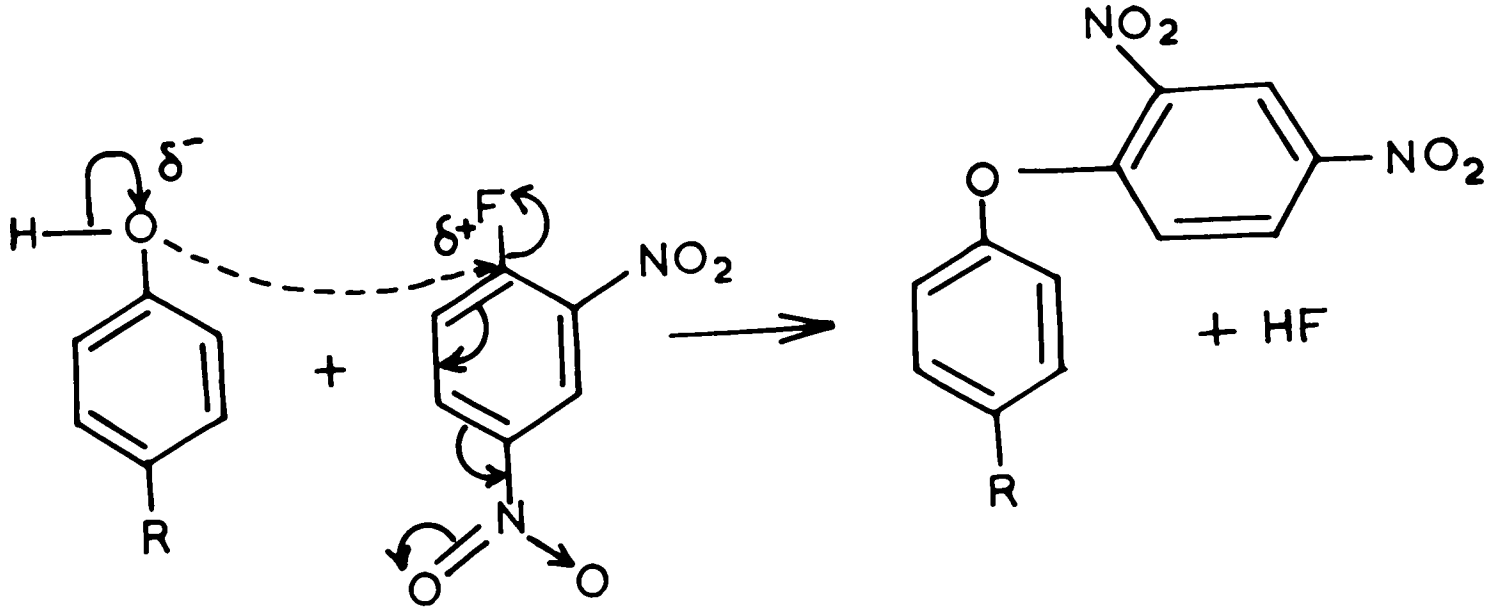
(1) Introduction

The reaction of nitroso groups with phenols forms the basis of the long-established Millon's (1849) reaction which has been finally adapted for visible light histochemistry by Baker (1956). According to Gibbs (1927), Millon's reaction proceedes in two stages. First an o- or m-nitrosophenol (I) is formed, followed by the formation of a coloured mercury chelate incorporating both the phenol and nitroso groups. Another comparable reaction is Morel and Sisley's (1927) reaction. Lillie (1957) adapted this reaction for histochemistry by allowing excess nitrous acid to convert the substituted nitroso group to a diazonium nitrate which can then couple with amines in alkaline solution to form coloured ccmplexes.

The colorimetric assay for tyrosine which is based upon its reaction with 1-nitrosonaphthol in the presence of nitric acid and a trace of nitrite probably depends upon a similar initial mechanism (Udenfriend and Cooper,1952). The composition of the final products of nitrosonaphthol and tyrosine or tyramine are, however, unknown. Waalkes and Udenfriend(1957) found the product with tyrosine was fluorescent in solution.



I



II

The excitation maxium occurred at 460 mμ which resulted in an uncorrected fluorescence maximum at 366 mμ was about half that at 460 mμ . The nitrosonaphthol reaction was therefore investigated as a possible technique for fluorescence histochemistry.

(2) The histochemical method

Tissue sections were treated for 30 minutes at 60° with an alcoholic solution of 1 - nitrosonaphthol freshly mixed with an equal proportion of dilute nitric acid containing a little sodium nitrite. At the end ofthis time, excess nitrosonaphthol was extracted by washing the sections in ethylene dichloride. This would also extract any interfering acidic metabolic products of tyrosine that have survived the fixation and embedding processes. These products would include hydroxyphenylacetic acid, hydroxyphenypyruvic acid and homogentesic acid (Udenfriend, 1962).

After the rinse in ethylene dichloride the sections were washed in 97% alcohol and examined as dry mounts. Fig.17 shows the greenish-yellow fluorescence of the tyrosine conjugate in the chromidial areas of the mouse pancreas. The fluorescence fades completely after about 10 minutes exposure to ultra-violet light.

The intensity of the fluorescent colours depicted in photomicrographs is inevitably less than initially obtained in freshly prepared sections. The fluorescence faded even more

Fig. 17. Mouse salivary gland. Fixed in neutral calcium-formalin.

Nitrosanaphthol + nitrous acid, 60°, 30 mins. Dry mount, X 180.

The chromidial regions containing phenolic groups emit an unstable yellowish-green fluoresence.

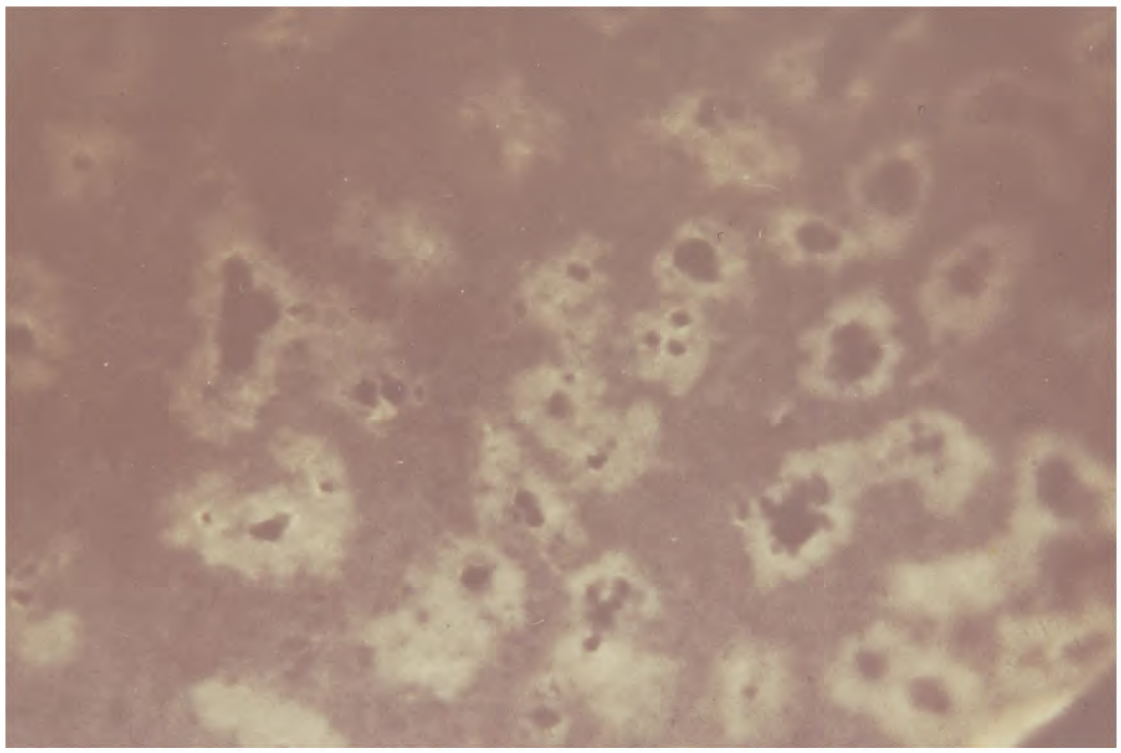


Fig. 17

rapidly if the sections were examined in water, dilute nitric acid or in Fluoremount after alcoholic dehydration. Despite this technical difficulty, the sensitivity of the reaction is much greater than that of Baker's (1956) variation of Millon's reaction for visible light histochemistry.

(3) Confirmation of the histochemical reaction.

The fluorescence reaction in tissue sections can be prevented by iodinating the phenolic nucleus (I) with the dilute alkaline solution of iodine originally used by Landing and Hall (1956) for blocking the reaction of tyrosine residues with Millon's reagent. Unfortunately, the specificity of this iodinating reagent is questionable. Histidine derivatives which might conceivably have an activated nuclear centre capable of reacting with the nitrosonaphthol reagent, is also iodinated, although according to Ramachandra (1956), its rate of iodination is much slower than that of tyrosine.

Alcoholic solutions of iodine should be more specific for tyrosine. Richards and Speakman (1953) found that the tyrosine side chains in Keratin were completely iodinated after 72 hours at 25°. Histidine was affected to a neglible degree, and tryptophane and proline not at all. However, in my hands, iodine did not dissolve in alcohol to the strength (0.78N) recommended by Richards and Speakman (1956).

A much weaker solution did block the nitrosonaphthol reaction, which therefore seems to be specific for the phenol groups of tyrosine.

3. The DNFB reaction

Thiols, but not protonated amines, react with 2,4 - dinitro - 1 - fluorobenzene (DNFB) in vitro at a pH below 5.5 (Zahn and Traumann, 1954) provided the temperature is not too high. Above this pH, other groups including phenols and amines react, the reaction being optimal at about pH 9 in the presence of bicarbonate buffer (Sanger, 1945, 1950; Porter 1950).

Only one previous instance of the reaction of DNFB with phenols below pH 5.5 has been recorded. Zuber, Traumann and Zahn (1955) found that DNFB reacted with thiol, phenol and amine groups at 60° at pH 5.2, but at 2° only thiol groups reacted. It has now been found that sites in tissue sections known to contain such groups will also take up DNFB below this pH. Any interfering thiol groups can be blocked beforehand with iodoacetate (sec. 4.4). In tissue sections which contain high local concentrations of thiol groups (e.g. skin and hair) the DNFB reaction at pH 5.3 is completely blocked by prior treatment with iodoacetate.

The nitro groups of the resulting conjugate (II) can be reduced with sodium dithionate to amines, which can be diazotised and coupled with H - acid to form a visible purple - red product. Unfortunately, neither the reduced nitro groups nor the amine groups of the tissue proteins would give a fluorescent product with the Konig - Sassi or Solochrome Black - aluminium techniques (exp. secs. 3 • 1 and 3.8).

Presumably not all the nitro groups are reduced; those that are left would quench the potential fluorescence of compounds absorbed in their neighbourhood. The DNFB treated sections appeared completely colourless after reduction in solutions of sodium dithionate, but as the DNFB - phenol conjugates are themselves colourless in vitro (Sanger, 1945), the absence of a yellow colour cannot be used as a guide to the complete reduction of the phenol conjugates.

It was thought that the alkaline solution of DNFB normally used in histochemistry (Burstone, 1955) could also be used for the specific demonstration of phenolic groups if the amine and thiol groups were suitably blocked as originally suggested by Danielli (1950). This possibility was tested by blocking the amine groups with a Konig-Sassi reaction and the thiols with iodoacetate before treating the sections with DNFB dissolved in alcohol buff red with bicarbonate. The sections were visibly stained yellow by this treatment, although the intensity of staining was much less than that of control sections. As phenol conjugates are colourless, the yellow colouration indicates that DNFB can react with other groupings besides amines, thiols and phenols at an alkaline pH. Pearse (1960) has pointed out that this possibility should be considered. There are three additional centres which DNFB can attack at alkaline pH levels; these are the aliph tic hydroxyl groups of proteins and polypeptides (Freudenberg, 1958), heterocyclic base nitrogen atoms (Kronhnke and Leister,

1958) and derivatives of imadazoles whose ring is ruptured (Zahn and Pfannmuller, 1958). The reaction with aliphatic hydroxyl groups is unlikely to occur in the histochemical reaction; Triethylamine is required as a catalyst for reactions <u>in vitro</u> (Whalley, 1950: Zahn and Gerstner, 1955; Zahn and Zürn, 1958) where bicarbonates specifically failed. Because of the complexity in devising various blocking reactions for the last two groups, an alkaline solution of DNFB is not a suitable reagent for the selective demonstration of phenols.

4. <u>The attempted blocking of phenol groups</u>

As the acidic DNFB method is the only direct one for the demonstration of phenols, it is valuable in principle, despite the fact that it has not been perfected for fluorescence histochemistry. However, no confirmatory tes s for this reaction have yet been found. The most obvious test would be to show that the DNFB reactions is negative after methylating the phenolic group. Unfortunately, all the reagents that have been tried for doing this, including methanolic thionyl chloride , had no effect on the DNFB reaction in tissue sections. This is probably due to a unique resistance of the phenol groups in tyrosine towards methylation. Even the efficient methylating reagent, alkaline methyl sulphate, does not methylate the phenolic groups of tyrosine <u>in vitro</u> (Corti, 1949), despite the fact that multivalent phenols and alcohols are readily methylated under comparable conditions (Brendereck, Jonnig and Ran, 1953).

5. Catechols

In fresh frozen tissues, catechols may also be present in substantial concentrations in addition to the tyrosine residues of proteins. They should give positive reactions by the previous two methods, but they also have two additional specific reactions; These are the fluorescent formalin method of Bränkö and the periodic acid - salicyloylhydrazide reaction, which are discussed in sec. 12.3/2.

Experimental Section 6

Phenols

1. The nitrosonaphthol method (f)

Method

(1) Take dewaxed sections to 50% alcohol.

(2) Immerse in a freshly prepared mixture of equal volumes of 0.05% 1 - nitrosonaphthol in 95% alcohol and 1% sodium nitrite in 10% nitric acid for 20 - 30 mins at 60°.

(3) Wash in ethylene dichloride for 1 min. to remove excess nitrosonaphthol.

(4) Rinse twice in 97% alcohol and allow to dry in air.

(5) Examine for fluorescence as a dry mount, and if necessary, photograph at once.

RESULT

A bright green-yellow fluorescence is seen initially in sites containing phenolic groups, but it fades after about 10 minutes exposure to ultra-violet light.

2. The DNFB Method (v)

Method

(1) Take dewaxed sections down to the water.

(2) Treat with 2% solution of iodoacetic acid (whose pH has been adjusted to 8.0 with dilute sodium hydroxide) for 24 hours at 37°. This blocks thiol groups.

(3) Wash with water and take to 95% alcohol.

(4) Treat with a fresh 1% solution of dinitrofluoro-benzene (DNFB) in 95% alcohol saturated with sodium bicarbonate, whose pH has been adjusted to the range 5.0 - 5.5 with the

minimal amount of hydrochloric acid, for 12 - 16 hours (overnight).

(5) Wash twice in alcohol, and take down to water.

(6) Reduce in a fresh 5% solution of sodium dithionate for 2 - 4 hours at 37°.

(7) Wash in water.

(8) - (11) As stages (6) - (9) in exp. sec. 12.12.

RESULT

An intense reddish-purple colour is produced in sites containing phenolic groups.

Section 7 Carboxyl groups.

1. Introduction.

This section will be confined to the detection of the carboxyl groups in proteins; the uronic acid groups of acid mucopolysaccharides will not be included (see sec. 10). The usual histochemical test for carboxyl groups is to show that, once nucleic acids and acid mucopolysaccharides have been excluded, they take up basic dyes only at a pH above 4.0 (Spicer 1960). This method is unsound theoretically and has been shown to be so in experiments using fluorescent dyes in conjunction with extraction techniques for RNA (sec. 9.7/2).

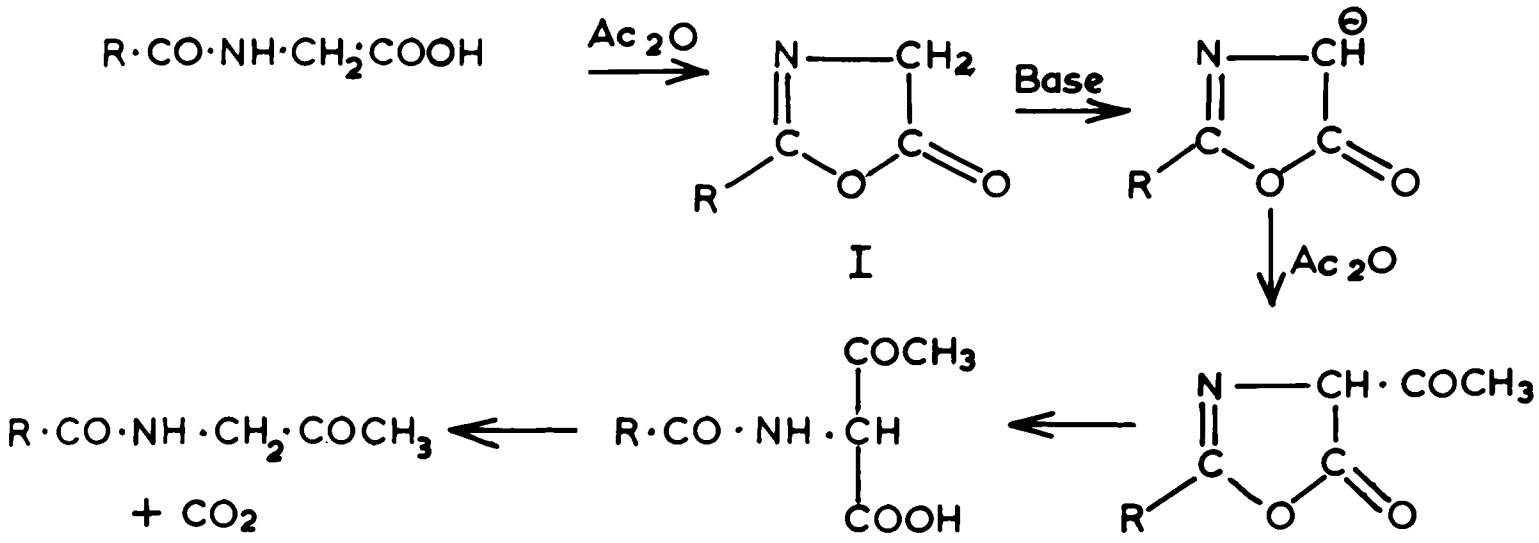
C-terminal carboxyl groups can be converted to methyl ketones (with hot acetic anhydride and pyridine) which are demonstrable as the intensely fluorescent zinc complexes of their salicyloyl hydrazones. The remaining side-chain carboxyl groups can be demonstrated by their basophilia as suggested in the previous paragraph. If the side-chain carboxyls are present in a low concentration, the colour and intensity of the fluorescence of sections stained at pH 2-3 with coriphosphine will differ from that of sections stained at a pH greater than 4. On the other hand, if the concentration of C-terminal carboxyl groups is small, the colour and intensity of the fluorescence in sections heated with acetic anhydride and pyridine will be the same after staining at both pH 5 and pH 2.

2. The acetic anhydride-pyridine reaction for C-terminal Carboxyl groups.

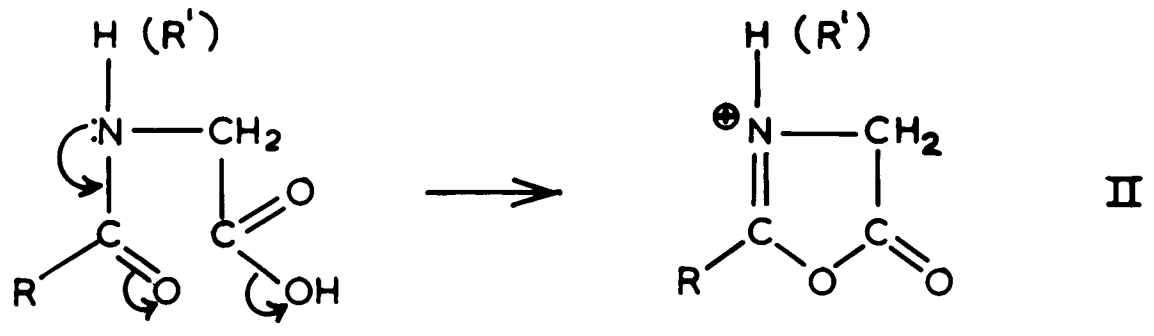
(1) Introduction. Barrnett and Seligman (1958) devised a method for the histochemical demonstration of C-terminal (α-acylamidc) carboxyl groups which was based upon their conversion to acylamido ketones by hot acetic anhydride in the presence of a suitable base such as pyridine. This reaction has been widely studied (Wood and du Vigneaud, 1945; Wiley, 1943) since Dakin and West's (1928 a, b) original discovery.

The mechanism generally accepted for this reaction involves an azlactone intermediate, I (Levene and Steiger, 1927, 1928; Attenburrow et al., 1948). Wiley (1950) later claimed that that substitution of N-CH_3 for N-H prevented the formation of an azlactone. Consequently he proposed two alternative mechanisms. The first involved decarboxylation due to the base, caused by the electrophilic nature of the α-amino group, and an attack of the carbanion thus formed with acetic anhydride. This hypothesis was challenged by Cornforth and Elliott (1950) and by Buchanan et al. (1957) who emphasised that the presence of a hydrogen atom on the α-carbon was more important than the hydrogen on the nitrogen atom. Cornforth and Elliott (1950) proposed the formation of a transitory oxazolium (azlactone) cation (II) as the intermediate. This is followed by acetylation on the α-carbon, ring opening and decarboxylation. In addition these authors showed that heating acetylglycine in pyridine did not cause decarboxylation as required by Wiley's

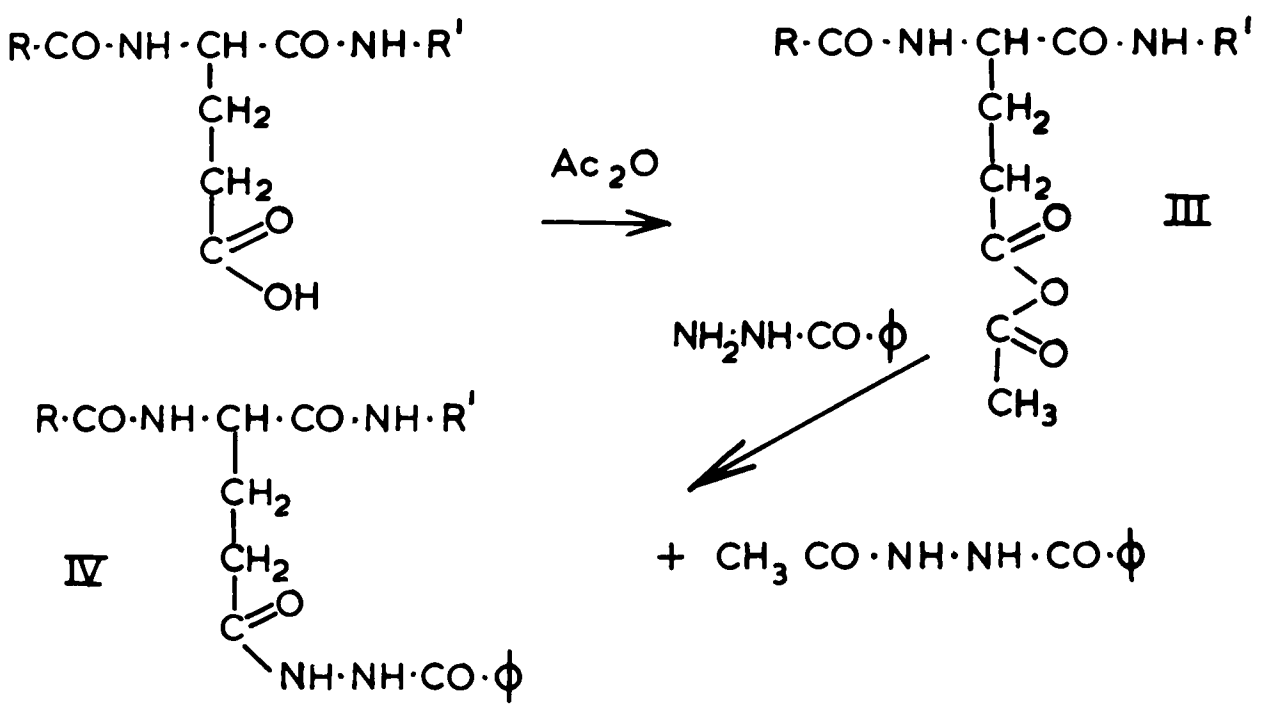
The Azlactone mechanism:



The Cornforth and Elliott mechanism:



The mixed Anhydride hypothesis:



(1950) mechanism. As a less likely alternative, Wiley(1950) suggested that the acylamino acid undergoes C-acylation as an active methylene compound which is a generalisation of the azlactone mechanism.

(2) The histochemical method. Barrnett and Seligman (1958) visualised the methyl ketones produced from the C-terminal carboxyl groups in tissue sections by condensing them with 2-hydroxy-3-naphthoic acid hydrazide and coupling the hydrazones so formed with a diazonium salt. A slightly different technique has now been devised for fluorescence histochemistry: the methyl ketones were condensed with salicyloyl hydrazide to form non-fluorescent hydrazones (Cp. the fluorescence of aldehydo-hydrazones, sec. 12.19) which were rendered fluorescent by treating them with a solution of a zinc salt. The experimental details are given in exp. sec. 7.1.

The particularly strong fluorescence reaction in the kidney of the guinea pig is illustrated in Fig. 18. It should be compared with Fig. 19 which shows a moderate reaction in the muscle of the gut taken from the same animal and the negligible reaction in the lamina propia and epithelium. An interpretation of these illustrative examples is discussed in sec. 7.3.

(3) Further investigations into the specificity of the reaction. Although Barrnett and Seligman (1958) assumed that the side-chain β- and γ-carboxyl groups of the glutamic and aspartic acid residues of proteins would not react, and that only the C- terminal carboxyl groups would give ketones, they

Fig. 18. Guinea pig gut. Fixed in glycol ether.

Acetic anhydride + pyridine, 60°, 1 hr. : salicyloyl hydrazide : zinc acetate. Fluormount, X 180.

The muscle exhibits a more intense blue fluorescence than is apparent in this photomicrograph. (Cp. Fig. 19).

Fig. 19. Guinea pig kidney. Fixed in glycol ether.

Acetic anhydride + pyridine, 60°, 1 hr.: salicyloyl hydrazide : zinc acetate. Fluormount, X 180.

The C-terminal carboxyl groups emit an intense blue fluorescence.

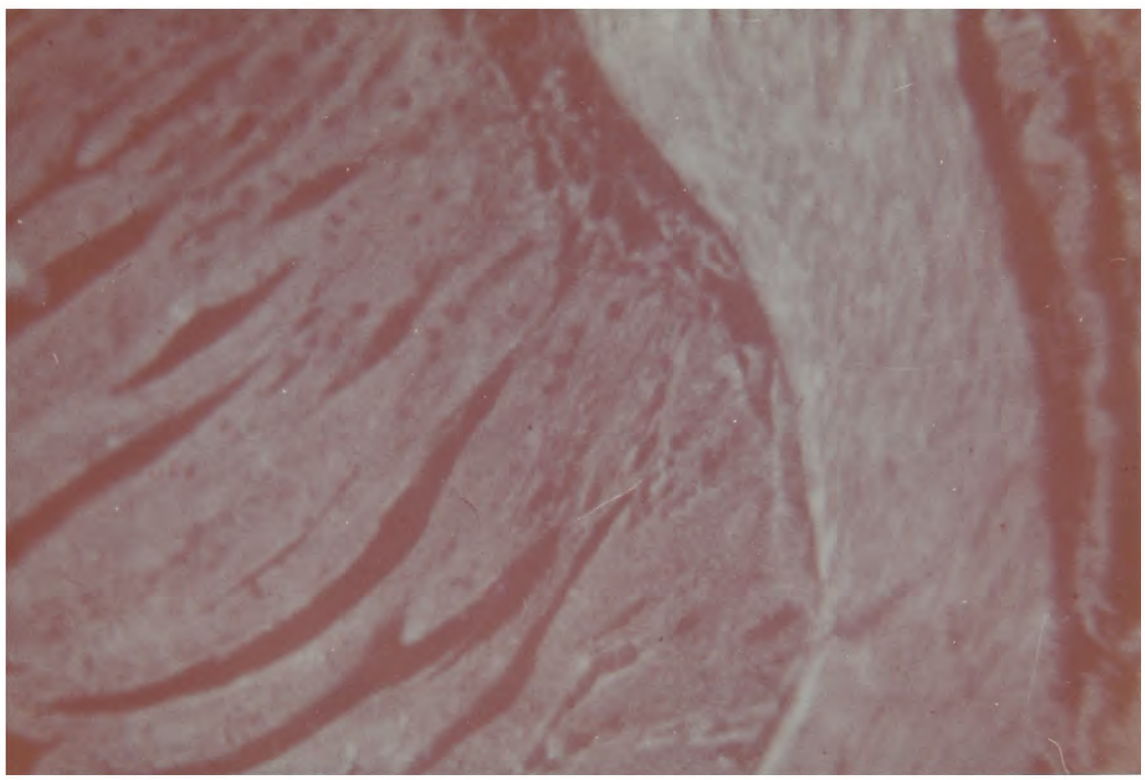


Fig. 19

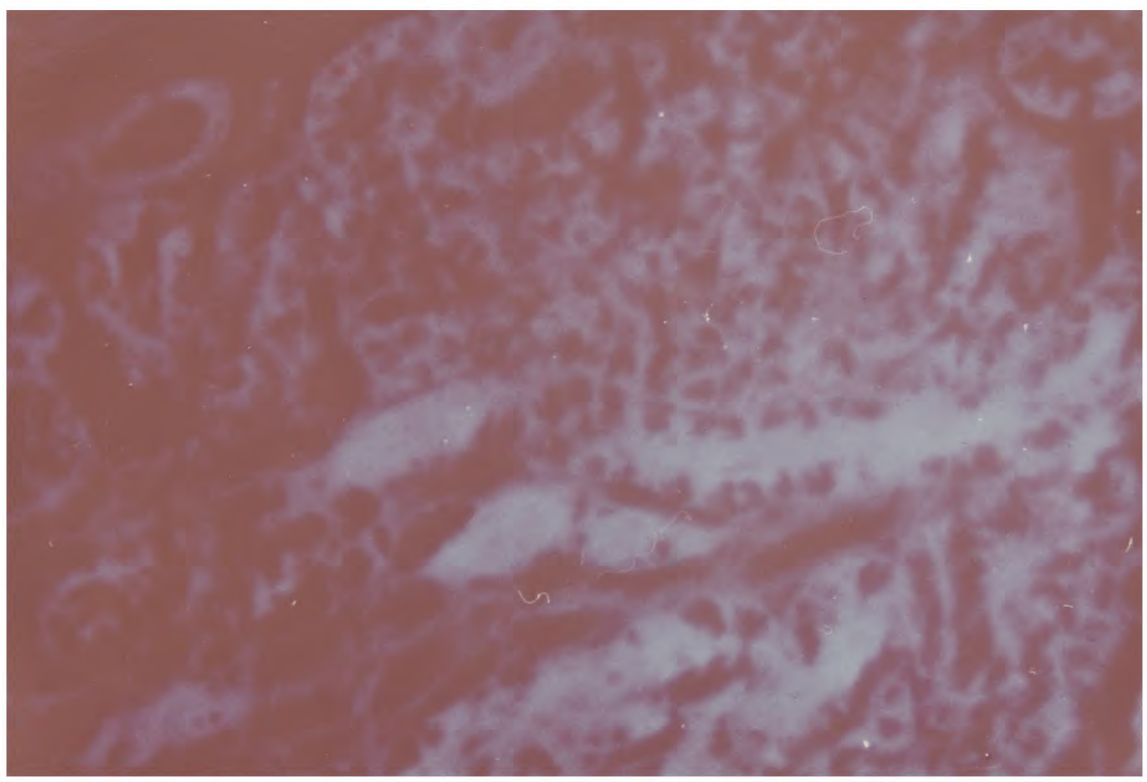


Fig 18

did not exclude the possibility that the former might yield reactive carbonyl groups in the presence of acetic anhydride and pyridine by some mechanism other than that proposed by Wiley (1950) for the latter. Karnovsky and Fasman (1960) suggested that non-ketonic side-chain carboxyls are converted to mixed anhydrides (III) by a reaction of the type (Vander Haar, 1929; Breslow and Hauser, 1939):

Anhydride of A + salt of B $\rightleftharpoons$ Salt of A + mixed anhydride of A and B.

In the comparable Perkin-Fittig reaction, the anhydride is essential, but the sodium salt of the carboxylic acid can be replaced by other bases such as pyridine (Kalnin, 1928).

Moreover, Karnovsky and Fasman (1960) claim that there are few C- terminal Carboxyl groups adjacent to "electrophilic nitrogen" atoms in most tissue proteins, and that the strong reaction obtained by Barrnett and Seligman (1958) cannot be due to those groups alone. They suggest that the strong reaction is more likely to be due to the formation of mixed anhydrides which can acetylate naphthoic acid hydrazide. The ultimate reaction produce (IV) would couple with the diazonium salt and form a product of a similar colour to that which would be produced if ketones were present.

As proof of their hypothesis, Karnovsky and Fasman (1960) suggested that the mixed anhydrides should be destroyed by a strong base (sodium hydroxide), with a consequent failure to form a dihydrazide (IV) and a negative colour reaction after

attempting to couple with a diazonium salt. Such was found to be the case in vitro. The methyl ketones derived from C-terminal carboxyls were considered to be inert towards alkali and would therefore be still capable of demonstration by the hydrazide -diazonium salt coupling sequence. That they were never able to obtain any positive colour reaction in any tissue after the strong base treatment was not regarded as vitiating their hypothesis. Instead they inferred that the negative reaction indicated a sparce concentration of C-terminal carboxyl groups in the proteins of average or high molecular weight which would be expected in tissue sections. This inference was considered especially relevant to the intense reaction obtained in hyaline cartilage (Karnovsky and Fasman, 1961) which they attributed to the presence of large, high molecular weight aggregates of chondromucoproteins, rather than to a high concentration of low molecular weight protein or polypeptide.

The following evidence will now be put forward against Karnovsky and Fasman's (1960) mixed anhydride theory, but which is in favour of Barnett and Seligman's (1958) earlier ketone theory:

(i) There is no reason a priori why C-terminal carboxyl groups should be scarce in all tissue proteins. The comparative analysis of the amino-acid constituents of commonly occurring proteins suggest that the opposite may be just as plausible (Edsall, 1943).

(ii) Ketones are certainly formed in high yields in some

tissues such as the Kidney (Fig. 18) after the acetic anhydride -pyridine treatment as can be shown by the intense fluorescence which is produced when their salicyloyl hydrazones are complexed with zinc ions. Camber (1954, 1957) showed that, in vitro, the salicyloyl hydrazones of simple ketones, unlike those of aldehydes, are non-fluorescent or are only weakly so, but they can be converted to an intensely fluorescent form by treating them with a solution of a zinc salt.

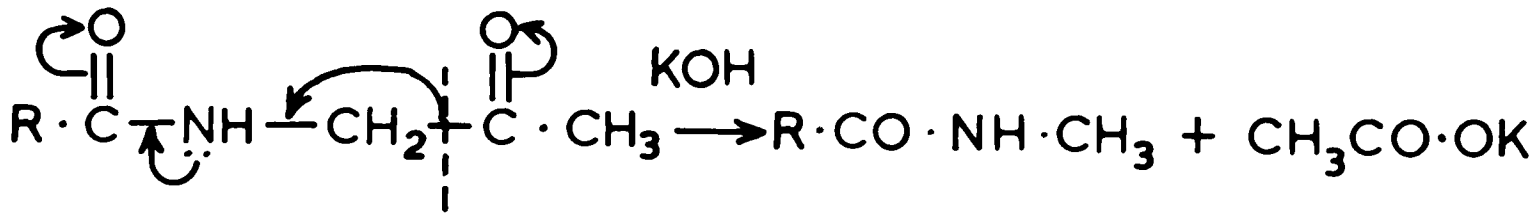
(iii) Acid anhydrides could also react with salicyloyl hydrazide, but as the structure of the resulting dihydrazide (IV) differs from that of the hydrazone (sec. 12.21), it is unlikely that it would have the same fluorescent properties. To test this point, tissue sections were acetylated in conditions similar to those which favour the formation ofmixed acid anhydrides in vitro (Clarke and Rahrs, 1929) before subjecting them to the salicyloyl hyrazide-zinc routine. This was done by incubating them in hot acetic anhydride containing a trace (0.1%) of phosphoric acid. Only a weak purple fluorescence could be elicited subsequently from sections of guinea pig kidney in contradistinction to the intense fluorescence of sections treated with acetic anhydride and pyridine or acetic anhydride alone. This means that if mixed anhydrides are formed, and there is no reason why they should not be, then the fluorescence of their zinc - salicyloyl hydrazones is negligible.

(iv) If the mixed anhydride theory were correct and anhydrides did form fluorescent zinc complexes with salicyloyl hydrazide, then the fluorescence reaction in tissue sections would be abolished by treating sections with amines or alcohols between the acetic anhydride and salicyloyl hydrazide stages. Experiments in the field of peptide synthesis suggest that mixed anhydrides react smoothly and efficiently with aniline dissolved in a hydrocarbon (overnight at 8^{o}) or with methyl alcohol in the presence of pyridine (Vaughan and Osato, 1951). Ketones, on the other hand, form derivatives with simple primary amines only with difficulty (Mannich and Hieronimus , 1942) and do not react at all with methyl alcohol.

The fluorescence of the salicyloyl hydrazide - zinc reaction was unaffected by leaving tissue sections in either 5% aniline dissolved in xylene or 5% pyridine in methanol for nine days at room temperature after the acetylation stage. This confirms the supposition that reactive acid anhydrides are not formed in tissue sections.

(v) The most important experimental fact in favour of the mixed anhydride theory is the abolition of the hydrazide - diazonium salt coupling reaction after treating the "acetylated" sections with sodium hydroxide. If this theory is correct, the original carboxyl groups should be regenerated by the hydrolysis of the anhydride. Therefore if the saponified sections are again subjected to the acetic anhydride - pyridine procedure, a positive hydrazide - coupling reaction should be obtained. However, no such fluorescent salicyloyl hydrazide

zinc reaction was produced in "acetylated" sections of guinea pig kidney that had been treated with 0.1N sodium hydroxide in 70% alcohol for 25 minutes. The aqueous N/10 solutions of caustic soda used by Karnovsky and Fasman (1960) was found to remove the sections from their glass slides. Karnovsky and Fasman (1960) contend that methyl ketones do not react with alkai, and therefore as the hydrazide reaction was abolished after saponification, the ketone theory cannot be correct. However, it is by no means certain that acetoamido ketones are inert towards alkalies. It is just as plausible that the engendered ketones can undergo hydrolytic cleavage by a mechanism similar to the "acid hydrolysis" of acetoacetic esters by strong alkali.



Alternat vely, the ketones may undergo a Knoevenagel - type condensation with active methylene groups in other protein side chains.

(vi) The fluorescent salicyloyl hydrazide - zinc reaction is abolished by deaminating tissues beforehand with the diazotisation - deazotisation sequence (exp. ser. 3.9) Karnovsky and Mann (1961) similarly found that the reaction was prevented by prior treatment with nitrous acid. This means that an NH -group, which must be capable of acetylation,

is essential for the reaction and supports Cornforth and Elliot's (1950) mechanism.

(vii) The possibility that the carbonyl group of the acetylated amine or hydroxyl groups in proteins or mucopolysaccharides was not considered a possible source of the positive hydrazide reaction by either Barrnett and Seligman (1958) or Karnovsky and Fasman (1960). There is no specific evidence that hydrazines and hydrazides can react with such groups <u>in vitro</u> (sec. 4.2/3), but this does not mean that such a reaction is impossible.

The absence of fluorescence in sites known to contain neutral mucopolysaccharides (Fig. 19) rules out the possibility that - O. CO. CH_3 groups are responsible for the salicyloyl hydrazide - zinc reaction. It is unlikely that many hydroxyl - groups are acetylated in the reaction conditions used for these experiments; Usually higher temperatures are required for their complete acetylation (Lillie, 1954).

Proof that the - $NH.CO.CH_3$ groups are not reactive is more difficult to obtain. Although the deamination of sections abolishes the salicyloyl hydrazide reaction, this is because the amines are required for the formation of "intermediates". The following experiments were therefore carried out:

(a) Liver sections containing glycogen were treated with methanolic thionyl chloride (sec. 11.4). This leads to the methylation of the carboxyl groups and the abolition of the

acetylation - salicyloyl - hydrazide - zinc reaction. This does not prove that the - $NH.CO.CH_3$ groups were not reacting with the hydrazides; the protein amine groups are also methylated (sec. 11.4) and/or are probably not amenable to acetylation.

(b) The methylated sections were oxidised with periodic acid and left in a solution of p - phenylene diamine overnight. This results in free amine groups in the mucin sites (exp. sec. 12.12). After treatment with acetic anhydride containing a small amount of pyridine, no positive salicyloyl hydrazide - zinc reaction was produced in the mucin sites, thus proving that - $NH - CO.CH_3$ do not form fluorescent conjugates. The acetylation of the amine groups was checked by diazotising and coupling them with H - acid (exp. sec. 12.12). A positive colouration was obtained before, but not after, acetylation.

It is essential to limit the amount of pyridine in the acetylation mixture when carrying out experiments on methylated sections. A positive zinc - salicyloyl hydrazide reaction is obtained if 50:50 mixtures of acetic anhydride and pyridine are used on such sections. This is due to the partial demethylation of the carboxyl (and amine groups?) as can be shown by the partial restoration of their basophilia towards azure A.

(viii) The uronic acid groups of acid mucopolysaccharides do not give a positive reaction with hydrazides after the acetic anhydride - pyridine treatment either <u>in vitro</u> or <u>in situ</u>

(Karnovsky and Fasman 1960). This is illustrated by the absence of fluorescence in the gastric mucin sites of the guinea pig gut shown in Fig. 16. This negative result suggests that special conditions are required for the conversion of carboxyl groups to methyl ketones (i.e. the presence of an - NH.R. CH_2 - group). Karnovsky and Mann (1961) suggested an alternative explanation for the failure; mixed anhydrides are still formed from the uronic acid groups, which then react with the many adjacent hydroxyl groups to yield esters, thus preventing the reaction with the hydrazide. In view ofthe evidence cited earlier, Karnovsky and Mann's (1961) explanation now seems unlikely.

3. The demonstration of side-chain carboxyl groups

If C - terminal carboxyl groups are converted to non-basophilic ketones, the remaining side-chain carboxyl groups in tissue sections should be stained by basic fluorescent dyes provided that the phosphate groups of nucleic acids (particularly RNA) have been extracted previously.

Another difference between C-terminal and side-chain carboxyl groups is the relative values of their acid dissociation constants (sec. 9.1/3). The former have a pK_a value of about 4 whereas that of the latter is about 2. Therefore at pH 2 - 3 only the latter are still appreciably dissociated. At pH 4, both types of carboxyl group are fully dissociated.

If sections which contain proteins having a relatively high proportion of C-terminal carboxyl groups are stained with a polyfluorochromatic dye such as coriphosphine, then the colour and intensity of the fluorescence in those protein areas should differ after staining at these two pH levels. In practice, nearly all tissue proteins contain both types of carboxyl group, and it has been found impossible to distinguish them by such a fluorometric "titration" procedure.

However, the effect can be exaggerated by "blocking" the C-terminal carboxyl groups beforehand with the acetic anhydride pyridine procedure. Sections of guinea pig kidney, for example, then exhibit a light blue fluorescence after staining with coriphosphine at pH 2.7 (Fig. 2D) which reverts to the "normal" green fluorescence when they are stained at pH 5. (Fig. 2I). In both cases the interfering RNA is extracted with hot perchloric acid (sec. 9.7/2) before or after the acetic anhydride - pyridine procedure. On the other hand, sections of guinea pig gut, which contain relatively few C - terminal carboxyl groups exhibit practically the same shade and intensity of green fluorescence after staining at either pH (Figs. 22 and 23).

4. Conclusion

The evidence cited in secs. 3 and 4 does not support the hypothesis that there is a widespread conversion of side-chain carboxyl groups to mixed anhydrides when sections are treated wit acetic anhydride and pyridine. Instead, when a reaction does

Fig. 20. Guinea pig kidney. Fixed in glycol ether.

Acetic anhydride + pyridine, 60°, 1 hr.; perchloric acid, 60°, 8 mins.: 0.01% coriphosphine, pH 2.3. Fluormount, X 180.

The side-chain carboxyl groups of the protoplasmic proteins emit a blue fluorescence of a stronger intensity than is apparent in this photomicrograph.

Fig.21. Guinea pig kidney. Fixed glycol ether.

Acetic anhydride + pyridine, 60°, 1 hr.0.01% coriphosphine, pH 5. Water mount, X 180.

The protoplasmic side-chain carboxyl groups fluoresce a light bluish-green colour.

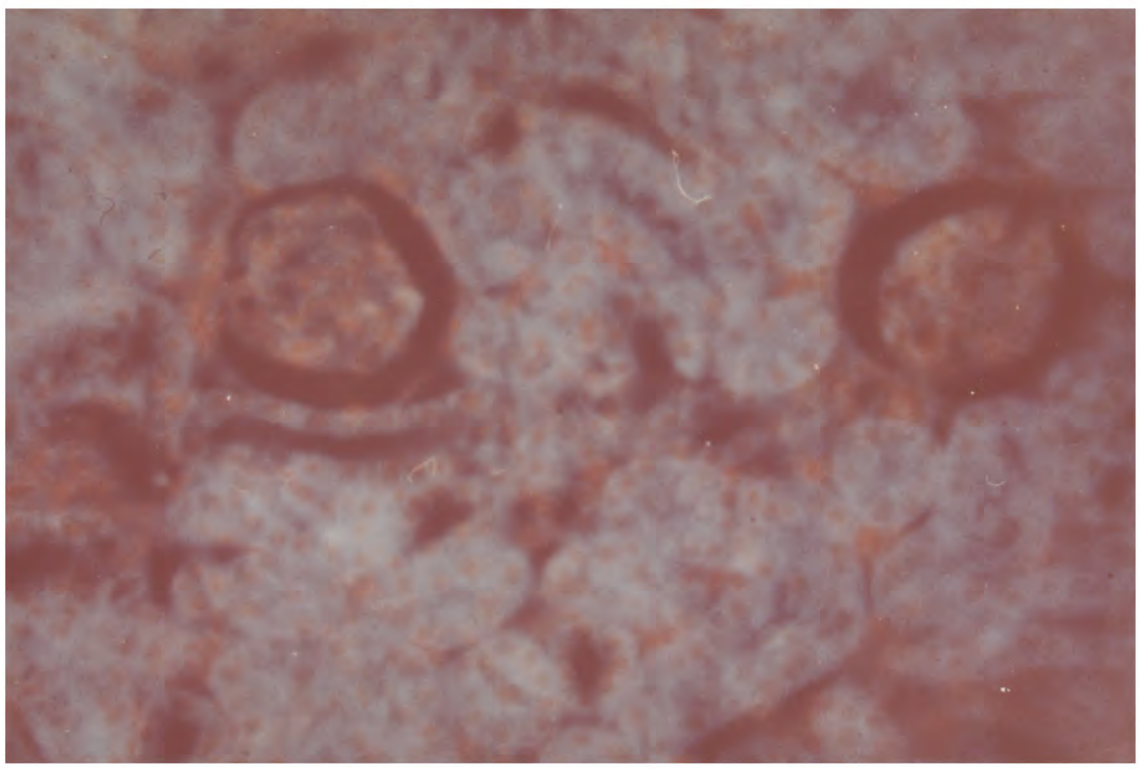


Fig. 20

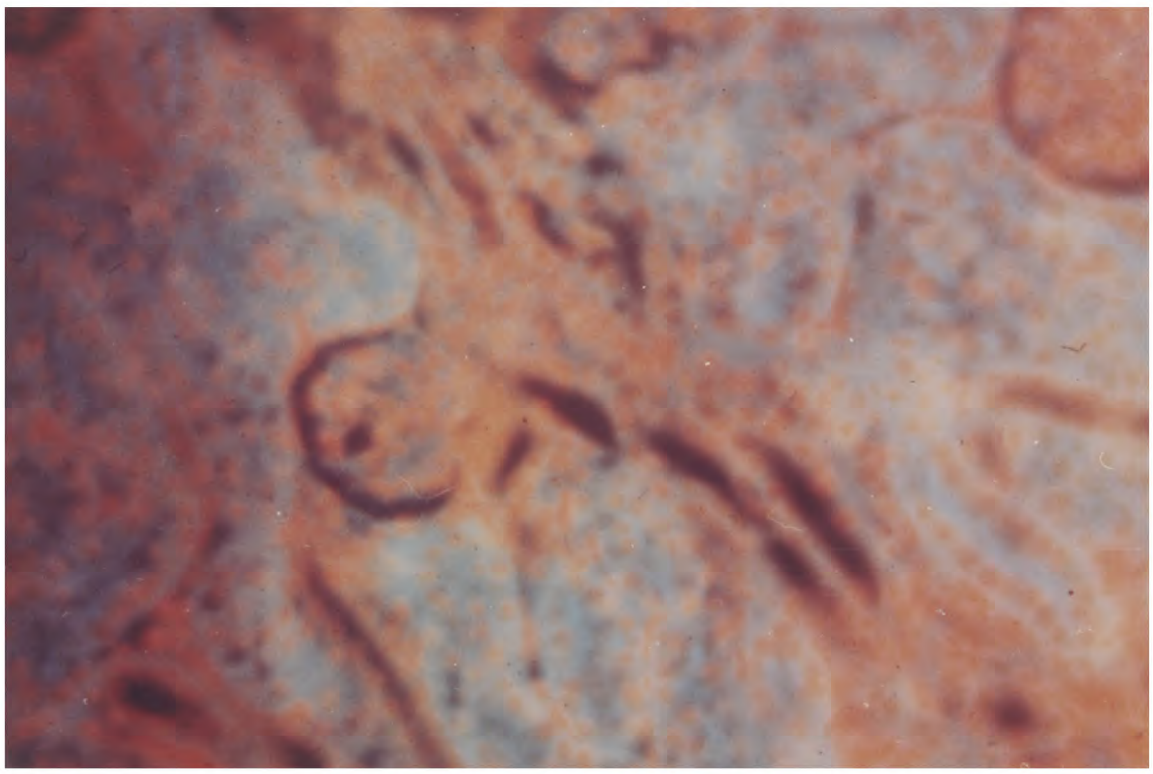


Fig. 21

Fig.22. Guinea pig gut. Fixed in glycol ether.

Acetic anhydride + pyridine, 60°, 1 hr.: 0.01% coriphosphine, pH 2.3. Water mount, X 180.

The protoplasmic proteins emit a light green (not blue) fluorescence. The orange-brown fluorescence of the nuclei is weaker than that exhibited by the nuclei in control sections.

Fig. 23. Guinea pig gut. Fixed in glycol ether.

Acetic anhydride + pyridine, 60°, 1 hr.: 0.01% coriphosphine, pH 2.3. Water mount, X 180.

The muscle exhibits practically the same pale green fluorescence as it does in control sections. On the other hand, the fluorescence of the nuclei and acid mucopolysaccharides is less distinct and is of a weaker intensity.

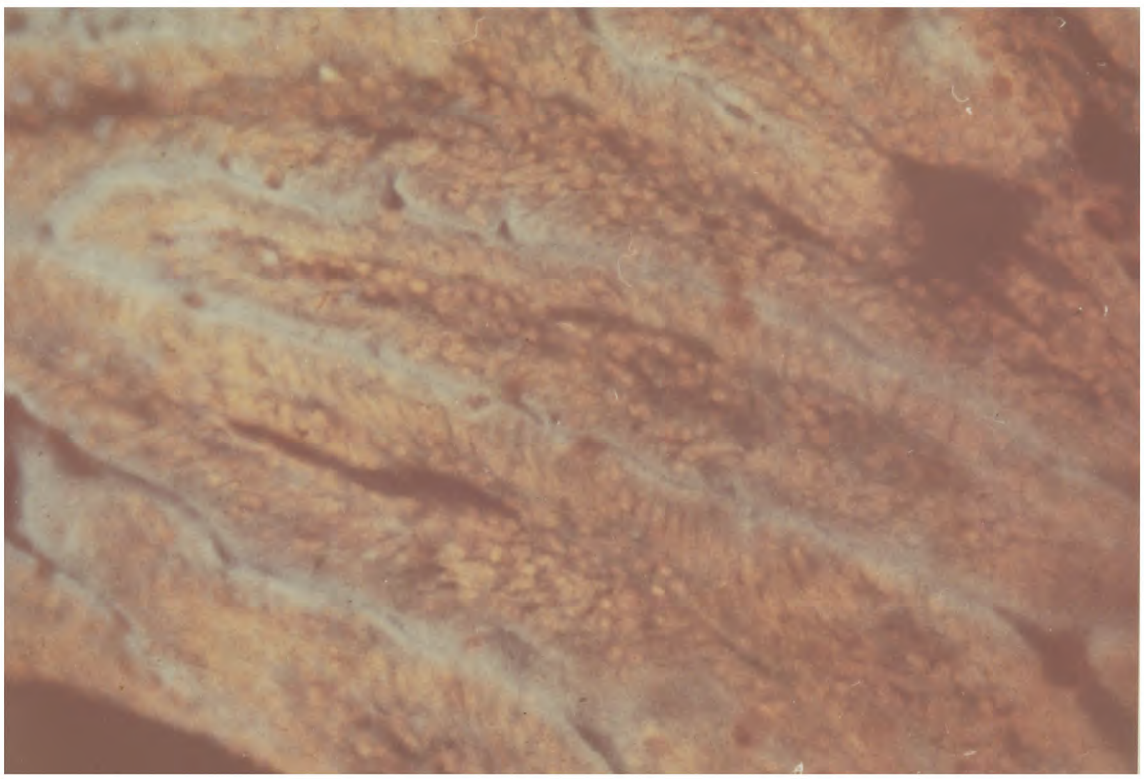


Fig. 22

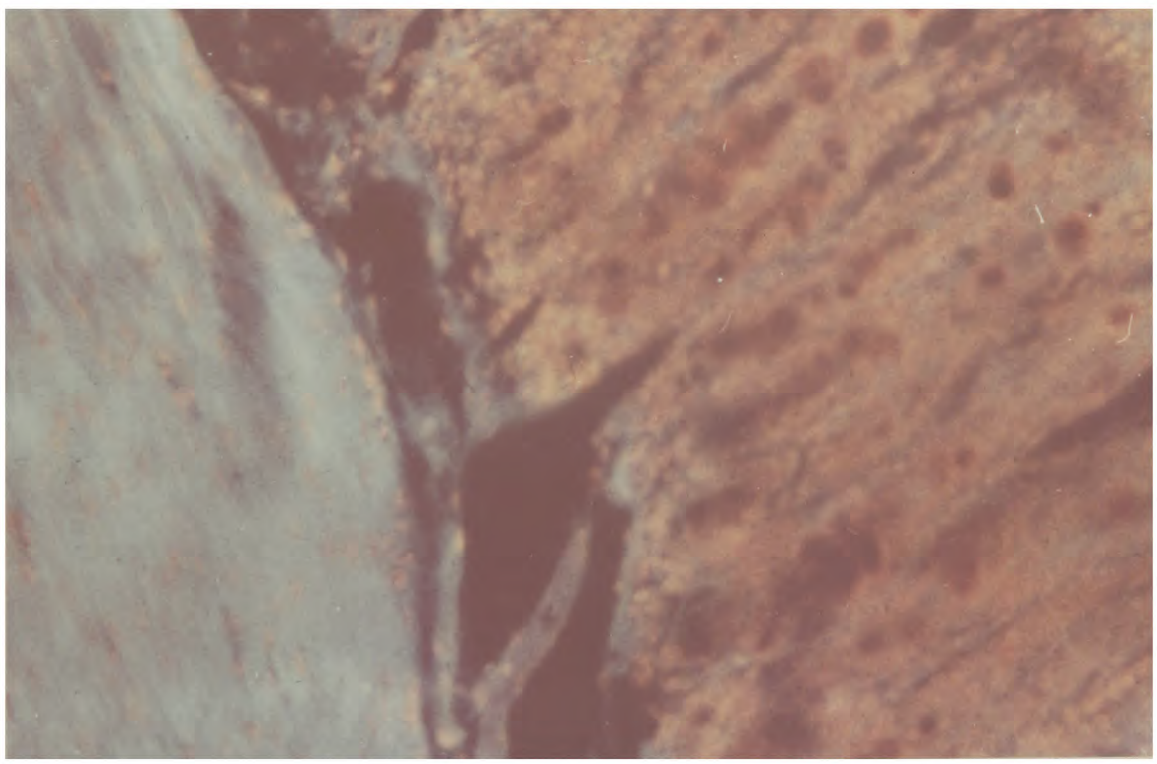


Fig. 23

take place, methyl ketones are formed from C-terminal carboxyl groups.

Experimental Section 7

Carboxyl Groups

1. The acetic anhydride - pyridine method for C-terminal carboxyl groups (f)

Method

(1) Rinse dewaxed sections twice in alcohol.

(2) Wash twice in acetic anhydride, 2 mins. each.

(3) Incubate in a 1:1 mixture of acetic anhydride and pyridine (previously dried over barium oxide for 1 hour at 60°.

(4) Rinse in acetic anhydride for 2 mins.

(5) Wash in alcohol for 2 mins. and take down to water.

(6) - (9) As stages (4) - (7) in exp. sec. 12.14.

(10) Immerse in 1% zinc acetate for 5 - 10 mins.

(11) Rinse in water, through the alcohols, clear in xylene, and mount in Fluoromount.

RESULT

Sites containing high concentrations of C-terminal carboxyl groups are non-fluorescent after stage (9) but exhibit an intense blue fluorescence after stage (11).

No fluorescence is developed if sections are treated with 0.4% sodium hydroxide in 70% alcohol after stage (5).

2. The coriphosphine staining method for side-chain carboxyl groups (f)

Method

(1) Two serial sections are treated separately as far as stage (10) of the previous method.

(2) Rinse both in water.

(3) Incubate both in 5% perchloric acid at 60° for 5 mins. to extract RNA.

(4) Wash both sections once each in dilute (1%) sodium carbonate, and water.

(5) stain separately in 0.01% solutions of coriphosphine of (a) pH 2.5-3.0 and (b) pH 5 for 20 mins.

(6) Rinse in water, and examine for fluorescence as water mounts.

RESULTS

Sites containing a low concentration of side-chain carboxyl groups and a high concentration of C-terminal carboxyl groups exhibit a green fluorescence after staining at pH 5 and a blue fluorescence after staining at pH 2.

Tissues which contain a high concentration of side-chain carboxyl groups and a low concentration of C-terminal carboxyl groups fluoresce green after staining at either pH. Sometimes the green fluorescence after staining at pH 2 has a bluer hue.

Section 8

Nucleic Acids

1. Introduction

Nucleic acids are best studied as separate entities in systematic fluorescence histochemistry because any scheme which is based solely upon the dectection of their functional groups can lead to complications. A typical example which illustrates this difficulty is the commonly used method which is based upon the basophilic staining of the nucleic acid phosphate groups by potentially polyfluorochromatic dyes, such as coriphosphine or acridine orange. Distinctive and different fluorescence colours can be observed in the sites of nucleic acids in sections which had been stained with dilute solutions of these dyes, but the actual colour in any structural feature is markedly dependent on the type of fixation used for the tissue. It is not possible to assign a particular fluorescence colour unambiguously to a given nucleic acid.

In frozen sections, nuclei (DNA) fluoresce green after staining with acridine orange, whereas RNA fluoresces red (vom Bertalanffy and Bickis, 1956). On the other hand, the fluorescence colours in fixed sections are often the reverse. The typical fluorescence appearance of a fixed and stained tissue is illustrated in Fig. 24 in which the nuclei are usually reddish-orange and other features are green or yellow. Hicks and Mathaei (1955) have published similar observations and commented that red fluorescence is hardly ever observed

in fixed tissues. However, Armstrong (1956), amongst others, has found that in tissues fixed in Carnoy, Zenker's or corrosive-acetic fluids, cytoplasmic inclusions (RNA) fluoresced a fiery-red colour, whereas nuclei were yellow. Only after fixation in Bouin's fluid was there no distinctive fluorescence of cytoplasmic RNA: This is understandable in view of the quenching properties of the picric acid content in Bouin's fixative, but it is difficult to understand how Armstrong (1956) obtained bright fluorescent colours with fixatives containing heavy metals, such as mercuric chloride and potassium bichromate in Zenker's fluid, as they should quench the fluorescence of dyes absorbed onto basophilic sites.

The variation of the fluorescence colours of tissues fixed in different ways may be due to the partial extraction of diffusable substrate in the tissue (e.g. RNA) and to the denaturation of some proteins. Moreover, basophils other than nucleic acids form fluorescent complexes of similar fluorescence colours. For example, the difficulty in distinguishing the fluorescence of the acid mucopolysaccharides and the nuclei in sections of gut has been discussed elsewhere (sec. 9.4 etseq). The fluorescence due to the uptake of acridine orange or coriphosphine by acid mucopolysaccharides can be quenched completely by pretreating tissue sections with alcian blue (sec. 10.2): the nuclei can then be seen much more clearly as discrete entities (Fig. 24 Cp Fig. 3 . Most of the

Fig. 24. Guinea pig gut. Fixed glycol ether.

Alcian blue : 0.01% coriphosphine, pH 5.
Water mount, X 180.

The fluorescence of the coriphosphine adsorbed onto sites of acid mucopolysaccharides is completely quenched. The nuclei (DNA) fluoresce an orange colour. The protoplasmic proteins fluoresce a light green colour.

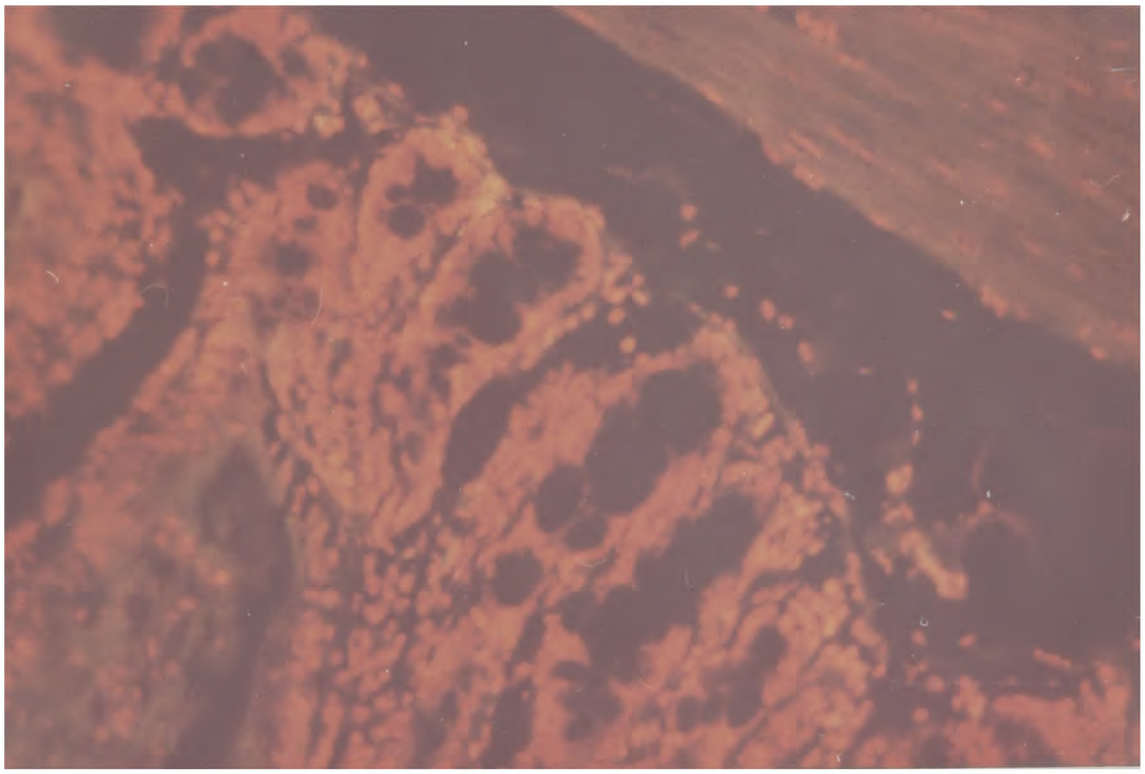


Fig. 24

green fluorescence in cytoplasmic sites is due to protein carboxyl groups (sec. 9.7/2) and not to RNA as assumed by previous workers.

The one important lesson from this profusion of conflicting experimental observations is that the polychromatic fluorescence of sections stained with acridine orange cannot be attributed solely to RNA and DNA. The differential fluorescence staining can be used as a guide to the presence of these two nucleic acids, but no firm conclusions can be drawn.

The presence of DNA can be confirmed by carrying out a Feulgen hydrolysis and condensing the exposed aldehydes with salicyloyl hydrazide. RNA, however, is difficult to confirm.

Methods based upon specific fluorochemical reactions of either the phosphate groups or heterocyclic bases are not very successful for the detection of nucleic acids.

2. <u>The detection of phosphate groups in nucleic acids</u>

Serra and Queiroz Lopes (1945) were partially successful in demonstrating the phosphate groups of nucleic acids in tissue sections by hydrolysing the phosphates off their parent molecules and immediately precipitating them as a molybdate complex, which can be converted to a coloured form with benzidine. This method is not very suitable for general use in histochemistry: the prolonged hydrolysis which Serra and Queiroz Lopes used is likely to lead to artefacts due to the diffusion of phosphate ions (Langeron, 1949).

A different approach has been tried for demonstrating the phosphate groups for fluorescence histochemistry. One important property of free and organic phosphates is the insolubility of their zirconium salts (Millard and Freund, 1946; Millard and Hahn, 1949), a fact which is utilised in the "phosphate separation" procedure in the qualitative analysis of metals. As zirconium ions can also form intense fluorescent complexes with morin and other organic hydroxy compounds (Kallistratos etal, 1960), it seemed feasible that the zirconium - nucleic acid phosphate complex would react with morin in situ. The reaction would only take place either where there are excess zirconium ions absorbed onto the nucleic acid complex or if the zirconium ions incorporated into nucleic acid complex itself form a chemically stronger complex with morin than with phosphates. The only objection in principle to a histochemical reaction of this type would be an interference from the reaction of zirconium ions with protein α - hydroxycarboxyl groups, if any, (Jonckers, 1950) and the absorption of morin onto acidophilic protein amines. The first interfering reaction is unlikely and the second can be avoided by using alcoholic instead of aqueous solutions of morin. Carboxylic acids show little tendency to complex with zirconium ions (Connick and McVey, 1949).

Solutions of zirconyl nitrate completely blocked the visible basophilia of tissue sections towards azure A within a few minutes. The specificity of the zirconium ions for

phosphate groups can be demonstrated by washing the impregnated sections with N/10, or N/100 potassium dihydrogen phosphate for 5 mins. at room temperature; all the zirconium ions are removed and the basophilia of the sections towards azure A is completely removed. On the other hand, N/10 solutions of sodium sulphate do not restore the basophilia to zirconium impregnated sections.

Zirconium ions adsorbed onto the tissue sections produce a yellow-green fluorescence in nuclear and cytoplasmic sites when treated with 0.1% alcoholic solution of morin. Sections that have been subjected to a preliminary Feulgen hydrolysis exhibit a more diffuse fluorescence. This supports the objections raised against Serra and Querioz Lopes' (1945) technique regarding the diffusion of liberated phosphate ions. RNA phosphate groups are almost certainly completely extracted from the section during the Feulgen hydrolysis (sec. 9.7/1). There was no fluorescence in the sites of sulphated acid mucopolysaccharides, thus showing that sulphates do not interfere with the specificity of the phosphate - zirconium - morin reaction.

Contrary to the abolition of visible basophilia, zirconium treated sections which have been stained with coriphosphine exhibit anomalous fluorescence colours. In sections of guinea pig kidney, for example, the fluorescence was a dull blue colour after staining at pH 5, while staining at pH 2 resulted in a much enhanced blue fluorescence (Figs. 25 and 26).

Fig. 25. Guinea pig kidney. Fixed in glycol ether.

2% zirconium nitrate, 20 mins, : 0.01% coriphosphine, pH 5, 20 mins. Water mount, X 180.

The section emits a weak, blue fluorescence.

Fig. 26. Guinea pig kidney. Fixed in glycol ether.

2% zirconium nitrate, 20 mins. : 0.01% coriphosphine, pH 2.7, 20 mins. Water mount, X 180.

The whole section emits an intense blue fluorescence.

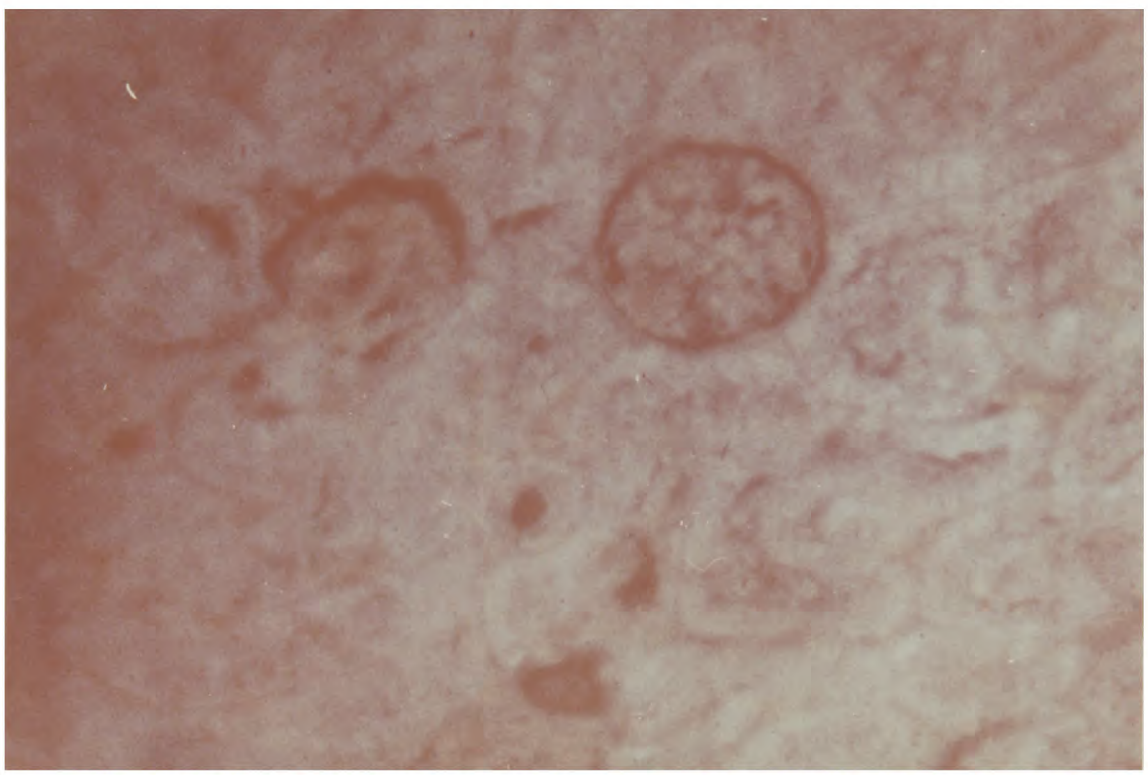


Fig. 25

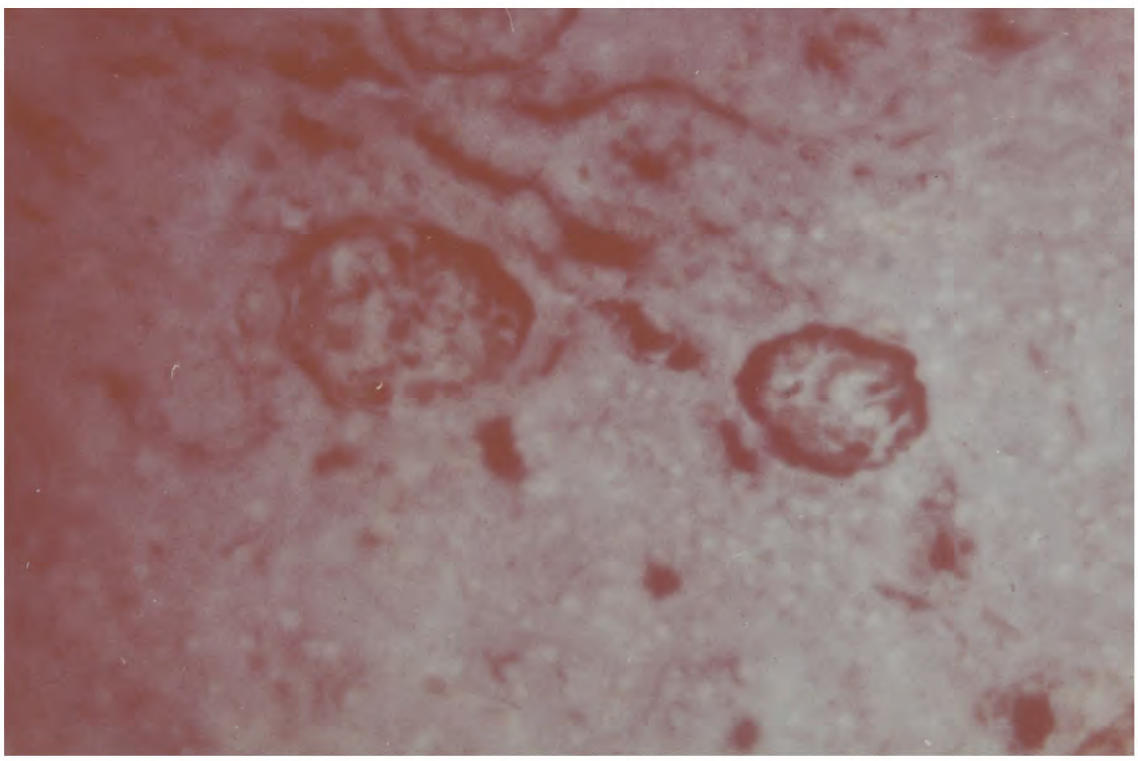


Fig. 26

As this tissue normally exhibits a green fluorescence when stained at pH 5 after its cytoplasmic RNA has been extracted with perchloric acid, some of the zirconium ions must be taken up by some of the protein carboxyl groups. Therefore the zirconium - morin method is not strictly specific for phosphate groups. Why the intensity of the fluorescence of the zirconium - treated sections should be increased after staining at pH 2 is mystifying. Perhaps at pH 2, the zirconium ions are incorporated as a chelated complex with undissociated (C-terminal?) carboxyl groups and are less likely to quench the fluorescence of coriphosphine absorbed onto neighbouring (side-chain?) carboxyl groups. If this theory is correct, the zirconium carboxyl linkages might revert or remain in a salt-like form in coriphosphine solution of pH 5. In this state, the "free" zirconium ions would, like other metal ions, tend to quench the fluorescence of adsorbed coriphosphine.

3. Confirmatory test for phosphate groups of nucleic acids

Phosphate groups in the form of their methyl esters can be specifically hydrolysed off by lithium aluminium hydride in hot dioxane (sec. 9.9). This reduction procedure can be used in conjunction with staining tests to follow their removal.

4. Reactions for purine and pyrimidine bases.

1. Introduction

The only test which has been used in visible light histo-

chemistry for the demonstration of purine and pyrimidine bases is the diazonium salt coupling reaction after benzoylation or acetylation (Mitchell, 1942; Barnard and Danelli, 1956). This method was based on the assumption that only the activated nuclear centres of heterocyclic bases would couple. Barnard and Danelli (1956) made a comprehensive study of this reaction and came to the conclusion that it cannot be used for the identification of nucleic acids, but it remained useful for demonstrating histidine residues. The reaction was therefore not considered worth adapting for fluorescence histochemistry. For this reason, other chemical reactions were investigated; unfortunately none of them could be successfully exploited for histochemical purposes.

2. <u>Reactions for pyrimidines and tryptophanes</u>

Many 2 - substituted pyrimidines give a red colouration when heated <u>in vitro</u> with 2 - thiobarbituric acid and dilute acetic acid at 60° (Shepherd, 1946). The colour of the product is reminiscent of that given by the complex formed between thiobarbituric acid and N - acetyl sialic acids that have been oxidised with periodic acid <u>in vitro</u> (sec. 12.4/2)

A moderately intense <u>blue</u> fluorescence with a slight green tinge was obtained in tissue treated with a 5% solution of the hot acid for 30 mins. No visible colouration was observed. The intensity and colour of the fluorescence was exactly the same as that induced in tissues (e.g. gut) by 70% perchloric acid

at room temperature. Serial sections of the same tissues gave a strong visible dimethyl aminobenzaldehyde - nitrite reaction (Adams, 1957) indicative of tryptophane residues. This suggested that the blue fluorescence in sections treated with perchloric or thiobarbituric acids might have arisen from a reaction with the tryptophane residues rather than with the pyrimidine fractions of RNA, especially as Tauber (1948) had previously found that tryptophane gave a yellow-green fluorescence *in vitro* when treated with 70% perchloric acid at room temperature. Gordon and Mitchell (1949) later found that tryptophane could be estimated in unhydrolysed proteins by a similar reaction thus implying that it was specific to tryptophane. However, the production of a predominantly blue fluorescence *in situ* instead of the yellow-green fluorescence *in vitro* would appear to argue against the tryptophane reaction in tissues, until it is remembered that the fluorescence colour of products formed in other fluorochemical reactions *in situ* are always shifted towards the blue with respect to the colours formed *in vitro*. A comparable example is the fluorescence of the Konig - Sassi complex with amines which is green *in situ* but red or orange when the reaction is carried out in solution or on filter paper.

Proof that the fluorescence reaction between tissue sections and 2 - thiobarbituric acid is not due to a reaction with the pyrimidine bases of RNA was obtained by extracting both nucleic acids from the section beforehand with perchloric acid

at 50° for 30 minutes (sec. 9.7/2). There was no change in the fluorescence reaction of the tissue when it was subsequently treated with thiobarbituric acid.

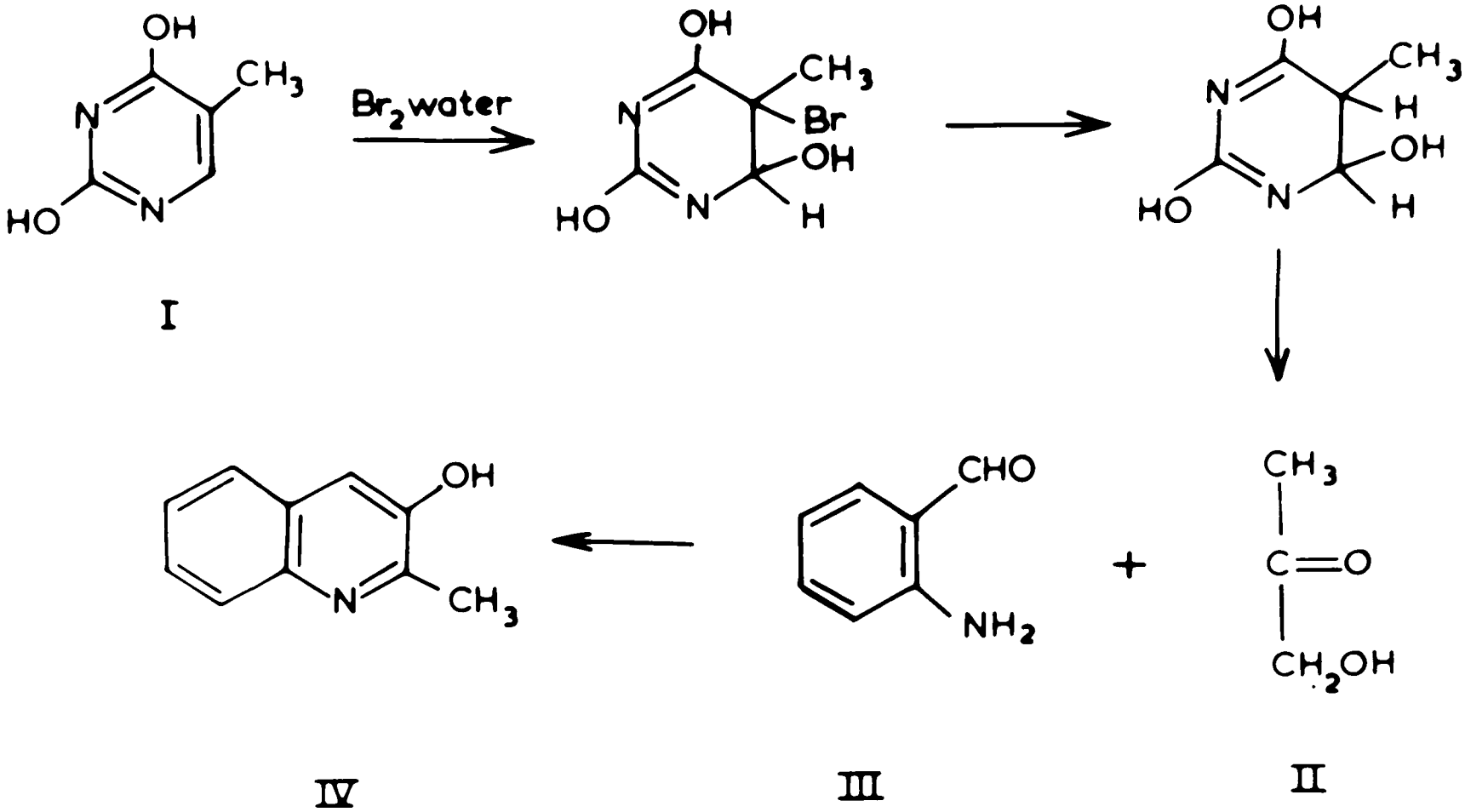
The failure of thiobarbituric acid to react with the pyrimidine bases is probably connected with the 4, 5 or 6 substituents in the pyrimidine nucleus (Shepherd, 1948).

3. <u>The Wheeler - Johnson test for the uracil and cystosine residues of nucleic acids</u>.

In this test (Wheeler and Johnson, 1907), uracil and cytosine residues are first converted to 5,5[1] - dibromo - 4 - hydroxy uracil with bromine water. The substituted bromine atoms are then removed with barium hydroxide to form as intermediates, isodialuronic and dialuric (2, 4, 5, 6 - tetrahydroxy-pyrimidine) acids, whose barium salts are purple. When tissue sections were subjected to a comparable routine, no visible staining was ever produced. Presumably the uracil and cytosine residues in nucleic acids are not accessible for this reaction.

4. <u>Reactions for the thymine residues of DNA</u>

The thymine (I) content of DNA can be oxidised with hydrogen peroxide or bromine water to acetol (II) and assayed fluorometrically by converting II to the fluorophor 3 - hydroxyquinaldine (IV) with O - amino-benzaldehyde, III (Roberts and Friedkin, 1958).



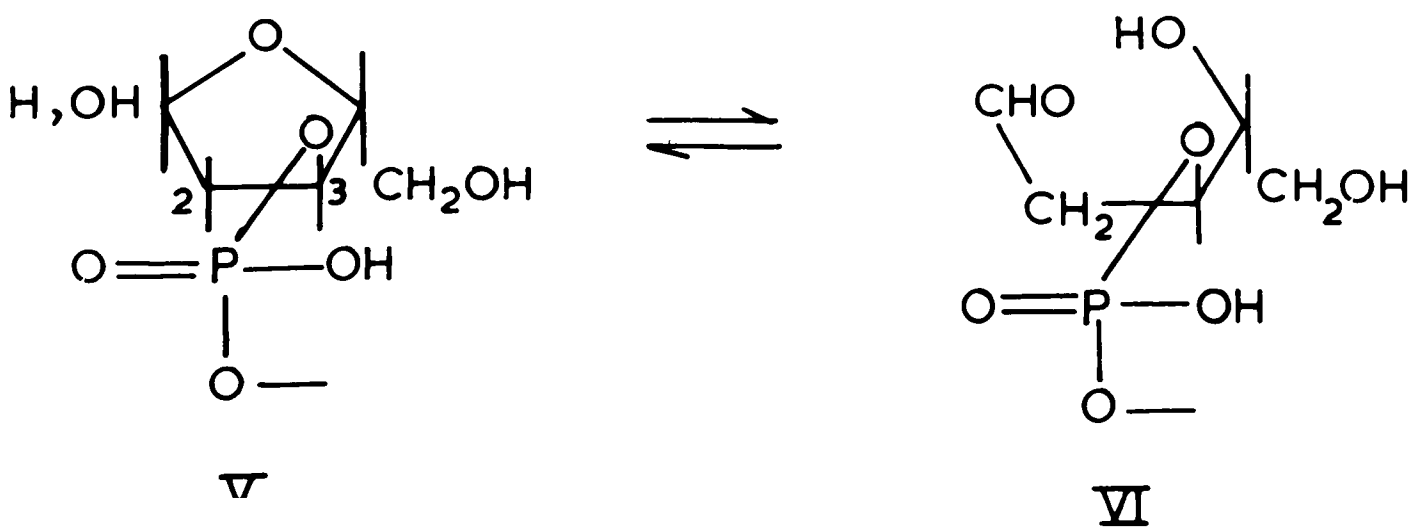
Potassium permanganate oxidises thymine residues slightly differently, the final products being urea and oxaluric acid (Heinrich and Wilson, 1950)

III is not readily available in this country. It is a relatively unstable compound being prone to self condensation. It was therefore thought worthwhile investigating other methods for converting the oxidation products of thymine to fluorophors. As II and oxaluric acid each have a ketonic group, they should form fluorescent zinc salicyloyl hydrazones (sec. 7.2/3). In practice however, no discrete fluorescence was ever discernable in nuclei after such an oxidation and condensation. One explanation for this failure is that the original oxidation products rapidly diffuse out of the nuclei before they can react <u>in situ</u> with salicyloyl hydrazide.

5. The Feulgen reaction for DNA

(1) Introduction

This reaction was devised by Feulgen and Rossenbeck (1924) as a specific test for thymonucleic acid (DNA). It depends on the mild acid hydrolysis of fixed tissues which releases aldehyde groups from the sugar moiety of DNA. A considerable amount of controversy has been associated with the explanation of the mechanism of the Feulgen reaction in tissues. Stacey's group (Stacey et al, 1946; Overend and Stacey, 1949) have put forward the most plausible mechanism: first, acid hydrolysis rapidly breaks the sugar linkages engaged in polymeric bonding and secondly it ruptures the glycosidic linkages between the sugar and purine bases. The deoxyribose compounds (V) thus revealed are attached through phosphate linkages to the main nucleic acid chain where they are firmly held in the furanose form. The arrangement at C_3 of the sugar after removal of the base includes a - CH_2 CHO grouping (VI). The ribose sugars, with a hydroxyl group at C_2 in place of the second hydrogen atom of the deoxysugar are not hydrolysed by hydrochloric acid and therefore no aldehyde groups are exposed.



V VI

Further acid hydrolysis removes the histones and nucleic acids (vide sec. 9.7/1) together with the exposed sugar aldehyde attachments (Li and Stacey, 1949). Therefore a histochemical Feulgen reaction is a compromise between the opti/mal exposure of aldehydes and the progressive removal of the nucleic acid itself. The optimal times for the hydrolysis of variously fixed tissues have been worked out by Bauer (1932). Sections fixed in the glycol ether which has been used predominantly in this investigation required about 5 minutes hydrolysis.

2. Condensation of the exposed aldehyde.

The following reagents have been investigated for visualising the exposed aldehydes in DNA sites for fluorescence microscopy.

(i) Pseudo- Schiff reagents

The uses and disadvantages of pseudo - Schiff reagents have been discussed in sec. 12.7/3. They are not selective for demonstrating the aldehydes exposed in nuclear sites as they also react with the residual basophilic phosphate groups of the nucleic acid backbone.

(ii) Salicyloyl hydrazide

This reagent (sec. 12.19) is very satisfactory for demonstrating hydrolysed DNA. The intensity ofthe blue fluorescent conjugate varies from tissue to tissue (Figs 12 and 27) and is not always as intense as might be expected by comparison

Fig. 27. Guinea pig gut. Fixed in glycol ether.

N Hydrochloric acid, 60°, 5 mins.: salicyloyl hydrazide. DPX mount, X 180.

An intense blue fluorescence is emitted by the nuclei.

Fig. 28. Guinea pig gut. Fixed in glycol ether.

N Hydrochloric acid, 60°, 5 mins. : salicyloyl hydrazide : solochrome black + alum. Fluormount, X 180.

The acidophilic protoplasmic and muscle proteins fluoresce red. Some nuclei (particularly those in the muscle) retain the blue fluorescence they emitted before the solochrome black + alum treatment while others fluoresce a red or sometimes purple colour.

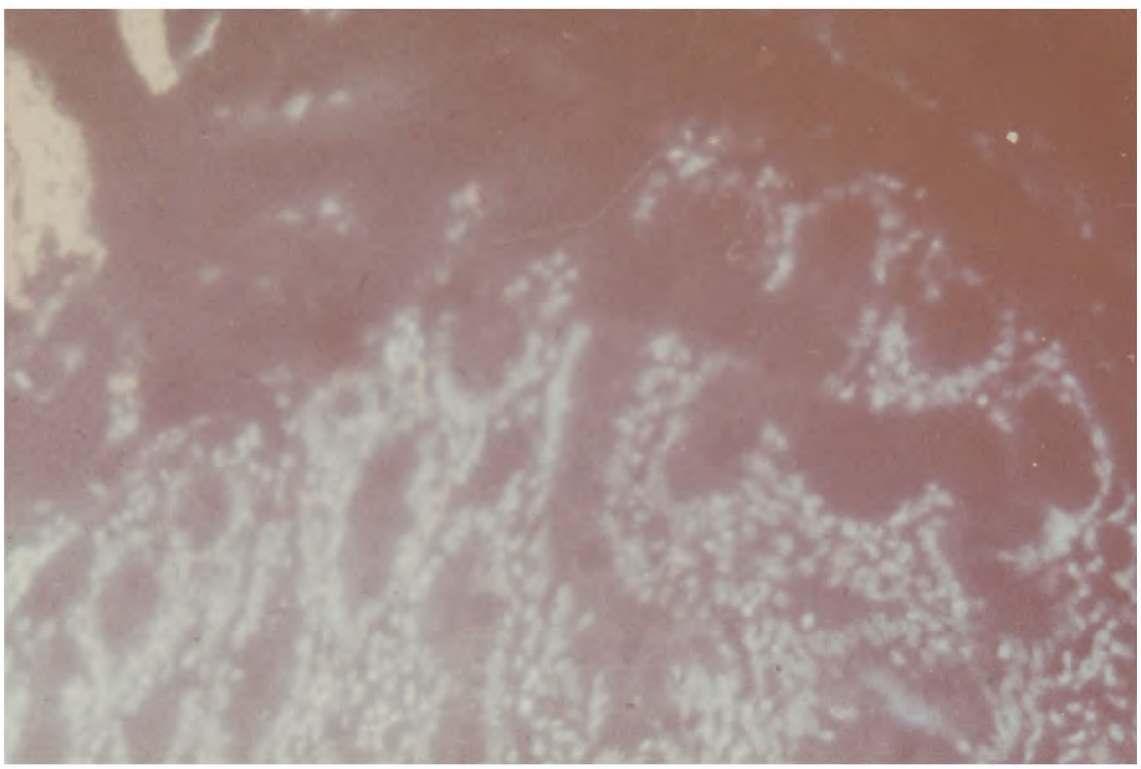


Fig. 27

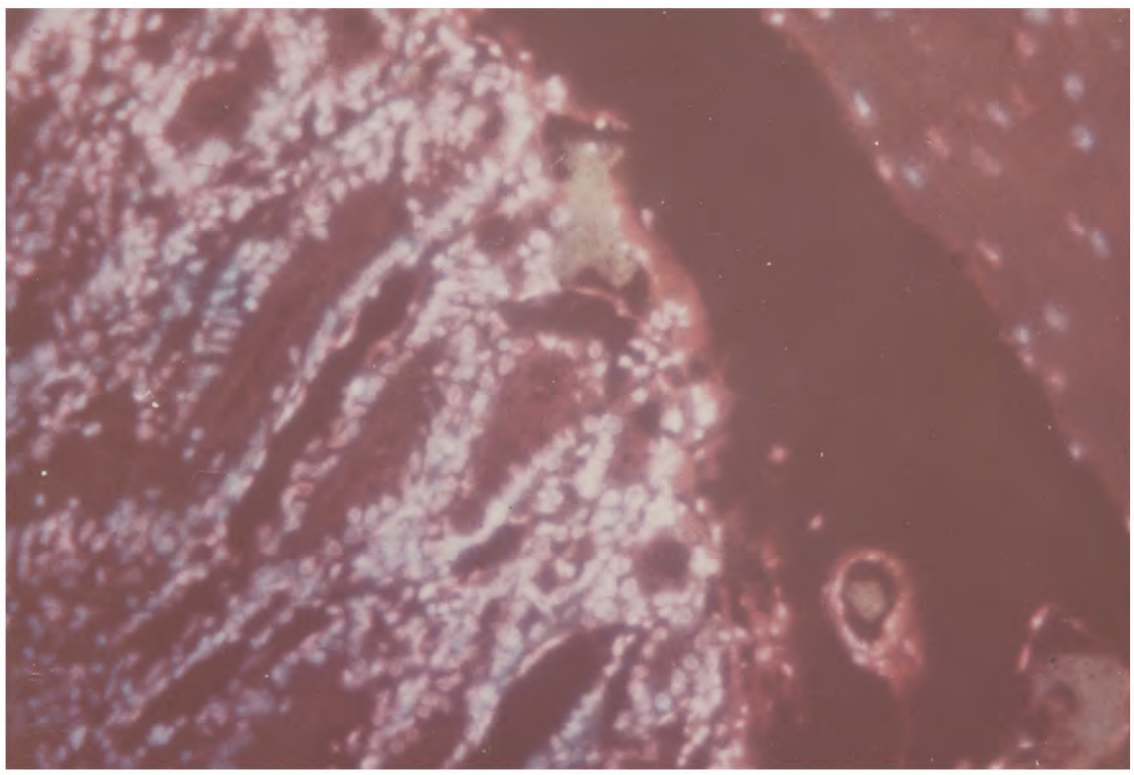


Fig. 28

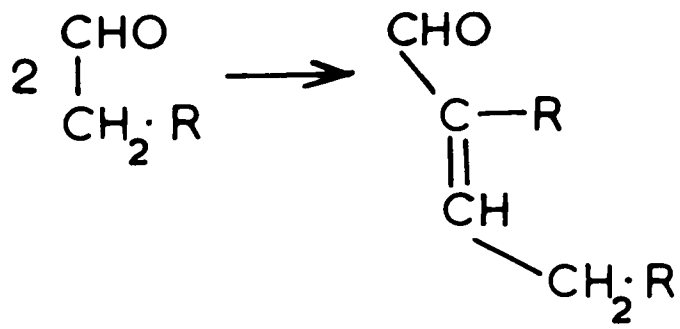
with the "dialdehyde" conjugates formed from periodate - oxidised neutral mucopolysaccharides (cp. Figs 50 and 51). The reasons for the variation of fluorescence output are unknown.

The exposed nuclear aldehydes completely resist methylation (sec. 11.4) and give a practically unchanged reaction with salicyloyl hydrazide after a methylation sequence. One peculiarity of the hydrolysed nuclear sites after condensation with salicyloyl hydrazide is their residual acidophilia. Approximately the same percentage of nuclei persistently exhibit a stronger and overwhelming acidophilic fluorescence towards the solochrome black - alum complex (sec. 3.10) after the salicyloyl hydrazide condensation than do others (Fig. 28).

The basophilic "background" tissue cannot be counter-stained with fluorescent basic dyes after sections have been condensed with salicyloyl hydrazides: The blue fluorescence of the nuclear hydrazone is completely obliterated by the characteristic fluorescent staining from the dye.

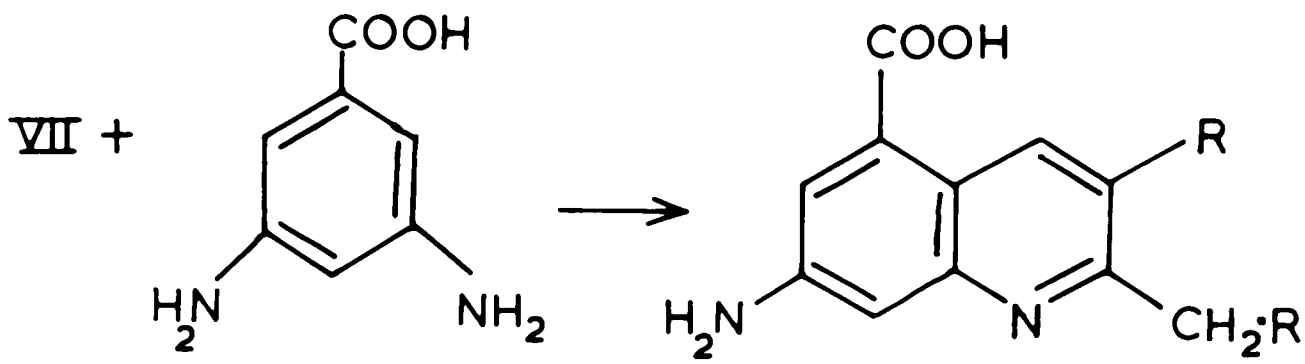
(iii) <u>3: 5 - diaminobenzoic acid</u>.

Missane and Robins (1958) developed an extremely sensitive fluorescence assay method for DNA based upon the formation of a fluorescence quinaldine (IX) by a Doebner - Miller condensation of the CH_2CHO grouping from the deoxyribose moiety with 3 : 5 diaminobenzoic acid (VIII) as first suggested by Velluz <u>etal</u> (1948 a,b). Aldehydes which do not contain an α - methylene group do not react with VIII. This restriction also



VII

includes
/pentoses and hexoses (Pesez. 1950).



VIII IX

Therefore VIII should be particularly specific for the demonstration of the aldehydes exposed in nuclear DNA as it is unlikely that other aldehydes of the type $R.CH_2CHO$ would ever be engendered in other structural features in tissue sections.

Unfortunately no specific fluorescence was formed in nuclear sites in Feulgen - hydrolysed tissue sections after they had been treated with acidified 1% solutions of VIII at room temperature or at 60° for 30 mins. Unsuccessful attempts were also made to form a fluorescent conjugate as soon as the nuclear aldehydes were exposed by incubating unhydrolysed sections in the acidified solutions of VIII at 60° for 5 or 30 minutes. Only a non-specific diffuse greenish-blue fluorescence could be seen over the whole tissue section after any of these variations. The failure of VIII to form fluorescent compounds with aldehydes engendered <u>in situ</u> matches the general failure of other aromatic amines, including m - phenylene diamine which is chemically similar to VIII, for the reasons

discussed in sec. 12.21.

6. RNA

RNA is difficult to detect unequivocally by fluorescence histochemistry for the reasons outlined in the introduction. In visible light histochemistry, it can be detected by observing those sites which lose their basophilia towards azure A or toluidine blue after sections have been digested with RNase or extracted with hot perchloric acid (secs. 9.8/2 and 9.7/2). However, most tissue sections stained with fluorescent dyes exhibit practically the same fluorescence colour (but not intensity) in sites containing RNA both before and after the digestion or acid extraction procedures.

Experimental Section 8

Nucleic Acids

1. The zirconium - morin method for phosphates (f)

Method

(1) Take sections to water.

(2) Immerse in 1% zirconyl nitrate for 10 - 30 mins.

(3) Wash in two changes of water.

(4) Take through the alcohols to 95% alcohol.

(5) Immerse in 0.1% morin in 95% alcohol for 10 mins.

(6) Wash in alcohol, dry in air and examine for fluorescence as dry mount.

RESULT

Sites which have adsorbed zirconium ions fluoresce a bright-yellowish-green. These sites correspond to nucleic acids.

2. Attempts at a Wheeler - Johnson test for uracil and cytosine residues in situ

Sections which had been taken down to water were oxidised in dilute (2%) bromine water for 30 mins. After rinsing the oxidised sections briefly in water, they were immersed in 1% barium nitrate solution for 30 mins. A purple colouration was not formed in cytoplasmic (RNA) or other sites.

3. Attempted ozidations of thymine residues of DNA.

Section which had been taken to water and then oxidised either with 0.5% potassium permanganate for 30 minutes or with 100 vol.

hydrogen peroxide at 37° for 30 minutes. The oxidised sections were condensed with salicyloyl hydrazide as per stages 6 - 11 in exp. sec. 7.1. No discrete blue fluorescence was subsequently observed.

4. The Feulgen - salicyloyl hydrazide method for DNA (f)

Method

(1) Take dewaxed sections to water.

(2) Hydrolyse in N hydrochloric acid at 60° for 5 - 10 minutes.

(3) Wash once in N hydrochloric acid at room temperature

(4) Rinse briefly in two changes of water.

(5) - (8) As stages 4 - 7 in exp. sec. 12.14.

(9) Dehydrate through the alcohols, clear in xylene and mount in Fluoromount. Examine for fluorescence.

RESULTS

Sites exhibiting a bright blue fluorescence contain DNA (nuclei).

Section 9 Sulphate Groups

1. Introduction

There are no direct chromogenic chemical tests for sulphates or sulphonic acids. The only characteristic property of sulphates *in vitro* is the insolubility of their barium salts in water or alcohol. Even sulphated acid mucopolysaccharides have been isolated from crude homogenates in this way, but *in situ* they do not appear to take up barium ions; when sections were immersed in solutions of barium nitrate, very little barium could be detected subsequently in the acid mucin sites when sodium rhodizinate and hydrochloric acid (Waterhouse, 1951; Pearse, 1960) or 1% alizarin red S (Rozenburg and Viasova, 1959) were used as visible complexing agents. Before attempting to complex the adsorbed barium with either reagent, sections were washed in alcohol to remove barium ions adsorbed onto nucleic acid phosphates: barium phosphates, unlike barium sulphates, are insoluble in water but are soluble in alcohol.

If the barium ions are *firmly* bound to the sulphate groups, they should not react with organic complexing reagents. Therefore the failure to demonstrate barium adsorbed onto acid mucins might be due either to a strong barium-mucin bond or alternatively to the non-adsorption of barium ions in the first place. The second explanation is probably correct; the basophilia of sections towards azure A after treatment with solutions of barium nitrate was hardly altered; the acid

mucopolysaccharides exhibited their normal ϰ- meta-chromasia, although the chromasia of the nuclei changed from alpha to beta.

As aromatic sulphonic acid groups can condense with aromatic hydrazines in vitro (Latimer and Best, 1937), it was thought that this reaction could be applied as a histochemical test for the isomeric sulphate groups of acid mucopolysaccharides, especially as Bonomi (1951) and Pearse (1951) had successfully used a similar reaction for the detection of oxidised thiol groups in tissues sections. However no visible coloration could be observed in the acid mucin sites in sections of guinea pig gut, for example, after they had been incubated in a mixture of phenylhydrazine and acetic acid, nor could any fluorescence be detected after they had been immersed in an acidified solution of salicyloyl hydrazide (Sec. 5.2/2iv). When the phenylhydrazine-treated sections were stained afterwards in azure A, it was found that the basophilic chromasia of the acid mucin sites was exactly the same as that in control sections. This confirmed the suspicion provided by the earlier experiments that hydrazines do not react with sulphated mucopolysaccharides in situ.

Although histochemical methods which rely upon the conjectured affinity of sulphate groups for barium ions or aromatic hydrazines are unsuccessful, techniques based upon the basophilia of sulphate groups towards basic dyes or colloidal inorganic cations are much more reliable. These methods can be made specific for sulphate groups by devising

techniques for blocking or extracting other types of basophils likely to be present in tissue sections.

Using the method introduced by Pischinger (1926, 1927), Dempsey et al. (1946; 1947; 1950) and Pearse (1949, 1950) found that the degree of basophilia of tissue components varied with pH. The results of these investigations led Pearse (1953, 1960) to conclude that if "nucleic acids (phosphate groups) can be excluded, the capacity to bind basic dyes (e.g. methylene blue) below pH 4 almost certainly indicates sulphate groups and thus acid mucopolysaccharides". This statement has formed the theoretical basis for recent histochemical investigations of acid mucopolysaccharides (e.g. Spicer, 1960). However, there are both theoretical and practical objections to its validity.

(1) In my experience, solutions of basic thiazine dyes (e.g. toluidine blue or azure A) at, or below, pH 3 never stained nucleic acids or acid mucopolysaccharides in different types of tissue which had been fixed by freeze-drying, glycol ether, formalin or alcohol. This failure may be due to idiosyncracies of the particular batches used in my experiments, but it serves to illustrate the unreliability of using such dyes as an absolute guide. Other workers, such as Ball and Jackson (1953), have also reported that the staining properties of toluidine blue vary from batch to batch.

(2) Stoichiometric studies on the uptake of basic dyes by acid mucopolysaccharides in vitro have shown that even at pH 3 approximately half the uronic acid groups of hyaluronic

acid bind basic dyes (Szirmai and Balazs, 1958).

The stoichiometry in tissues is even more complicated. Under optimal conditions, the molecular ratio of dye bound per anionic group of acid mucopolysaccharides in whole tissue sections usually exceeds that in tissue extracts; in the case of nasal septum cartilage, ratios of 1.2 to 1.3 have been found (Szirmai and Doyle, 1961). Purification of extracts by deproteinisation brings the ratio closer to unity showing that the extra uptake is due to protein carboxyl groups.

At a low pH, dye binding by sulphate groups in tissues may be suppressed by relatively large amounts of surrounding protein possibly because of a competing or repulsive effect of their cationic amine groups (Szirmai and Doyle, 1961). Several observations in the past have suggested such an interfering effect on the metachromatic shifts in the absorption spectra of dye solutions (French and Benditt, 1953; Hamerman and Schubert, 1953; Kelly, 1955).

(3) Carboxyl groups in tissues are usually thought to be undissociated below pH 4, or at the very least, below pH 3 (Springall, 1954). However, some amino-acid constitutents of tissue proteins have acid dissociation constants (pKa) of the order of 10^{-2} and therefore their carboxyl groups will be dissociated approximately 50% at pH 2. Even at this low pH, there are still sufficient ionized carboxyl groups capable of taking up basic dyes.

Although no instances of the uptake of basic dyes at low acid levels of pH have been recorded for tissues examined by

transmitted white-light microscopy, a green (or sometimes, blue) fluorescence has always been observed in the protein sites of tissue sections which have been stained with a dilute (0.01%) solution of coriphosphine at pH between 2 and 3, even when RNA has been previously extracted from the section with hot perchloric acid. This is particularly noticeable in smooth muscle (similar to Fig.23) whose principal proteins, myosin and tropomysin, have a relatively high proportion of carboxyl groups (Dubuisson and Hamoir, 1943). The principal amino acids of both these proteins are lysine, leucine, glutamic and aspartic acids (Bailey, 1948) whose first pK_a values vary from 1.8 to 2.36 (Edsall, 1943). At pH 2.7 their percentage dissociations would therefore range from 69 to 87.

By staining tissue sections at a pH well below 2, the uptake of basic dyes by carboxyl groups can be largely suppressed. However, even at pH 1.5, the proteins of the muscle are still stained by coriphosphine as is apparent by their bright blue fluorescence (Fig.33). At this pH, the dissociation of the carboxyl groups can vary from 30% to 12%.

Pearse's (1953, 1960) hypothesis, that only phosphate and sulphate groups take up basic dyes below pH 4.0, is therefore not correct: the dissociation and basicity of carboxyl and uronic acid groups progressively decrease as the pH is lowered from 4 to 2 and they will still take up basic dyes within this range.

(4) Certain lipid complexes can also take up basic dyes

at an acid pH (Ries, 1935; Holftreter, 1947). Fluorescent dyes are particularly lipophilic (Volk and Popper, 1944). This complication should not arise, however, with paraffin-embedded sections.

2. The visible metachromasia towards thiazine dyes

Acid mucopolysaccharides are visibly stained red (called γ-metachromasia originally by Michaelis, 1947 and introduced into histochemical terminology by Pearse, 1953) in situ by dilute solutions of thiazine dyes. This test has been used as a standard by which the efficacy of other techniques for demonstrating acid mucopolysaccharides could be judged. Nuclei (DNA) and cytoplasmic RNA are stained blue (orthochromasia) or sometimes purple (β- metachromasia), but partially depolymerised DNA can also exhibit γ-metachromasia (see 9.8/2).

In agreement with Kelly (1956), Pearse (1953, 1960) and others, I have found that γ-metachromasia is destroyed by washing sections in alcohols stronger than 30%. Sylven's concept (1941, 1945) that alcohol resistant γ-metachromasia is "true" and alcohol-labile metachromasia is "false" cannot therefore be upheld, despite Kramer and Windrum's (1954, 1955) experiments and assertion that alcohol never reverses metachromasia due to ester sulphates.

In view of the variable results reported by others, a standardised procedure was adopted for this investigation as follows: sections were stained in 0.02% azure A dissolved in unbuffered 30% alcohol (pH 5 - 7) for 20 - 30 minutes,

rinsed briefly in 30% alcohol, examined and, if necessary, photographed as water mounts. If the sections were mounted in Aquamount or glycerine, the γ-metachromasia was immediately destroyed.

The inability to make permanent mounts or to obtain high resolution is a serious disadvantage of methods based on metachromasia. Dyes other than thiazines can also stain metachromatically but the colour shift is usually less (Kelly, 1956).

3. The usefulness and limitations of fluorescent dyes

All acid mucopolysaccharides *in situ* take up basic fluorescent dyes, but with the exceptions of acridine orange and coriphosphine, their fluorescence is usually quenched. Acriflavine, for example, has been used as a visible stain in conventional histochemistry for sulphated acid mucopolysaccharides (Takeuchi, 1962), but the fluorescence of the mucin-dye complex is very weak. Even auramine O, which forms extremely intense fluorescent conjugates with all other basophils, has its potential fluorescence quenched. On the other hand, neutral mucopolysaccharides that have been sulphated by sulphuryl chloride vapour, forms strong fluorescent complexes with most aminoacridine dyes *in vitro* but not *in situ* (sec.13.5).

Presumably the ionic charge density on the sulphate groups of acid mucopolysaccharides is so high that it distorts the symmetry of the π-electron system of the dye and thus destroys one of the essential properties required for a dye to

fluoresce. This is especially noticeable for fully sulphated glycogen that has been stained *in situ* with coriphosphine; the fluorescence of coriphosphine is reduced to very dull brown-red colour.

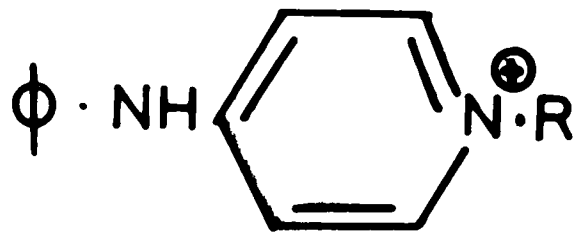
There is a tendency for coriphosphine or acridine orange adsorbed onto acid mucopolysaccharides to undergo photolytic decomposition to nonfluorescent products after a prolonged exposure to ultra-violet light. Coriphosphine is less susceptible than acridine orange, and the former has therefore been preferred. Keeble and Jay (1962) also noticed that the fluorescence of nucleic acids in tissue sections stained with coriphosphine was stable to ultra-violet light unlike that of sections stained with acridine orange.

The problem of avoiding the quenching of the fluorescence of dyes adsorbed onto acid mucopolysaccharides could be solved by using a dye in which the fluorescent moiety was far removed spatially from the cationic centre at which the dye is adsorbed onto the tissue basophil. Evans and Kent (1962) did this by conjugating or labelling basic polysaccharides isolated from cultures of *Aspergillus parasiticus* with fluorescein; such polyvalent cations form strong fluorescent complexes with acid mucins and to a lesser extent with nucleic acids and proteins. Unfortunately the preparation of such conjugates is neither simple nor convenient and attempts were therefore made to synthesise a more convenient compound.

The basic antibiotic Polymyxin B was coupled with Lissamine Rhodamine B (RB200) sulphonyl chloride (prepared

according to Chadwick et al., 1958), but it failed to stain acid mucopolysaccharides specifically. This was disappointing in view of its specific adsorption onto polymyxin - sensitive sites in bacteria with the DANS (1 - dimethylaminonaphthalene - 5 - sulphonylchloride) conjugate (Newton 1955).

Another approach which was tried was to use fluorescent derivatives of cationic detergents of the type:

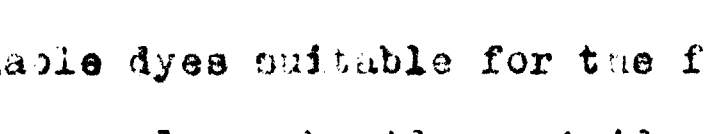


where ϕ is a fluorescent dye (e.g. RB200 sulphonyl chloride) and R is a long chain hydrocarbon. The structurally similar cetyl pyridinium salts form insoluble complexes with acid mucopolysaccharides in vitro (Scott, 1955 a, b) and have been used for fixing mucins in tissues (Williams and Jackson, 1956). Pretreatment of conventionally fixed sections in solutions of these salts promptly blocks all the tissue basophilia towards fluorescent dyes.

As a first step in an attempt to synthesise a dye of this type, p-aminopyridine was boiled with hexadecyl bromide according to an analagous synthesis for cetyl pyridinium bromide (Hauser and Niles, 1941). The ring nitrogen atom should be selectively alkylated (Chichibabin and Ossetrova, 1955). However the few colourless, monoclinic crystals which were deposited from a filtered aqueous extract of the resulting tarry mixture boiled with activated charcoal were believed to be unchanged p-aminopyridine: when dissolved in

water, they had no detergent properties nor did they block the basophilic staining of gut sections.

The effectiveness of polymethine dyes for staining acid mucopolysaccharides was also investigated. On the assumption that pyridine forms fluorescent polymethine dyes in the presence of cyanogen bromide and amines (sec. 3.3), a few drops of cyanogen bromide and ammonia solutions were added to aqueous solutions of pyridine or cetyl pyridinium chloride. Opalescent fluorescent solutions were formed, but both solutions failed to stain tissue basophils, so that dyes of the structure shown below could not have been formed.



At the present moment, acridine orange and coriphosphine are the only available dyes suitable for the fluorescent staining of acid mucopolysaccharides. Acid mucopolysaccharides stained with coriphosphine can be exposed just long enough to ultra-violet for a reasonable photomicrograph on High-Speed Ektachrome to be obtained. At the end of a longer exposure, as might be required for a photomicrograph on the slower Kodachrome II, nearly all the coriphosphine fluorescence is destroyed. Less photolysis takes place in water mounted sections than in dry mounts.

4. <u>The coriphosphine - thiazol yellow fluorescence method</u>

The fluorescence of nuclei in fixed sections that have been stained with either acridine orange or coriphosphine is of practically the same colour as that exhibited by acid

mucopolysaccharides, and a clear, diagnostic appearance cannot always be obtained. This confusing fluorescence appearance is illustrated in Fig. 31.

Fortunately all tissue basophils other than acid mucopolysaccharides also have basic (amine) groups as well as acidic groups. If sections are first overstained with the basic dye coriphosphine and then counterstained with a strong acidic dye of a different fluorescent colour, only the acid mucopolysaccharides will retain the fluorescent colour of the basic dye. The most suitable acidic dye found for doing this so far is thiazol yellow (Fig. 69) which is best used as a 0.001, solution. A 0.01% solution removes coriphosphine from acidophils within 2 seconds and starts to elute the basic dye from acid mucopolysaccharides after about 30 seconds.

The fluorescent appearance of guinea pig gut stained in this way is shown in Fig. 29. A few nuclei still have a slight orange tinge. A similar distibution of differently staining nuclei can be obtained with section that have been subjected to a Feulgen hydrolysis, condensed with salicyloyl hydrazide and counterstained with the acidic Solochrome Black-alum complex (sec. 8.5/2). Presumably the difference is due to partially depolymerised DNA in some nuclei having a lower nucleotide content than others.

5. <u>Selective basophilia.</u>

Despite the objections outlined in section I, the basophilia of sulphate groups towards coriphosphine at pH 1.5 - 2

and pH 5 has been adopted as the standard fluorescent histochemical test on tissues from which the phosphate groups have been selectively removed by the technique to be described later. When Pearse (1960) made his statement concerning the differential ionisation of tissue basophils (sec. 9/1), methods for selectively extracting nucleic acids in the presence of acid mucopolysaccharides had not then been devised. The reason for staining at a pH above and below 4 is to exaggerate the subsequent fluorescent appearance of the tissue. At both pH 2 and 5, sulphate groups (of acid mucopolysaccharides), fluoresce initially brown or brownish red. Protein carboxyl groups fluoresce blue or green after staining at pH 5 while at pH 2 the intensity of their fluorescent colour is usually much weaker. Uronic acid groups (e.g. of hyuluronic acid in cock's comb) take up no dye below pH 2.0 (sec. 9/1/2).

As it is sometimes difficult to distinguish the fluorescence of nuclei and acid mucopolysaccharides, it is essential to remove or block one before staining for the other.

Four different approaches have been investigated for the selective blocking or removal of phosphate groups in the presence of acid mucopolysaccharides. They involve the use of salts, mineral acids, enzymes or reducing agents.

6. <u>The influence of salts on tissue basophilia</u>.

It is well known that simple inorganic electrolytes (e.g. sodium chloride or buffer salts) dissolved in solution of basic

dyes can reduce the staining of tissues (Landsmear, 1951; Singer 1952; Holman 1961; Radden 1961). Presumably salts compete preferentially for tissue anionic sites or they cause stains to flocculate with a consequent reduction in diffusibility (Baker, 1958). For these reasons, all dye solutions in my experiments have been made up without the addition of buffer salts. The pH of each solution was adjusted with the minimum amount of hydrochloric acid and was checked frequently.

Electrolytes can also induce misleading staining-artefacts by extracting, for example, cytoplasmic RNA from the tissue (Stowell and Lorzoli, 1947; Opie and Lavin, 1946).

Recently, the differing equilibra between salts and basic dyes (acidine orange) have been applied to the identification of individual acid mucopolysaccharides (Saunders, 1962). Although such procedures may be theoretically justified, they have severe limitations in practice (Schubert and Hamerman, 1956), particularly in fluorescence histochemistry.

In an attempt to stain acid mucopolysaccharides selectively by blocking the nucleic acid phosphate groups with magnesium ions as suggested by Friedenwald (1947) or by repressing the dissociation of protein carboxyl groups (Scott, 1955), sections were stained in buffer salt-free solutions of fluorescent dyes (e.g. coriphosphine) saturated respectively with magnesium sulphate or aliphatic carboxyl acids such as oxalic acid. Unfortunately, neither substance significantly altered the

uptake of fluorescent dyes.

Nearly all cytoplasmic RNA can be extracted with physiological saline after 5 to 6 hours treatment at 37° (Opie and Lavin, 1946). For example, the visible basophilia towards azure A of the cytoplasm of guinea pig gut was abolished after this time, but an unchanged green fluorescence due to carboxyl groups was still observable in sections subsequently stained with coriphosphine (sec. 9.7/2). The staining of acid mucopolysaccharides was also reduced, showing that even they are dissolved out of sections during prolonged immersion in these solutions.

7. <u>The extraction of nucleic acids with mineral acids</u>

(1). <u>Hydrochloric acid</u>.

Sevag <u>etal</u> (1940) and Dempsey <u>etal</u> (1950) used dilute hydrochloric acid for the extraction of nucleoprotein of both types from tissue homogenates and sections respectively.

NA in my test sections was removed by N hydrochloric acid at 37° after one hour, but after that time, both DNA and acid mucopolysaccharides were removed simultaneously. After four hours, the azurophilia of the whole tissue had been abolished.

A five minutes immersion in N hydrochloric acid at 60° removed cytoplasmic RNA <u>and</u> some proteins from sections of guinea pig gut as shown by the <u>blue</u> fluorescence of their cytoplasm after staining in coriphosphine. When only the

RNA was removed from serial sections with R nase or hot perchloric acid (secs. 9.8/1 and 9.7/2), the cytoplasmic fluorescence was still green. The proteins extracted by hot hydrochloric acid are probably of the histone type (Stedman and Stedman, 1950).

Many of the DNA phosphate groups were also hydrolysed off and resulted in the exposure of the aldehyde groups from the sugar fraction (Feulgen hydrolysis). The nuclear fluorescence was weak when stained in acidic solutions of coriphosphine (pH 2.6). Neutral solutions of coriphosphine, however, stained nuclei much more strongly (Fig. 30). Moreover it was even more difficult to distinguish the fluorescence of nuclei from that of acid mucopolysaccharides in acid-extracted sections stained with 0.1% solutions of coriphosphine than in unextracted sections; the acid mucopolysaccharides often exhibited an enhanced fluorescence. The changes in the fluorescent appearance of both acid mucins and nuclei may be due to a partial removal of sulphate groups from the former with a consequent reduction of their quenching properties and/or to the removal of protective proteins from both basophils.

Both DNA and acid mucopolysaccharides were removed by hot hydrochloric acid from sections treated for periods longer than 5 minutes.

(2). Perchloric acid.

Erickson etal (1949) and Seshacher and Flick (1949)

Fig. 31. Guinea pig gut. Fixed in glycol ether.

N Hydrocholoric acid, 60°, 5 mins. : 0.01% coriphosphine, pH 5.0. Water mount, X 180.

The residual protein carboxyl groups in the protoplasm and muscle fluoresce a greenish-blue colour. The reddish-brown fluorescence of the acid mucopolysaccharides is difficult to distinguish from that of nuclei (DNA).

Fig. 32. Guinea pig gut. Fixed in glycol ether.

Perchloric acid 60°, 5 mins. : azure A. Transmitted white light. Water mount, X 165.

Acid mucopolysaccharides and nuclei (DNA) are stained whereas the protoplasmic protein sites (which originally contained RNA as well) are not.

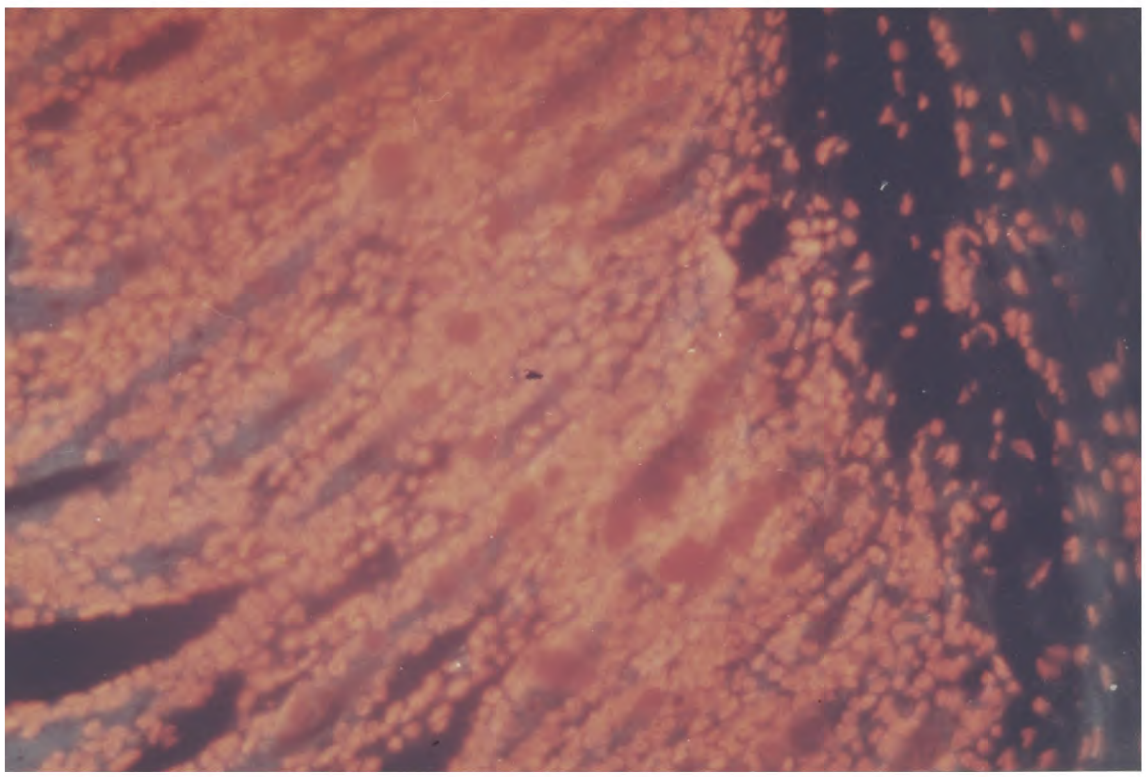


Fig. 31

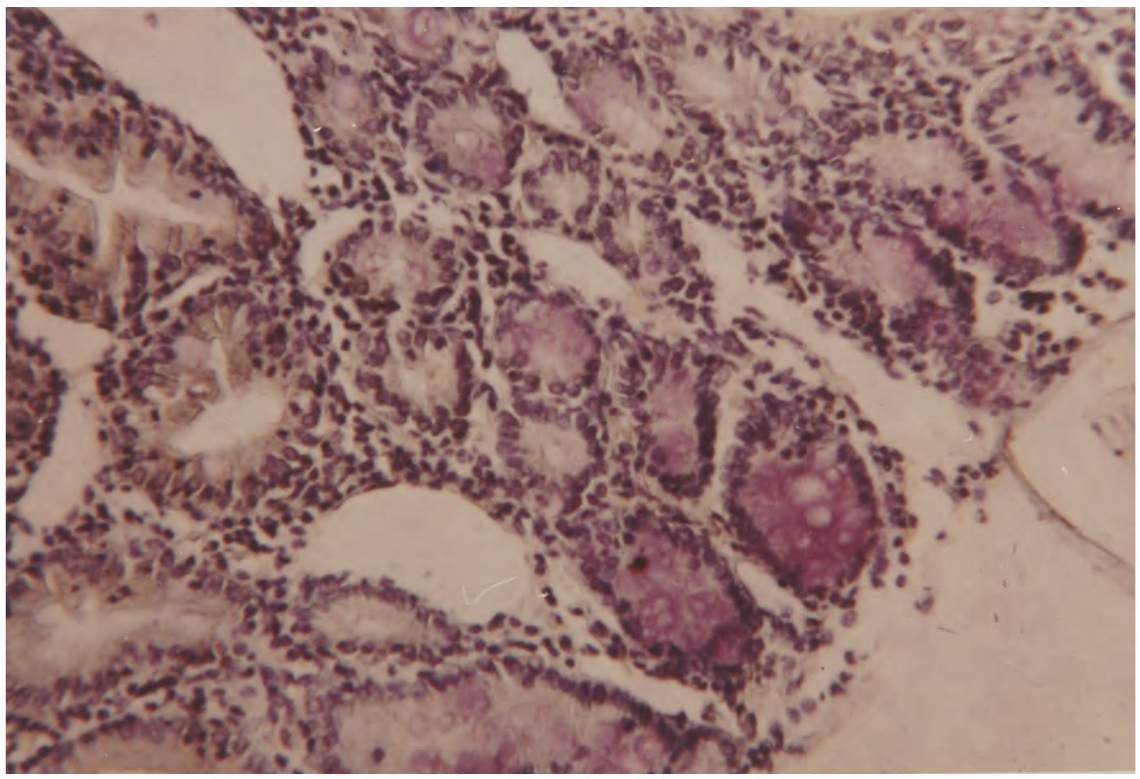


Fig. 32

employed perchloric acid of varying strengths to extract both types of nucleic acids from tissues. Cold perchloric acid extracted only RNA after 24 hours treatment. The affinity of DNA for methyl green was diminished after 16 hours, although the Feulgen reaction was still unaltered. In my hands, 5 - 10 minutes treatment with 5% perchloric acid at 60° abolished the visible azurophilia of cytoplasmic RNA and left the staining of nuclei and acid mucopolysaccharides unchanged (Fig. 32).

The interpretation of the changes in the fluorescence of sections that have been stained with fluorescent basic dyes after extraction with perchloric acid are more complex than would at first appear. In sections of guinea pig gut (fixed in gylcol ether) treated and stained in this way with C.C1, coriphosphine at pH 5, the cytoplasmic fluorescence was green and the muscle a bright yellowish green. On prolonged exposure to ultraviolet light, the colours changed a little, the cytoplasmic fluorescence becoming sea-green and the fluorescence of the muscle a bright light green.
The intensities of the fluorescent colours in both cases were considerably stronger than these observed in control sections.

The higher uptake of dye by the muscle, which gives rise to the strong fluorescence is probably due to the higher concentration of protein terminal carboxyl groups in that area (sec. 9.1/3). The reasonably strong fluorescence

reaction specific for C-terminal carboxyl groups (sec. 7.2) is shown for the muscle in Fig. 19.

Staining acid-extracted sections of gut with 0.01% coriphosphine at pH 2 resulted in a weak blue fluorescence in both the cytoplasmic and muscle areas (Fig. 34) showing that RNA has been extracted. The fluorescence of the nuclei was also reduced, showing that some DNA phosphate groups were hydrolysed off by perchloric acid. The uptake of coriphosphine by acid mucopolysaccharides was variable and often their fluorescence was destroyed after a photomicrograph had been taken.

Stronger solutions of coriphosphine (0.1% pH 2.7) stained sections that has been extracted with perchloric acid much more satisfactorily. The fluorescence of nuclei and acid mucopolysaccharides were much stronger, although the cytoplasmic and muscle fluorescence were still bluish-green (Fig. 35). When sulphur dioxide was present in the dye solution (pH 2.6), the fluorescence of the cytoplasm and muscle acquired a slightly greener tint which became more pronounced on longer exposure to ultra-violet light. (Fig. 36). The increase of the fluorescence of the nuclei is no doubt due to the hydrolysis of their DNA content to aldehydes which subsequently combine with the pseudo-Schiff complex. The intense fluorescence of the acid mucopolysaccharides is an unusual feature, an explanation for which has been offered in sec. 12.6/4.

Fig. 33. Guinea pig gut. Fixed in glycol ether.

0.1% coriphosphine, pH 1.5. Water mount, X 180.

The proteins in the protoplasm and muscle fluoresce blue. The acid mucopolysaccharides emit an orange fluorescence. The actual yellow fluorescence of the nuclei is more discrete than appears in the photomicrograph.

Fig. 34. Guinea pig gut. Fixed in glycol ether.

Perchloric acid, 60°, 6 mins. : 0.01% coriphosphine, pH 2.3. Water mount, X 180.

The protoplasmic and muscle proteins fluoresce a light blue colour. The brown-red fluorescence of the nuclei is weak. The acid mucins hardly fluoresce at all.

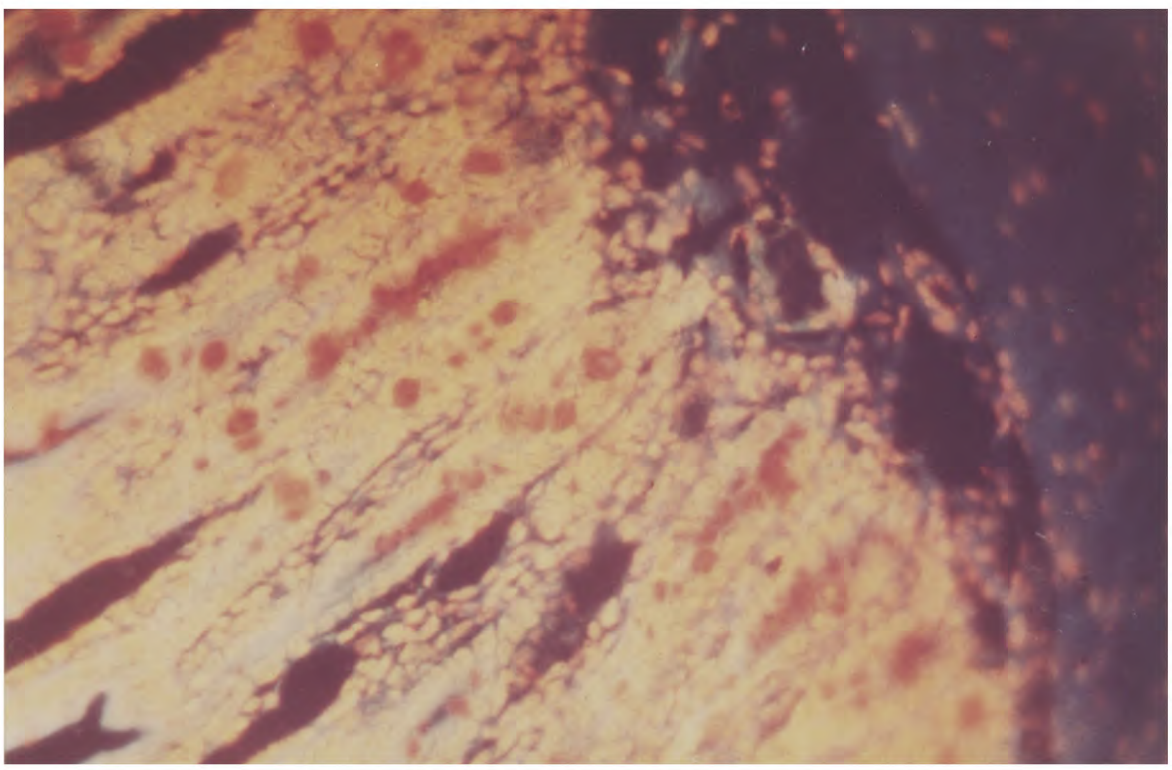


Fig. 33

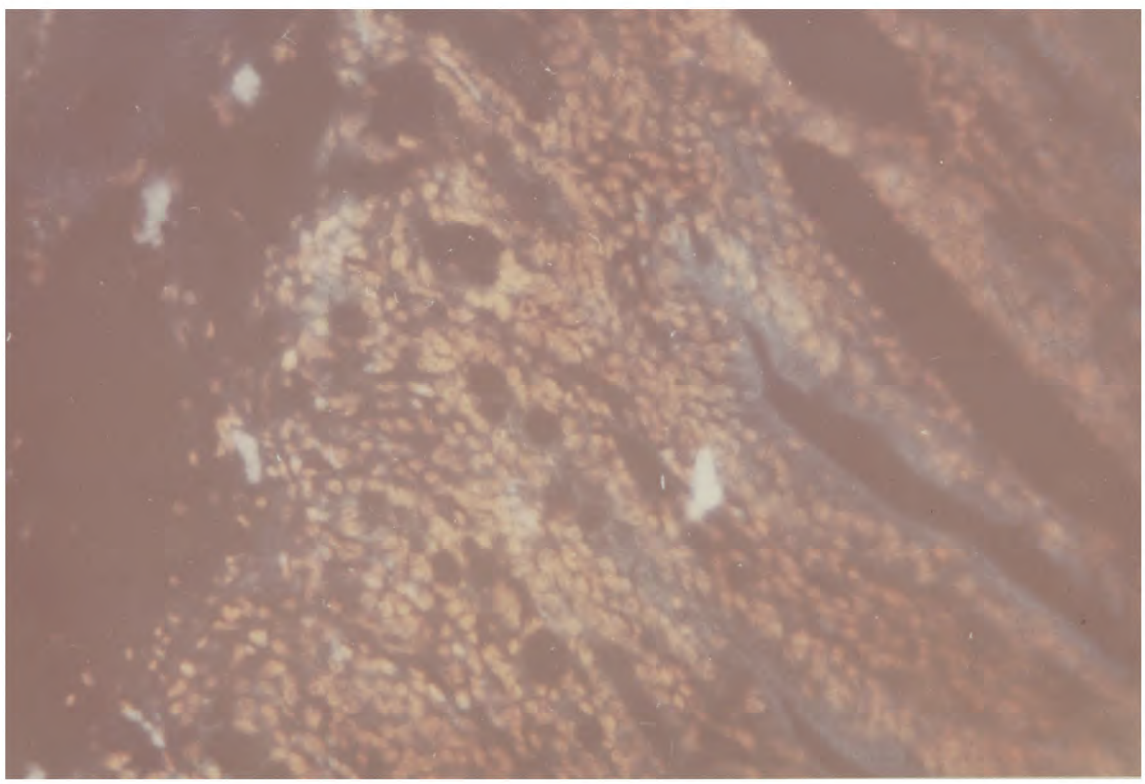


Fig. 34

Fig. 35. Guinea pig gut. Fixed in glycol ether.

Perchloric acid, 60°, 6 mins. : 0.1% coriphosphine, pH 2.7. Water mount, x 180.

The proteins in the muscle and the protoplasm fluoresce a bluish-green colour. The brown fluorescence of coriphosphine adsorbed onto acid mucopolysaccharides is destroyed during the course of the exposure required for this photomicrograph.

Fig. 36. Guinea pig gut. Fixed in glycol ether.

Perchloric acid, 60°, 6 mins. : 0.1% coriphosphine-SO_2, pH 2.7. Water mount, X 180.

The protoplasmic and muscle proteins fluoresce green. The acid mucopolysaccharides emit an anomalous bright orange fluorescence.

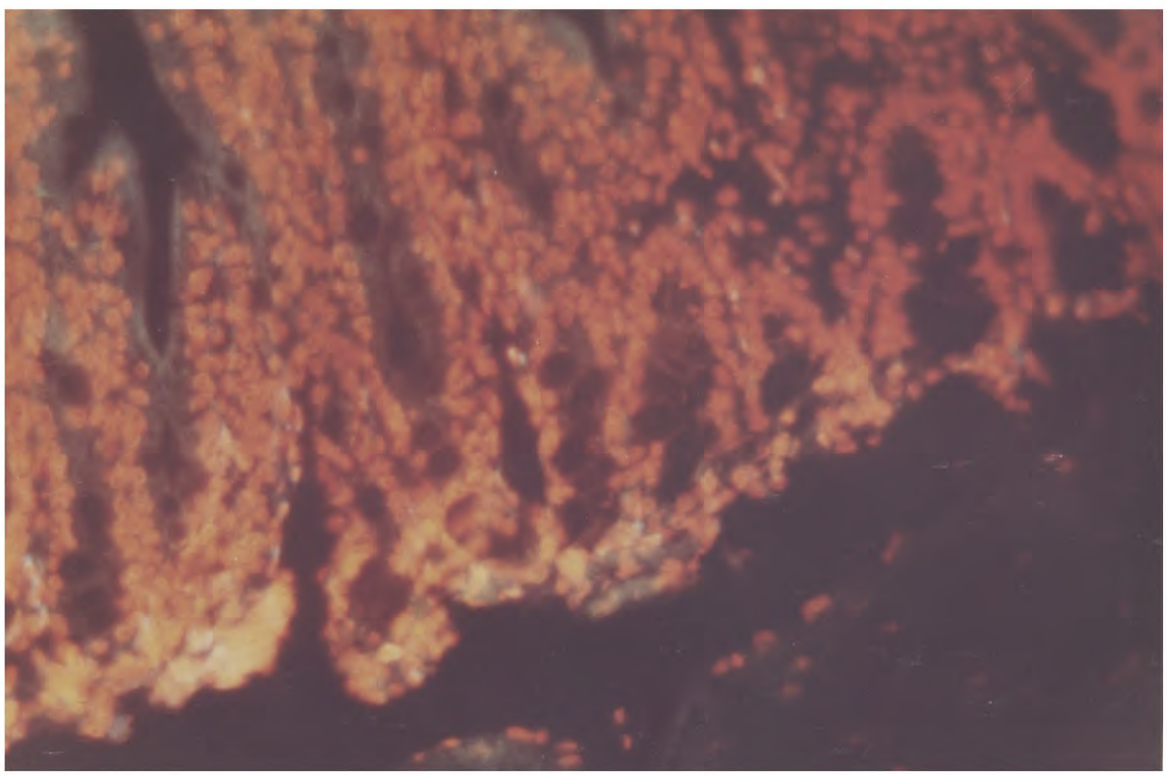


Fig. 35

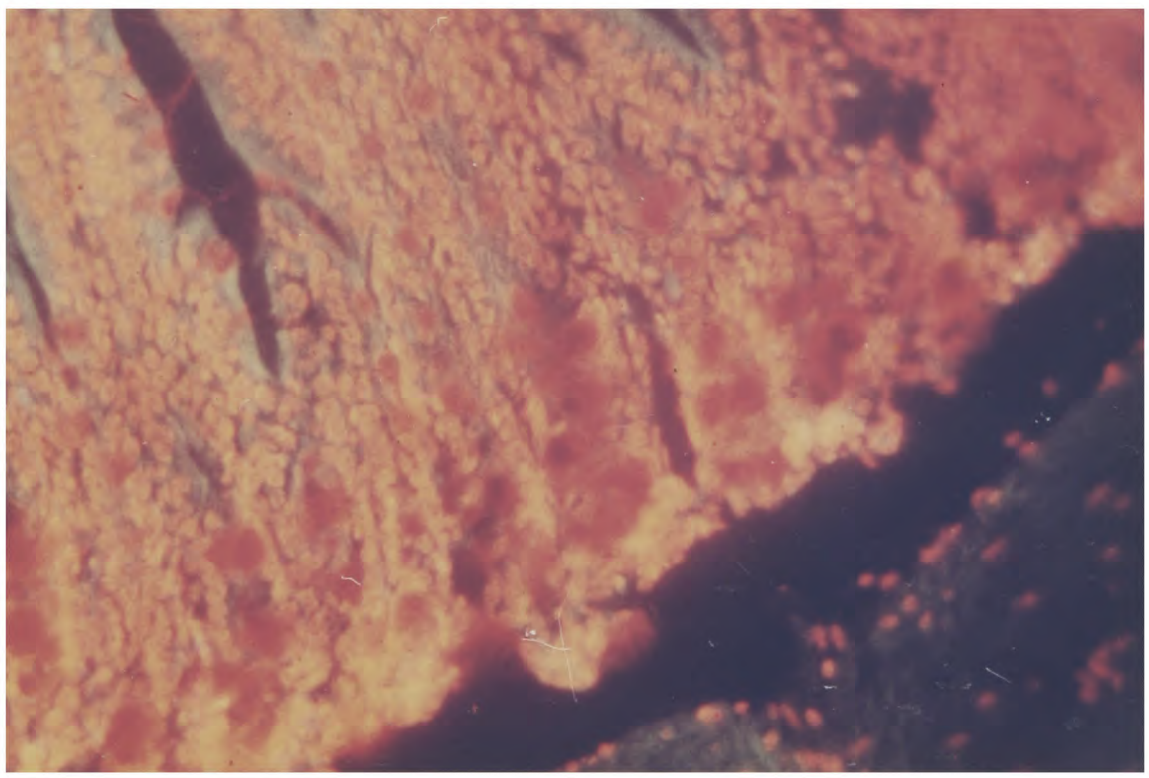


Fig. 36

Neutral 0.1% solutions of coriphosphine always overstained and gave only an orange fluorescence over the whole section.

The lack of a transition from green to blue fluorescence in sections treated with perchloric acid and stained in dilute coriphosphine at pH 5 and 2 cannot be taken as being conclusive for the absence of RNA in the original section. Sections of guinea pig liver (fixed in glycol ether), for example, exhibit the same light green cytoplasmic fluorescence after staining at both pH levels following extraction with perchloric acid. The intensity of the fluorescence after staining at pH 2 is, of course, less than that obtainable after staining at pH 5 (Fig. 37), but both are considerably stronger than the cytoplasmic fluorescence of control sections.

The stronger fluorescence following acid treatment may be due to the hydrolysis of peptide linkages which exposes more carboxyl groups or to the removal of protective amino acids of the kind suggested by Kelly (1955) and others. That protein terminal carboxyl groups are largely responsible for the residual green fluorescence following extraction in perchloric acid can be demonstrated by converting them beforehand to methyl ketones with hot acetic anhydride - pyridine (sec. 7.3). When sections treated in this way were stained in 0.01% coriphosphine at pH 2, a blue fluorescence was produced (Fig. 20) instead of the green fluorescence shown in Figs. 21 or 37. The green fluorescence was always obtained after staining at

Fig. 37. Guinea pig kidney. Fixed in glycol ether.

o

Perchloric acid, 60 , 8 mins. : 0.01%

coriphosphine, pH 5. Water mount, X 180.

The green fluorescence of the protoplasm and the orange fluorescence of the nuclei is brighter than is apparent in the photomicrograph.

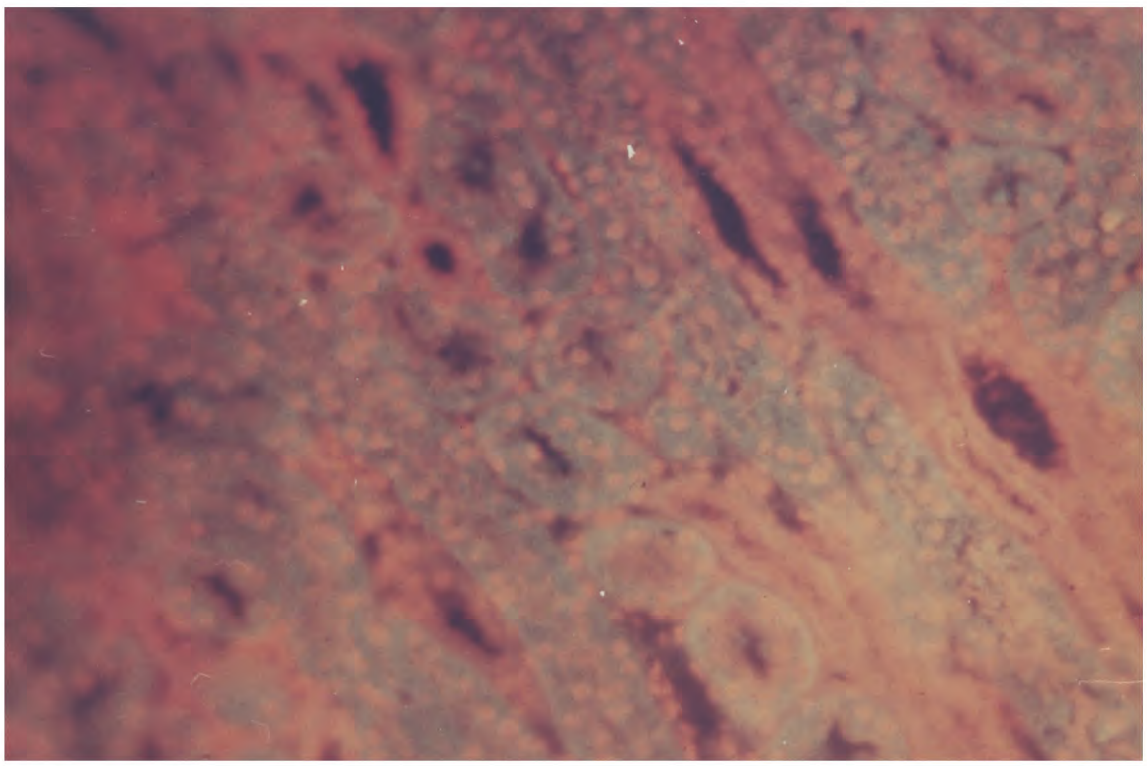


Fig. 37

p. 5. The intensity of the cytoplasmic green fluorescence was considerably less in sections not extracted with perchloric acid. Prolonged treatment (up to 16 hours) in hot acetic anhydride - pyridine made no difference to the ultimate fluorescence nor did reversing the order of the perchloric acid extraction and the acetic anhydride - pyridine treatment.

It could be argued that hot perchloric acid does not remove all the RNA within 5 - 10 minutes, so that the abolition of the green fluorescence after the acetic anhydride-pyridine treatment is due simply to the hydrolysis of RNA by pyridine. The ease with which alkali hydrolyses RNA at room temperature could be cited as supporting evidence (Markham and Smith 1952 a,b). This objection is vitiated by the similar appearance of the cytoplasmic fluorescence of sections previously incubated only in acetic anhydride - pyridine to that of control sections.

By hydrolysing sections in hot hydrochloric acid and perchloric acid respectively for about 5 - 10 minutes each, it was sometimes found that only the acid mucopolysaccharides were stained appreciably by coriphosphine, or at least, their fluorescence could be distinguished from that due to proteins. However, some of these acid mucins were undoubtedly removed. As the times required for the acid extractions varied from tissue to tissue, depending particularly on its fixation, the "double-acid" extraction technique is unreliable.

Other workers (Koenig and Stahlecker 1951, 1952; Frank etal, 1954; Goessner, 1954) have also found that fixation has a considerable effect on the stainable RNA after various times of extraction with perchloric acid. Although ribonuclease can be universally used for the extraction of RNA (Sec. 9.8/1), brief incubation in hot perchloric acid is more convenient as a routine technique once the effect of a particular fixative on a given tissue is known.

(3.) Trichloracetic acid.

Cohen (1944) and Schneider (1945) used hot trichloracetic acid for the extraction of nucleic acids from tissues. Both DNA and RNA are removed by this procedure, which has been applied by Kaufmann etal (1950) to onion root tips and by White (1950) to bone marrow smears. Both these authors have shown that Schneider extraction leaves behind a protein component of the nucleus which can be demonstrated by its increased affinity for acid dyes.

In my hands, hot trichloracetic acid extracted both nucleic acids and acid mucopolysaccharides simultaneously from sections of guinea pi gut. The structure of the tissue became distorted.

(4.) Conclusion

Mineral acids have little value for the selective extraction of nucleic acids in the presence of acid mucopolysaccharides, with the exception of perchloric acid, which can

be used for the selective extraction of RNA. Atkinson (1952) came to a similar conclusion.

8. Enzyme digestions

The use of enzymes seems the obvious solution to the problem of the selective extraction of nucleic acids from tissues so as to leave mucopolysaccharides intact. RNA can be removed specifically by enzymic digestion, but unfortunately the conditions required by deoxyribonuclease to hydrolyse its substrate also removes acid mucopolysaccharides.

(1) Ribonuclease (RNase)

RNase breaks the ester bonds between pyrimidine - nucleoside 3^1 - phosphate esters and the 5^1 - hydroxyl groups of neighbouring nucleotide residues and transfers the 3^1 - phosphate to the 2^1 - phosphate of the pyrimidine nucleotide itself to form a cyclic nucleotide.

RNase derived from pancreas, unlike leaf RNase, also catalyses the transfer of the phosphate group from the 2^1 - position in the cyclic phosphate in water; this results in the complete depolymerisation of RNA. (Markham and Smith 1952a; Brown etal 1952, 1956). The pH of optimal activity is 7.7 (Kunitz, 1940).

Brachet (1940 a,b,) first reported the use of the enzyme for the extraction of RNA in histochemistry. Later Stowell and Zorzoli (1947) drew attention to the f ct that the buffer salts in the enzyme solution could themselves remove variable

amounts of RNA (cp. sec. 9.6).

Protease-free RNase dissolved in glass-distilled water (Kearse, 1960) has been used as a standard by which the efficacy of other methods for extracting RNA could be measured. RNA was reckoned to be completely removed when the visible cytoplasmic azurophilia had been abolished. Nevertheless, 0.1% solutions of enzyme (20 - 40 Kunitz units per ml), although satisfactory when used at 37° on all my test sections, are not universally suitable; Leduc and Bernhard (1960) found that these solutions were not strong enough to affect the basophilia of liver and pancreas fixed in formalin. With higher concentration (0.7% and 1%), the cytoplasmic basophilia was completely abolished after 24 hours at 54°, but was irregular or incomplete at 37° (Leduc and Bernhard, 1960a).

(2) Deoxyribonuclease (DNase)

DNA is hydrolysed by pancreatic DNase to di- , tri- , and larger polynucleotides. Only about 25% of the 3^1 - phosphate ester bonds in DNA are hydrolysed. (Sinsheim and Koerner, 1952; Sincheim, 1954; Smith and Markham, 1952 a,b). The enzyme is stable in the pH range 4 - 9, and has its optimal activity at pH 6 - 7 (Kunitz, 1950 a,b,).

The cytochemical application of DNA has not been uniformly satisfactory and it is doubtful whether it ever will be. Leduc and Bernhard (1961) found that the Feulgen reaction of nuclear chromatin can never be abolished by prior digestion in

solutions in DNase (0.4% and 1.0%) containing magnesium ions. Following Peters and Stockenius's (1954) suggestion, Leduc and Bernhard (1961) pretreated formalin - fixed pancreas and thymus with pepsin before incubating with DNase. Only then were nucleo - protein and the chromosomes of dividing cells eventually removed.

However, the acid conditions required for protease digestions militate against their use as part of a routine method for removing DNA selectively; acid mucopolysaccharides would also be extracted. Even without a preliminary digestion with protease, solutions of crystallised DNase can remove acid mucopolysaccharides from tissue sections at the same rate as DNA.

In my hands, 0.1% solutions of the enzyme (4000 dornase units per ml) in distilled water (pH 7.0) containing 0.003M magnesium sulphate reduced the azurophilia, but not the Feulgen reaction of nuclei after 2 hours in sections of guinea pig gut that had been fixed in glycol ether. The staining of acid mucopolysaccharides and cytoplasmic RNA appeared unimpaired. Kaufman <u>etal</u> (1951) used 0.1-1.0% solutions in distilled water at pH 6 without adding magnesium, or 0.01 - 1.0% solutions at pH 6 with added magnesium salts as an activator.

The buffered solutions of DNase recommended by Pearse (1960) and Kurnick (1952) were better; only the acid mucopolysaccharides exhibited any substantial azurophilic staining

in visible light although the fluorescence of sections stained in coriphosphine showed some loss of acid mucopolysaccharides and a reduction in the basophilia of some nuclei.

The Feulgen reaction was almost abolished after 60 minutes digestion with 0.4% unbuffered solutions (16,000 dornase units per ml) of enzyme, but nearly all the nuclei stained metachromatically with azure A and could hardly be distinguished from acid mucopolysaccharides. The variance between the Feulgen reaction and theazurophilia shows how difficult it is to judge the completion of DNase digestions. Cerotti (1955) has also observed Feulgen - negative nuclear sites in routine sections which were stained metachromatically by toluidine blue. Earlier Lison (1949) had found that the metachromasia of thiazine dyes in both solutions and <u>in situ</u> could only be produced when the dye nucleic acid ratio was high. The metachromasia in my experiments is therefore probably due to the low concentrations of the residual undigested or de-polymerised DNA.

After 90 minutes digestion, the 0.4% solutions of the enzyme removed nearly all theDNA and acid mucopolysaccharides from sections of gut, leaving the cytoplasmic RNA practically intact. 1% enzyme solutions (43,000 dornase units per ml) extracted both nucleic acids and acid mucopolysaccharides simultaneously, both extractions being complete in one hour. The removal of acid mucopolysaccharides from the section could be reduced, but not prevented, by dissolving an acid mucin

coagulant, such as cetyl pyridinium chloride in the enzyme solution. Such solutions also removed some RNA.

9. **Selective reduction**

Phosphate groups (as their methyl esters) of nucleic acids have been found susceptible to reduction *in situ* by complex metal hydrides while sulphate groups are unaffected. The sulphate groups of simple sugar sulphates (previously methylated with diazomethane) can be removed *in vitro* by lithium aluminium hydride (LAH) in boiling dioxane (Grant and Holt,1960), but those on polysaccharides are unreactive (Holt and Mulvin,1962). The reduction of methylated carboxyl groups (of proteins and uronic acids) is discussed in secs. 10.2/2 and 10.4/1.

Methylated sections (sec. 11.4) were unaffected by LAH dissolved in ether at room temperature, but required a higher temperature (in dioxane at 60°) before any reduction took place. Subsequent saponification with alcoholic potash revealed only sulphate groups (and protein carboxyl groups), which could be demonstrated by their brown fluorescence after staining in coriphosphine, or by their visible γ- metachromasia after staining in azure A (sec. 10.2/2). The morphological structure of the sections was left in a good condition. By staining the reduced and saponified sections in 0.1% coriphosphine at a pH less than 2, only the sulphate groups should be evident. Unfortunately, the intensity of their brown fluorescence is

relatively poor when stained at this pH. Better results can be obtained with stronger solutions (0.1%) at a pH nearer neutrality: the sulphated acid mucopolysaccharides fluoresce brown which contrasts with the light green fluorescence background due to protein carboxyl groups. A few nuclei can sometimes be seen as a faint brown fluorescence. When the stained sections are exposed continuously to ultra-violet light as is required for a photomicrograph, the brown fluorescence of the acid mucopolysaccharides is rapidly destroyed (Fig. 40). Presumably less coriphosphine is taken up by acid mucopolysaccharides after LAH reduction than those in untreated sections. The lower uptake may be due to any or all of the following causes:

(a) Some sulphate groups may be removed by LAH.

(b) The saponification time may be insufficient to demethylate all the sulphate groups.

(c) Some of the sulphate groups may be removed by alcoholic potash as soon as they have been demethylated. O-sulphate groups are known to be labile towards alkaline hydrolysis in vitro (Jorpes, Boström and Mutt, 1950).

(d) The uronic acid groups may be reduced so halving the number of anionic sites in the acid mucopolysaccharide polymers. The evidence for this reduction is discussed in sec. 10.2.

No reduction of either phosphate or sulphate esters took

Fig. 39. Guinea pig gut. Fixed in glycol ether.

Methanolic thionyl chloride, 4 hrs. : lithium aluminium hydride + dioxane, 60°, 23 hrs: saponification : azure A. Transmitted white light. Water mount, X 165.

The acid mucopolysaccharides exhibit an unchanged γ-metachromatic staining. The orthochromatic staining formerly attributable to nucleic acids is abolished.

Fig. 40. Guinea pig gut. Fixed in glycol ether.

Methanolic thionyl chloride, 4 hrs. : lithium aluminium hydride + dioxan, 60°, 50 hours : saponification : 0.1% coriphosphine. Water mount, X 180.

Both the protoplasmic and muscle proteins emit a dull green fluorescence. There is no fluorescence in sites formerly containing nuclei. The brown-red fluorescence emitted by the acid mucopolysaccharides is destroyed during the course of the exposure required for this photomicrograph.

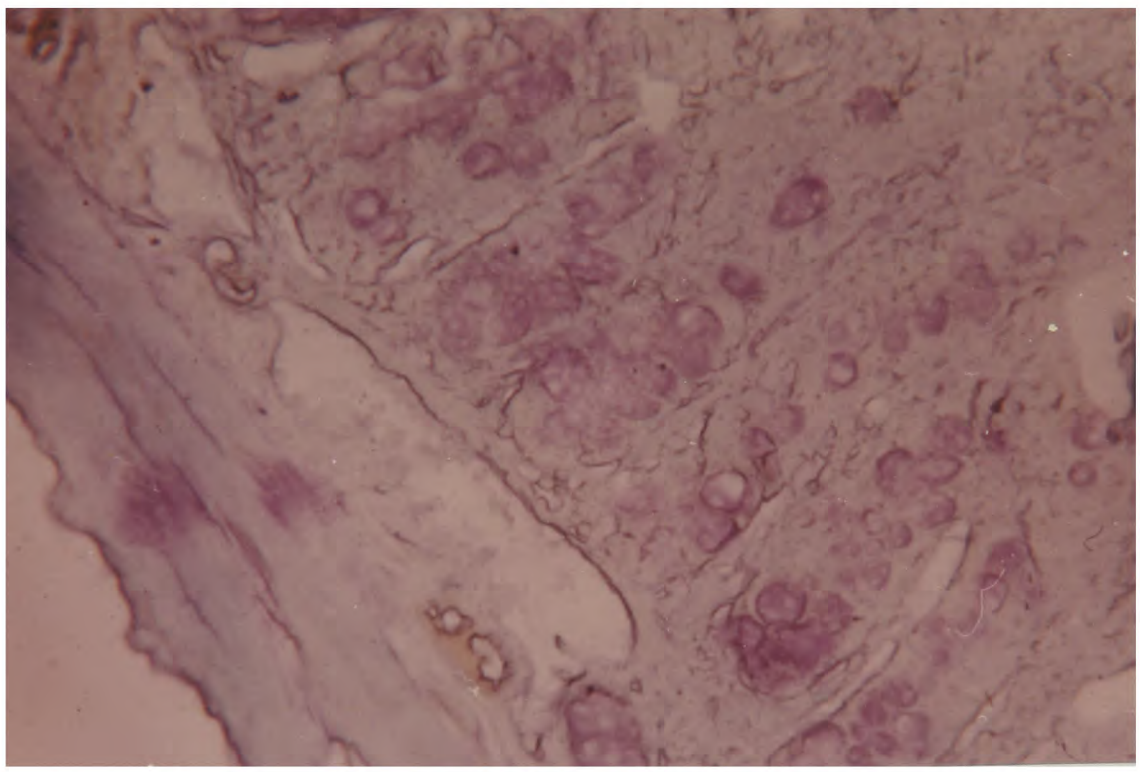


Fig. 39

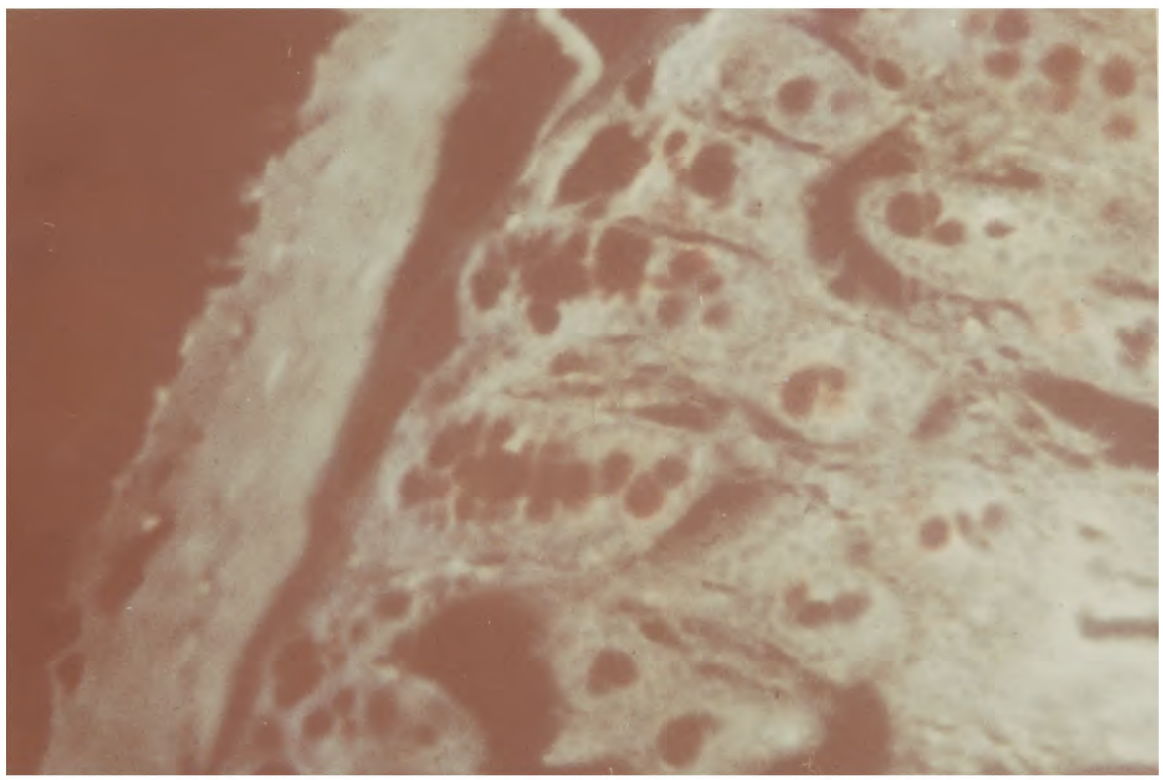


Fig. 40

place when a Lewis base, such as aluminium chloride (Brown, 1952) was added to the LAH solution, unlike other reductions *in vitro* which are promoted by its presence.

Unmethylated sections were unaffected by LAH in hot dioxane. Most reducing agents normally used in histochemistry, such as sodium dithionate, had no effect on either methylated or unmethylated sections. One exception was methanolic thionyl chloride: when sections were accidently left in this mixture at room temperature for a period longer than that required for methylation, nucleic acids were removed at a much faster rate than acid mucopolysaccharides. After six hours immersion in methanolic thionyl chloride, the nucleic acids possessed very little azurophilia after saponification (Fig.41). The effect of 12 hours treatment is shown in Fig. 42. The cytological structure is progressively distorted and is accompanied by an elution of acid mucopolysaccharides from the section after about 10 hours treatment. Unfortunately, the optimal time required for the selective extraction of nucleic acids without affecting acid mucopolysaccharides varied from tissue to tissue. Usually a time between 6 and 10 hours is sufficient.

Fig. 41. Guinea pig gut. Fixed in glycol ether.

Methanolic thionyl chloride, 6 hrs.: saponification : azure A. Transmitted white light, water mount, X 165.

The acid mucopolysaccharides exhibit an unchanged γ-metachromasia. A little structural distortion can be seen in the area indicated.

Fig. 42. Guinea pig gut. Fixed in glycol ether.

Methanolic thionyl chloride, 12 hrs: saponification : azure A. Transmitted white light, water mount, X 165.

The γ-metachromatic staining is less extensive than that shown in Fig. 41. The structural distortion is more pronounced.

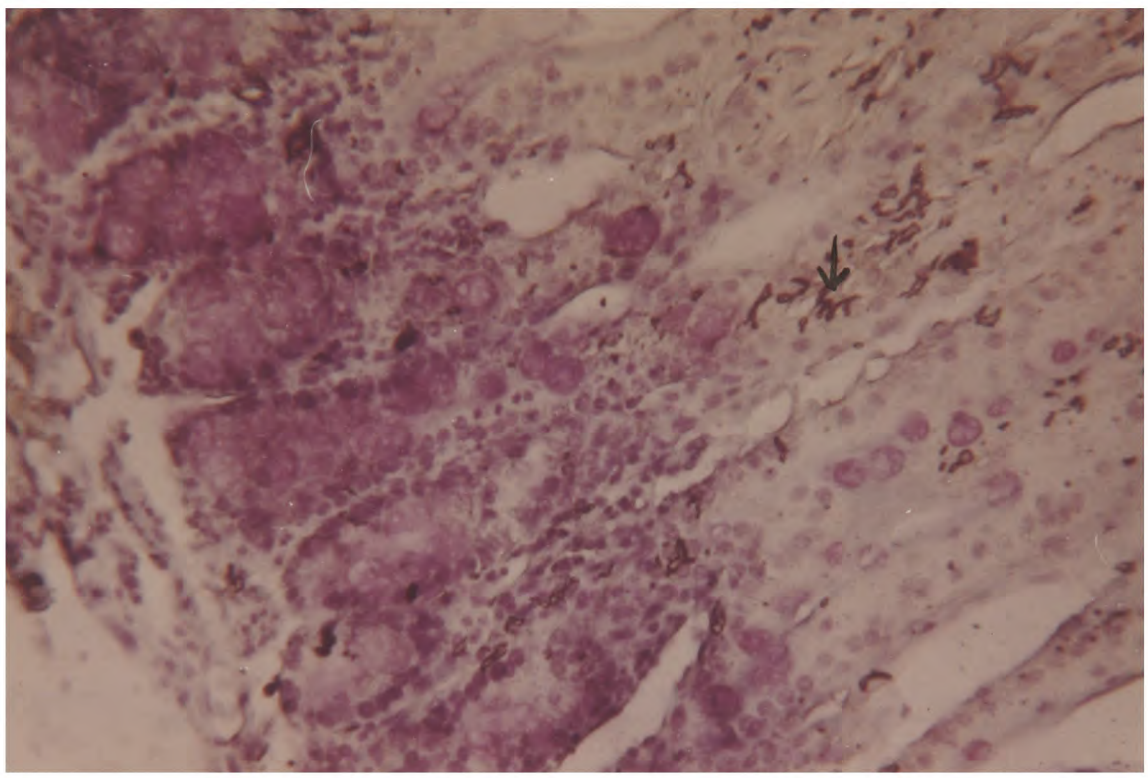


Fig. 41

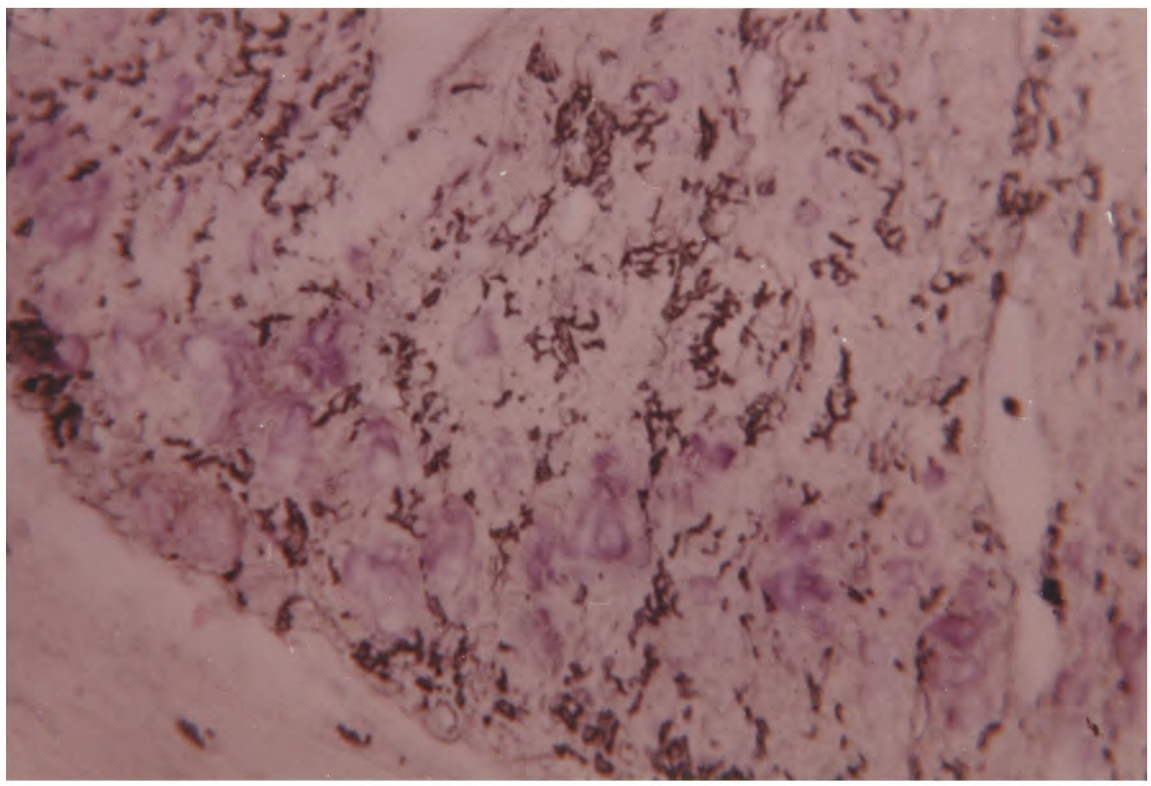


Fig. 42

Methods based on mordants.

10. Iron.

(1) Introduction

Hicks and Mathaei (1958) noticed that if acridine orange was applied to tissues after Masson's (1929) trichrome stain, epithelial mucins were apparently stained specifically so that they produced a bright orange-yellow fluorescence. This effect was attributed to the mordant. Similarly pretreatment of sections with ferric chloride or ferric alum quenched most of the potential fluorescence of tissues stained by acridine orange, but subsequent over-staining produced the specific staining of acid mucins.

The technique described by Hicks and Mathaei (1958) gave unsatisfactory results in my hands: using the iron solutions (pH 2.2) that they prescribed and 0.1% solutions of coriphosphine (pH 5.6), sections became overstained although acid mucin areas could sometimes be discerned as a slightly deeper and more intense orange fluorescence. Mordanting sections with ferric alum solutions of a pH less than 2 (e.g. 1.8), however, gave better differential staining: the acid mucin areas exhibited the normal brown or red fluorescence of coriphosphine which could be easily distinguished from the weak brown-red fluorescence of nuclei. The cytoplasmic RNA and proteins hardly fluoresced at all. Muscle, however, exhibited a light green fluorescence. During the long exposure to ultra-violet light required for

a photomicrograph, the fluorescence of the nuclei increased (Fig. 43). This is contrary to the behaviour of coriphosphine adsorbed onto acid mucopolysaccharides but even after a prolonged exposure to ultra-violet light, the fluorescence of the acid mucins could still be distinguished more easily than in unmordanted sections.

One important feature of this modified method is that it shows the internal elastic laminae of small arteries particularly well by their light green fluorescence. Without the pretreatment in ferric alum, their staining is often obscured.

(2) The specificity and mechanism of iron mordant methods.

There is no specific evidence that the adsorbed ferric mono-ions (I), can form complexes with acridine orange or other aminoacridine dyes as has been implied by Hicks and Mathaei (1958) or by the analogy with the dye complexes said (by Heath 1961, 1962) to be formed with aluminium adsorbed onto acid mucins. Numerous iron complexes have been prepared with simple heterocyclic bases, such as pyridine and quinoline, but little is known of their constitution (Sidgwick, 1950). However, as the affinity of ferric irons for nitrogen is small, the formation of a stable, insoluble complex with acridine orange

Fig. 43. Guinea pig gut. Fixed in glycol ether.

Ferric alum, pH 1.8 : 0.1% coriphosphine, pH 5. Water mount, X 180.

The intensity of the orange-brown fluorescence of the nuclei is much weaker than that of the sites containing mucopolysaccharides.

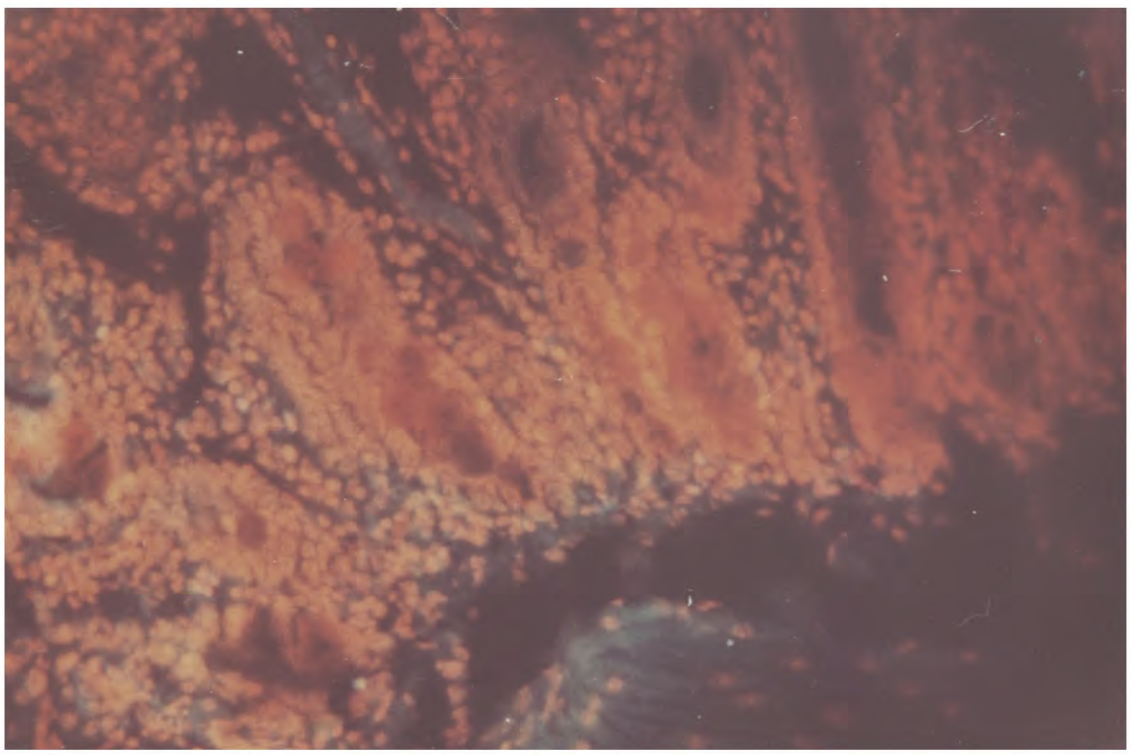


Fig. 43

or coriphosphine is improbable. What is more likely is that the dye replaces the iron initially adsorbed onto acid mucins. This has been confirmed experimentally by mordanting sections in colloidal iron, staining them in coriphosphine and finally treating with acidified ferrocyanide (Lison, 1926). Any remaining adsorbed ferric ions are converted to Perls's (1867) Prussian Blue complex by this treatment. Less Prussian Blue is deposited in the acid mucin areas of mordanted sections stained with coriphosphine (Fig. 46) than in unstained sections. Moreover, very little, if any, Prussian Blue is deposited in the acid mucin areas of sections that have been mordanted with non-colloidal ferric alum solutions (Figs. 47 and 48), even without the intermediate staining in coriphosphine. This means that acid mucopolysaccharides do not take up iron from the ferric alum solutions of Hicks and Mathaei (1958) and are accessible to staining by basic dyes in the normal way.

The reasons why a more specific fluorescence appearance is obtained with sections mordanted in ferric alum solutions of pH less than 2 may be connected with (i) a more specific uptake of iron by acid mucopolysaccharides, (ii) changes in the chemical structure of the ferric ions themselves above and below pH 2 and (iii) the relative affinity of iron between the tissue substrate and the basic dye or complexing agent used for visualizing the

Fig. 46. Guinea pig gut. Fixed in glycol ether.

Colloidal ferric iron, pH > 2 : 0.01% coriphosphine : acid ferrocyanide. Transmitted white light, water mount, X 165.

Prussian Blue is deposited in some, but not all, sites containing acid mucopolysaccharides. The limited staining is not restricted to acid mucins. The nuclei are stained brown by coriphosphine. The dull pink background is a photographic printing artefact.

Fig. 47. Guinea pig gut. Fixed in glycol ether.

Ferric alum, pH > 2 : acid ferrocyanide. Transmitted white light, DPX mount, X 165.

Prussian Blue is <u>not</u> deposited in the sites containing acid mucopolysaccharides. The nuclei are heavily stained. The pink background is a photographic printing artefact.

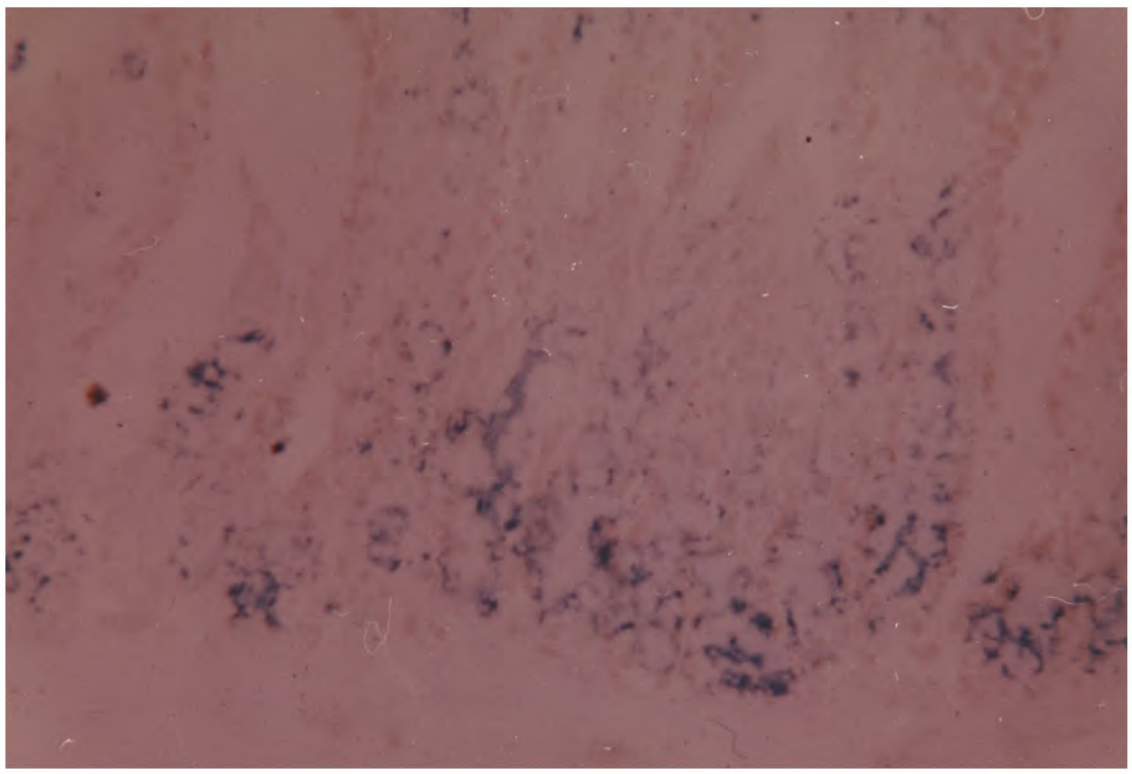


Fig. 45

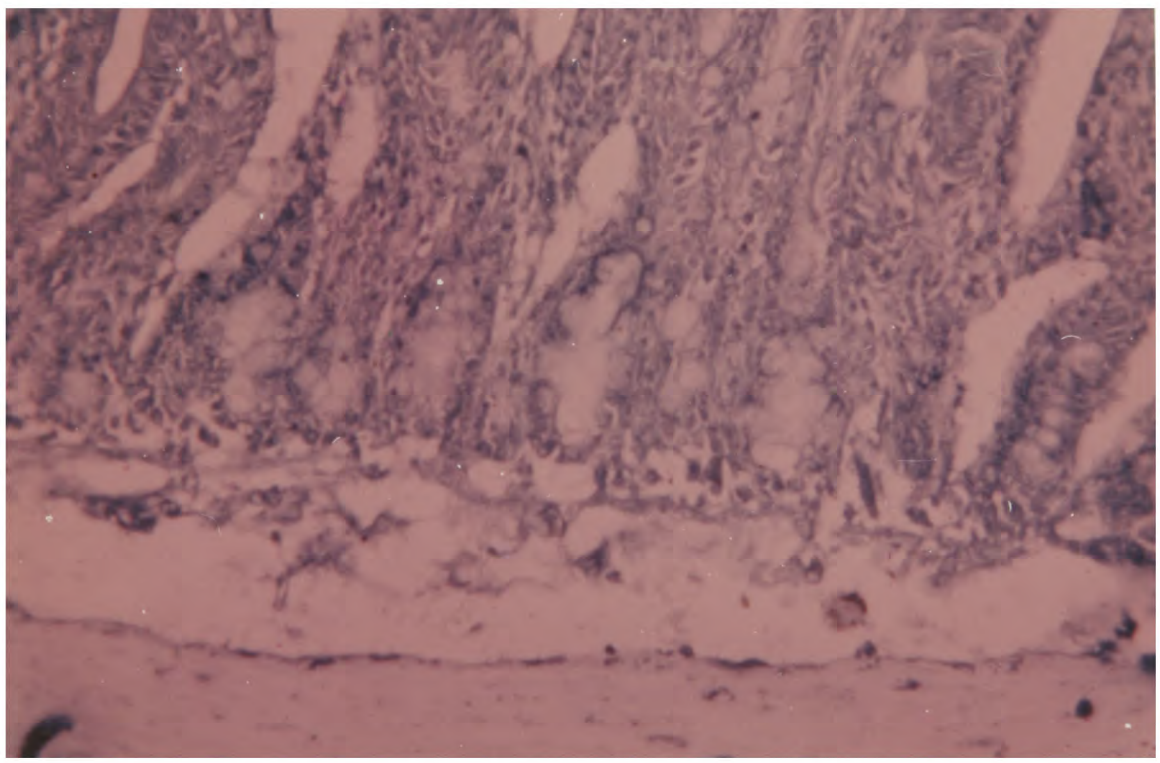


Fig. 47

Fig. 48. Guinea pig gut. Fixed in glycol ether.

Ferric alum, pH 1.8 : acid ferrocyanide. Transmitted white light, DPX mount, X 160.

The nuclei and the protein carboxyl groups of the protoplasm and the muscle are stained. There is no Prussian Blue deposited in the sites of acid mucopolysaccharides. The pink background is a photographic printing artefact.

Fig. 49. Guinea pig gut. Fixed in glycol ether.

Colloidal ferric iron, pH 1.8 : acid ferrocyanide. Transmitted white light, DPX mount, X 165.

Only the peripheries of the sites known to contain acid mucopolysaccharides are stained. The nuclei are stained weakly. The muscle is not stained.

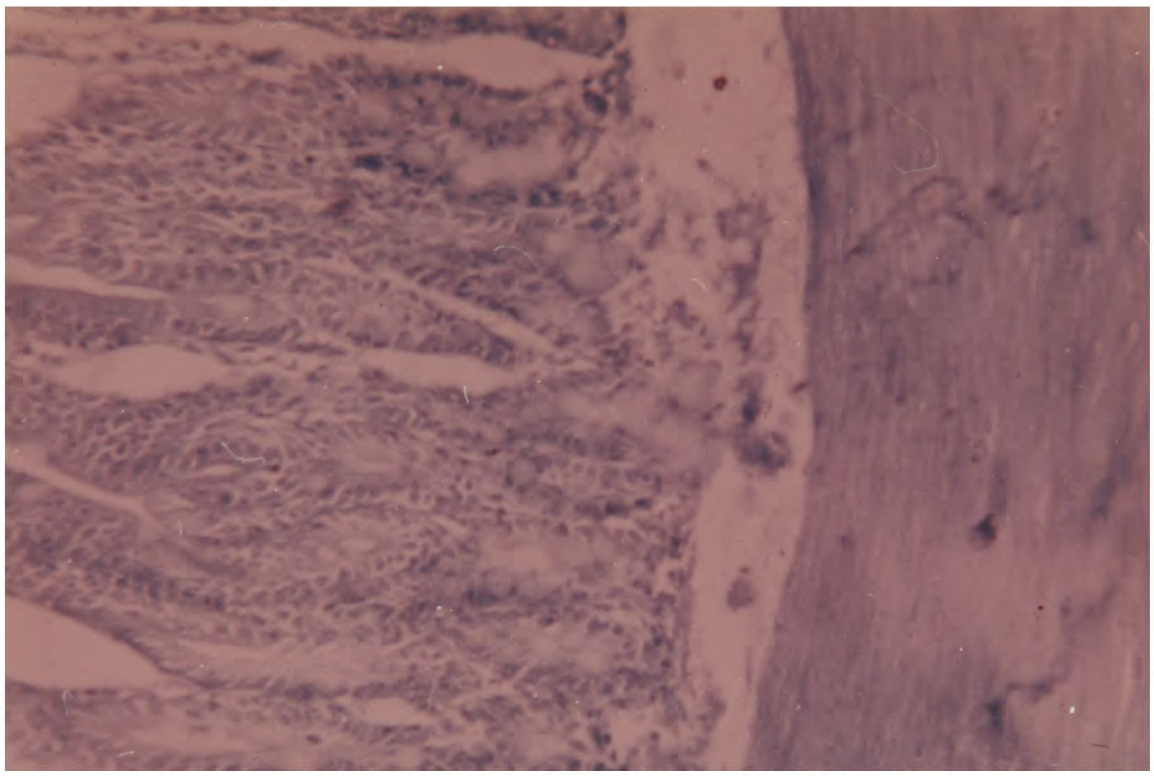


Fig. 48

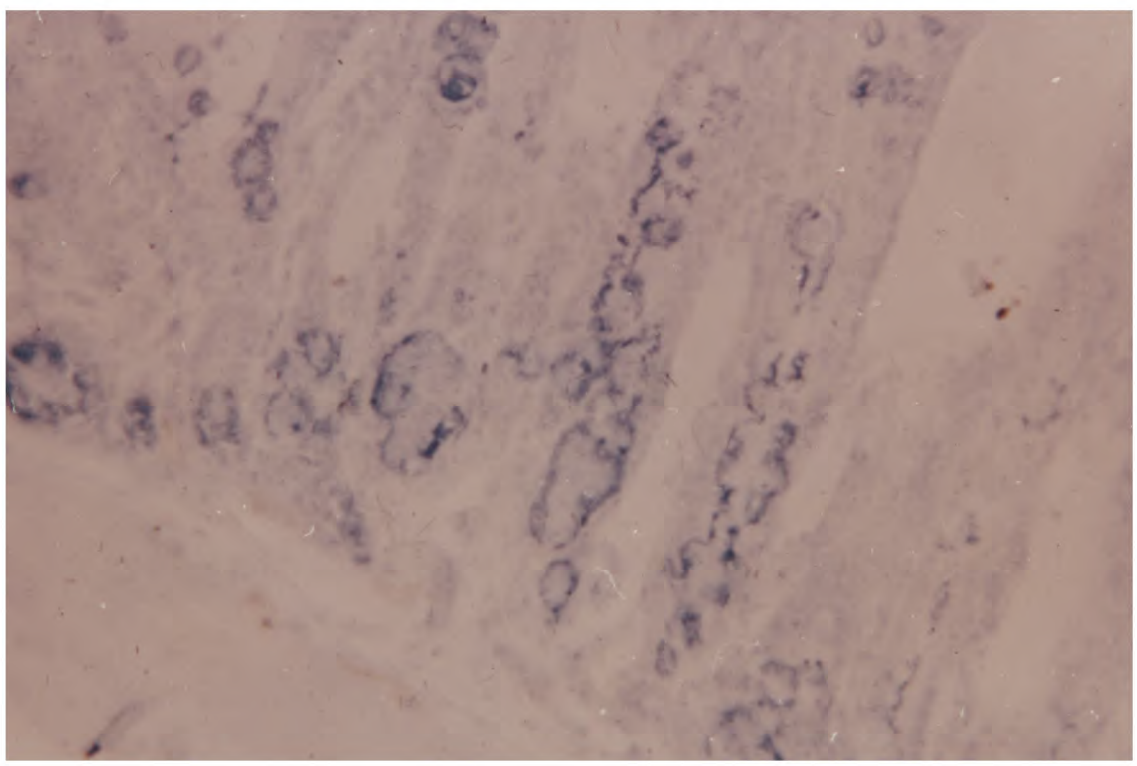


Fig. 49

adsorbed iron.

(i) Hale (1946) and Ritter and Olson (1950) claimed that colloidal ferric iron was specifically taken up by acid mucopolysaccharides and could be subsequently demonstrated as a Prussian Blue complex. Mancini (1950) and Gomori (1952) found staining was largely confined to those structures giving a metachromatic reaction towards toluidine blue. In my experience, however, good preparations of colloidal iron are taken up by most, but not all, acid mucin sites having a positive alcian blue or a γ-metachromatic reaction towards azure A. Moreover, basophils other than acid mucins also give a positive reaction (Fig. 49).

Pearse (1951) and Lillie (1954) also doubted the specificity of Hale's technique; for example, collagen and reticulin (Lillie and Mowry, 1949), and proteins (Lison, 1953; Braden, 1955) gave a positive reaction.

There has been much controversy over the binding of ferric ions to proteins. Cysteine thiol groups take up ferric ions but the binding decreases as the pH is lowered (Page, 1955). Bechtold (1928) found that constant amounts of ferric iron were firmly held by protein which he considered to be due to the amine and carboxylic acid groups. Others, such as Wigglesworth (1952), believe that the initial linkage is an electrostatic bond with ionized carboxyl groups which is followed by chelation. Most

carboxylic acids (e.g. oxalic acid) have a very strong affinity for ferric ions *in situ* and form chelates *in vitro* (Orgel, 1960; Starke, 1960).

Below pH 2, few carboxyl groups should be ionized and therefore the uptake of ferric ions should be limited to sulphated acid mucopolysaccharides and perhaps to nucleic acids. This has been confirmed by Rinehart and Abu'l Haj (1951). Using colloidal iron, they found that acid mucopolysaccharides stained specifically, with the exception of hyaluronic acid (Abood and Abu'l Haj, 1956) which gave a weak reaction. Ghiara (1954, 1956) and Woolman (1956) also obtained better specificity by lowering the pH, although the latter found that hyaluronic acid also stains. Since very few of the uronic groups of hyaluronic acid are ionized below pH 2 (Szirmai and Balazs, 1958), they should take up hardly any ferric ions below this pH and this would explain the variability of its cytochemical demonstration by workers who have used iron solutions of unspecified pH.

Nevertheless, nucleic acids should still take up ferric ions and should, and do, give a Prussion Blue reaction even with colloidal iron (Fig. 49). Perhaps if the ionic weight of colloidal iron could have been increased sufficiently, it would not have penetrated the comparatively dense nucleic acids and would not have been taken up by them

(Goldstein, 1961; 1962). Only the less dense sulphated acid mucopolysaccharides usually take up cations whose ionic weights exceeds, say 1,000, and give a specific Prussian Blue reaction.

On the other hand, colloidal iron is useless for the iron-coriphosphine fluorescence method. When mordanted with colloidal iron at pH below 2, the nucleic acids subsequently took up coriphosphine in the normal way. The colour of their fluorescence could hardly be distinguished from that of the acid mucopolysaccharides whose colours were variable. Some of the acid mucin sites in sections of gut exhibited a bright red fluorescence in addition to their normal, less intense brownish-red fluorescence. Both fluorescent colours were destroyed by prolonged exposure to ultra-violet light. The variation of fluorescent colours may be related to the strength and extent of the original acid mucin-iron bond. Not all the colloidal ferric ions were replaced by coriphosphine (Fig. 46) during the comparatively short period of staining recommended by Hicks and Mathaei (1958).

Unlike the alum solutions containing ferric mono-ions, mordanting with colloidal iron at a pH greater than 2 leads to some quenching of the fluorescence of coriphosphine taken up by nucleic acids. Presumably the increased negative charge on the nuclear phosphate groups as the pH

is increased leads to a larger uptake of colloidal iron. The selective staining and quenching of the old mordanted sections used by Hicks and Mathaei must therefore be regarded as fortuitous.

(ii) Although the intensity of fluorescent staining can be explained satisfactorily for sections mordanted with colloidal iron solutions of differing pH, the same arguments cannot be evoked for explaining the converse variation with the mono-ionic ferric alum solutions. Here iron is taken up in different hydrated forms that depend upon the pH of the mordant solution.

Bray and Hersey (1934) suggested that the following equilibria are set up in solutions of simple ferric salts:

$$\underset{\text{I}}{Fe^{3+}} + H_2O \rightleftharpoons \underset{\text{II}}{FeOH^{++}} + H^+$$

$$Fe^{3+} + 2H_2O \rightleftharpoons \underset{\text{III}}{Fe(OH)_2^+} + 2H^+$$

$$Fe(OH)_2^+ + H_2O \rightleftharpoons Fe(OH)_3 + 2H^+$$

These equilibria have been studied in detail by Lamb and Jacques (1938) and by Kaboniwitch and Stockmayer (1942). Later a further reaction was added (Hedström, 1953: Mulay and Selwood 1954).

$$2\,Fe^{3+} + 2H_2O \rightleftharpoons \underset{\text{IV}}{Fe_2(OH)_2^{4+}} + 2H^+$$

In solutions containing hydrochloric acid many other ions of the type $FeCl^{++}$ will be formed (Bent and French 1941).

At a pH above 2, the tissue basophils will take up iron principally in the forms II to IV, but the strength of binding will not be very strong as the ions carry a low net charge and would be readily replaced by coriphosphine. As none of these ions has the structure I, they cannot form a red complex with ammonium thiocyanate. Only the $FeCNS^{++}$ radicle is red (Bent and French). $Fe(CNS)_3$ or $FE(CNS)_6^{\equiv\equiv\equiv}$ are not coloured as was formerly thought (Schlesinger and Van Valtenburgh, 1931). Moreover, the ions which are bound to the tissue basophils form a stronger complex than the $FeCNS^{++}$ bond. This applies particularly to those ferric ions bound to phosphate groups which have an OH group adjacent to the anionic centre. This arrangement promotes chelation and is responsible for the well-known iron-phosphate complexes (Frommer and Hamler).

Such reasoning does not explain why the various hydrated ionic species of iron give a Prussian Blue reaction. The positive reaction is probably due to the acid present in the complexing ferrocyanide solution. Hydrogen ions tend to displace the adsorbed ions II to IV and will then, if the acidity is high enough, convert them to ferric

ions (I) which immediately react with ferrocyanide ions. As Prussian Blue is deposited, the various hydrolysis equilibria will move even more to the left until all species of iron are converted eventually to Prussian Blue. Hale' (1946) technique, in this respect, gives a fortuitous result.

The only method of demonstrating I as such, is to use neutral ammonium thiocyanate solution. This reagent gave a yellow colouration to muscle (carrying a relatively large number of dissociated carboxyl groups; Sec.9.1/3) when mordanted with ferric alum below pH 2. (Cp. the formazans which are red *in vitro* but yellow *in situ* Sec. 12.23). Below this pH, the colour of the mordant ferric alum solutions changes and the additional acid forces the hydrolysis equilibria to the left with an attendant rise in the concentration of I. The few remaining dissociated carboxyl groups form a loose bond with I and give rise to the weak yellow colour with ammonium thiocyanate. The Prussian Blue reaction is also positive in the muscle area when mordanted at pH below 2, but not at a pH greater than 2 (Figs. 47 and 48). Presumably at the latter pH, the ionic charge on II and IV is not high enough to form an electrostatic bond with the relatively weakly charged carboxyl groups.

Ferric ions (I) bound on nucleic acids will reduce

the amount of coriphosphine adsorbed onto them. This results in the quenching of the fluorescence of any dye that is bound by neighbouring ferric ions. Some of this iron can be displaced by washing with solutions containing a high concentration of another active cation. The usual procedure is an extensive washing in several changes of acetic acid (Mowry, 1958), but basic dyes such as neutral red (Hale, 1946) or coriphosphine can be used. Some ferric ions are still adsorbed onto nuclei after staining with basic dyes as is shown by their relatively weak fluorescence, but is probably bound so tightly that it cannot be released and deposited again as Prussian Blue complex or as a thiocyanate complex. Smythe and Schmidt (1930) also found that ferric ions bound to phosphoric acids *in vitro* will not complex with ammonium thiocyanate.

In view of the uncertainty of the exact ionic species of iron in mordant solutions and the extent of the colloidal state, their use for the selective quenching of nucleic acids in the presence of acid mucopolysaccharides is unreliable. Hicks and Mathaei's method therefore has no particular specificty and is much less easy to carry out than the coriphosphine-thiazol yellow method.

11. Aluminium

Heath (1961, 1962) claimed that when many heterocyclic dyestuffs, including aminoacridines, are dissolved in boiling solutions of aluminium sulphate, they visibly stain acid

mucopolysaccharides in tissue sections specifically.

In my experience, the presence of the aluminium salts hardly affected the normal basophilic fluorescent staining of tissues by solutions of coriphosphine or neutral red, and it is difficult to understand why Heath's method should be specific for acid mucopolysaccharides. Aluminium ions are only slightly attached to cytoplasm and hardly at all to elastin (Möllendorff and Tomita, 1927), and so should be taken up only by sulphated acid mucins with which they might form a coloured complex with the dyestuff. However, the nuclei and the cytoplasm should still take up the basic dye in the normal way and this has now been verified by examining sections for fluorescence after they have been stained with 0.03% coriphosphine dissolved in 5% aluminium sulphate. No improvement in the specificity of staining was obtained by preparing aluminium in a stable colloidal form according to the method described by Sen (1954).

Moreover, it can be easily shown that the acid mucopolysaccharides take up very few aluminium ions in the first instance; if sections are soaked in a solution of an aluminium salt, washed in water, and then stained in a solution of Solochrome Black, no red fluorescence due to an aluminium - Solochrome Black complex (sec. 3.10) could be seen in any of the basophil sites. Therefore the specificity of Heath's method for the visible staining of acid

mucopolysaccharides seems questionable.

12. The removal of O-sulphate groups.

If a method could be devised for selectively removing O-sulphate groups from acid mucins *in situ* without damaging the structure of the tissue, it would be very useful as a confirmatory test. However, the most notable feature of sulphate groups is their chemical unreactivity (Tipson, 1953) and so far, no universibly reliable method has been found for removing them even *in vitro*.

Solutions of hydrazine have been used with partial success for the desulphation of sugar sulphates *in vitro* (Wolfrom and Juliano, 1960; Dodgson, 1961), but the conditions would be too severe for desulphating tissue sections.

The following methods have been investigated for removing sulphate groups *in situ*:

13. Acetylative desulphation.

Wolfrom and Montgomery (1950) found that a mixture of absolute sulphuric acid and excess acetic anhydride simultaneously desulphated and acetylated mono- and polysaccharide sulphates *in vitro* after 2 to 3 hours at -10° to -20°. There was no Walden inversion, although some glycosidic hydrolysis took place.

The acid mucins in sections of guinea pig gut were unaffected by 4 to 24 hours treatment with various proportions of sulphuric acid and acetic anhydride at -20°, as was shown

subsequently by their unchanged γ-metachromatic staining towards azure A.

14. Reduction with complex metal hydrides.

The sulphate groups on monosaccharides can be slowly removed by complex metal hydrides in vitro (Bollinger and Ulrich 1952; Grant and Holt, 1960). Sulphated polysaccharides, however, are not desulphated by similar treatment, (Holt and Mulvin, 1962), neither are acid mucopolysaccharides [illegible] . (secs. 9.9 and 10.2/2). One exception to this generalisation is the desulphation of chondroitin sulphate in vitro by potassium borohydride in the present of alkali (Rees, 1961). In this instance, the hydride may only be accelerating the cleavage of the sulphate groups by the alkali (Jorpes et al., 1950). Unfortunately this method cannot be used for the desulphation of acid mucins in situ as the alkali would damage the tissues and remove them from their glass slides.

15. Hydrolysis with methanolic hydrochloric acid.

Although O-sulphates are comparatively stable towards methanolic hydrochloric acid (Wolfrom et al., 1953), prolonged treatment with this acid will remove them from acid mucins, particularly at high temperatures.

Dry hydrochloric acid dissolved in methanol will desulphate chondroitin sulphate completely in vitro (Kantor and Schubert, 1957) and heparin partly (Danishefsky et al.,

1960) within 12 to 24 hours at room temperature. Similarly, chondroitin sulphate B (dermatan sulphate) can be desulphated by an "aged" solution of acetyl chloride in methanol (Stoffyn and Jeanloz, 1960).

Methanolic hydrochloric acid has also been used at higher temperatures(60°) to methylate or block tissue basophils including sulphated acid mucopolysaccharides (Fraenkel-Conrat and Olcott, 1945; Fisher and Lillie, 1954). It is therefore conceivable that if tissues are methalyted with this reagent (as might be required when pseudo-Schiff reagents are used for the histochemical demonstration of aldehydes; Sec.12.7/9), acid mucins may be desulphated at the same time. This possibility has been investigated by Spicer et al. (1961) who used autoradiographic techniques to show that acid mucins labelled with S^{35} were removed completely from tissue sections by methanolic hydrochloric acid.

The desulphation of tissue sections can be followed histochemically by re-esterifying the sections afterwards with alcoholic potash (Spicer and Lillie, 1959), and then staining them in azure A to reveal the remaining basophils. If acid mucopolysaccharides are desulphated during the methylation process, their γ-metachromasia should be substantially reduced in comparison to that shown in control sections. On the other hand, acid mucins may also be eluted from the section by hot methanolic hydrochloric acid and

will result, after saponification, in the same loss of γ- metachromasia. However, desulphation and elution can be distinguished experimentally by carrying out the following tests:

(a) The alcian blue reaction at pH 2 (exp. sec. 10.1), before and after saponification.

If current ideas concerning the specificity of the alcian blue reaction for uronic acids are correct (sec. 10.2/2), a positive reaction, particularly after saponification, in sections where the subsequent γ- metachromasia is weak or absent would indicate desulphation and not elution. Some "methylated" sulphated acid mucopolysaccharides, particularly those in sections of gastric tissues, still give a positive alcian blue reaction before saponification although their basophilia is absent. In these cases, the uronic acid group is presumably shielded from attack by the methylating reagent.

(b) The Periodic acid - salicyloyhydrazide reaction (PA-SH; exp. sec. 12.14)

The net effect of the removal of anionic sulphate groups from most acid mucopolysaccharides is that the similarly charged periodate ions need no longer be repelled. The adjacent glycol groups in the mucin should therefore be cleaved much more rapidly (say within 10 minutes) than when sulphate groups are present. Usually 24 to 48 hours are required for the cleavage of glycol groups in acid

mucopolysaccharides by periodic acid (sec. 12.4/4).

When hydrochloric acid and methanol is used for methylating sections at 37° for 4 hours ("mild" methylation), the basophilia of acid mucins in most tissues is blocked. After saponification, the alcian blue and periodic acid reactions are normally restored (Spicer, 1960). In structures such as cartilage (containing chondroitin sulphate) and mast cells (containing heparin), the subsequent saponification failed to restore the azurophilia significantly. This may be due to desulphation by the alcoholic potash and not to the acid methanol: the O-sulphates groups of heparin are known to be labile towards alkalies in vitro (Jorpes et al., 1950).

After 4 hours methylation at 60° ("drastic" or "active" methylation), Spicer (1960) found that some acid mucins exhibited a marked increase in the intensity of their PAS reaction, which he attributed to the hydrolysis of the sulphate groups. Thus, in contrast to an initial feeble reactivity, the goblet cells in the upper part of the crypts and on the luminar surface in the rabbit-colon stained as darkly as those in the crypts after such treatment. Other sites which had a weak or negative PAS reaction, such as the mucinous acini in the laryngeal mixed glands, the glossopharyngeal mucous glands, and the Brunner's glands of the rabbit, also stained more strongly.

On the other hand, if sections of the guinea pig gut are methylated in this way for only two hours, the extent of the PA-SH reaction in the acid mucin sites is increased (Figs. 50 and 51) even when some of the nuclei are still weakly basophilic (i.e. incomplete methylation). After saponification, the affinity of the acid mucin sites for alcian blue and azure A is completely restored showing that none had been extracted during the methylation process.

The increase in the extent of the PA-SH reaction in the gut is particularly interesting as it may be absolute being due, not to an enhanced accessibility of the periodate ion, but to the removal of the sulphate group from the second hexuronic moiety of heparin (Fig. 3) and which leads to the formation of a <u>new</u> glycol group. Of the acid mucopolysaccharides whose structures have been studied so far, heparin is the only commonly occurring one in which this type of hydrolysis can occur. This hypothesis was tested by first oxidising control sections of guinea pig gut with periodic acid for 10 mins. and then incubating them with aniline (sec. 12.22) so as to block all the dialdehydes engendered from neutral mucopolysaccharides. This was followed by a second oxidation in periodic acid for 24 hours in order to cleave the "protected" glycol groups, if any (sec. 12.4/4). As no fluorescence could be detected in

Fig. 50. Guinea pig gut. Fixed in glycol ether.

Methanolic hydrochloric acid, 60°, 2 hrs.: periodic acid : salicyloyl hydrazide. Fluormount, X 180.

The extent of the blue fluorescence in sites known to contain neutral and acid mucopolysaccharides is much greater than that in control sections. (Fig. 51).

Fig. 51. Guinea pig gut. Fixed in glycol ether.

Periodic acid : salicyloyl hydrazide. DPX mount, X 180.

Sites containing oxidised neutral mucopolysaccharides exhibit an intense blue fluorescence. The muscle does not fluoresce. Cp. Fig. 55.

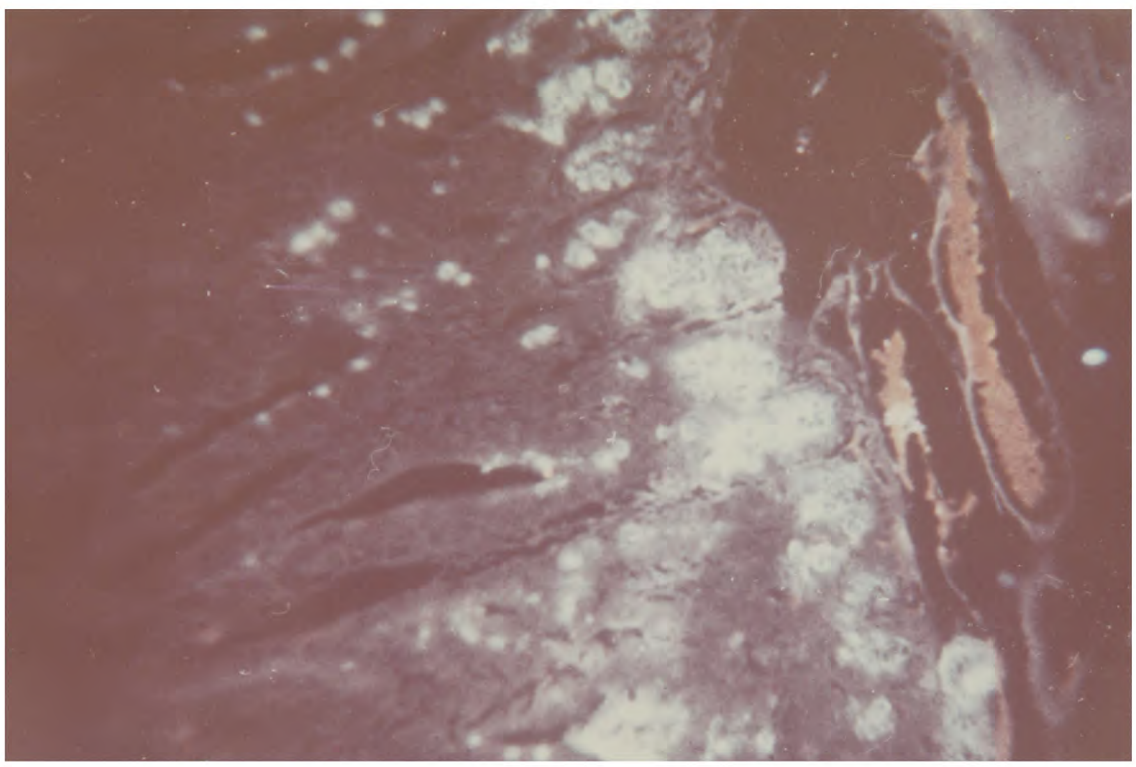


Fig. 50

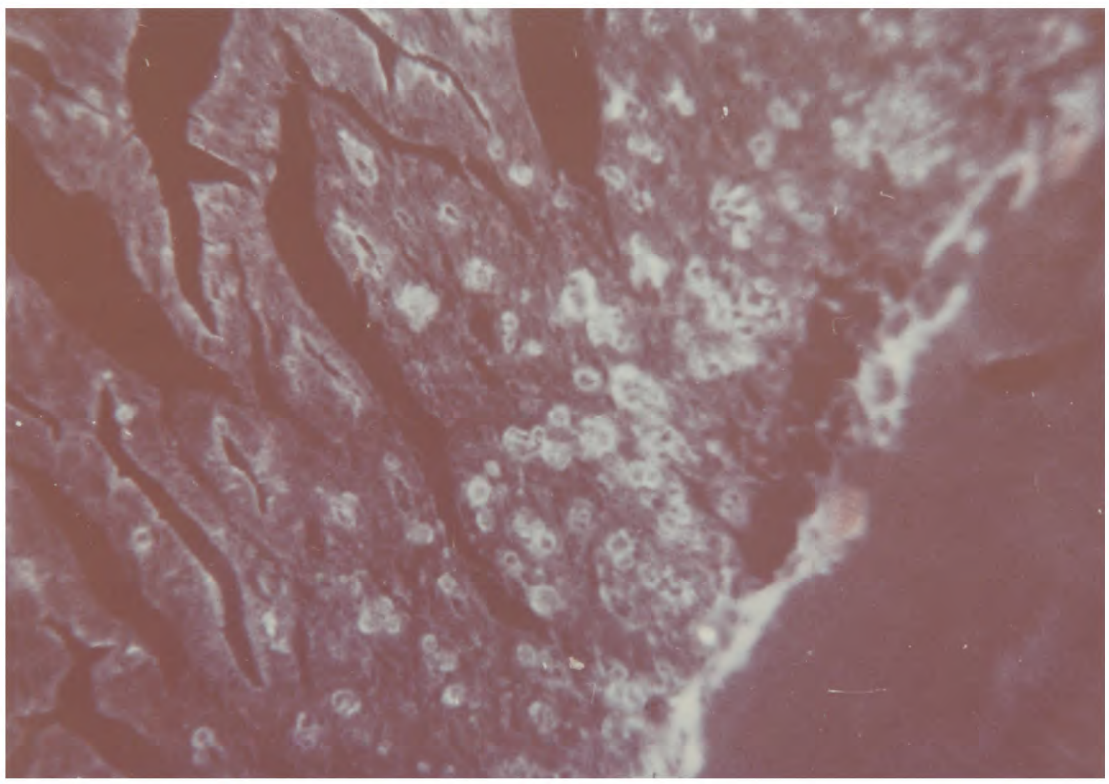


Fig. 51

acid mucin sites after incubating the doubly-oxidised sections subsequently in salicyloyl hydrazide, it implies that there are no sterically shielded glycol groups in the acid mucopolysaccharides of the gut. This is consistent with the chemical behaviour of heparin. Wolrom et al. (1950) found that heparin rapidly consumed only one mole of periodic acid per tetrasaccharide unit in vitro. The N-acetyl desulphated compound consumed a further 1 mole of periodate per disaccharide unit. No direct evidence has been sought for showing that the principle acid mucopolysaccharide in the particular specimen of guinea pig gut used in this investigation is actually heparin. Heparin has, however, been found in the gastric mucosa of other mammals (Smith, Gallop and Stanley, 1952).

16. Conclusion.

None of the methods investigated were completely satisfactory for selectively removing sulphate groups from tissues. The best hydrolytic agent was methanolic hydrochloric acid, but it could not be used with absolute certainty on tissues because it could remove the parent carbohydrate at the same time. Moreover it is uncertain whether methods based upon acidic hydrolyses will ever be completely effective. Experiments on the acid hydrolysis of methyl glycoside monosulphates in vitro have shown that

the sulphate group can stabilize the glycosidic link and that the 6-sulphate is more effective for doing this than the 3-sulphate. The parent sugar is the only one isolated, showing that no inversion of configuration occurs. These experiments show that the acid hydrolysis of sulphated polysaccharides should give some sulphated mono- and oligo-saccharides (Clamer and Turvey, 1961). In other words, when sulphated acid mucopolysaccharides are hydrolysed in situ, smaller sulphated oligo-saccharide fragments will be formed which would then probably diffuse out of the section because of their lower molecular weight. The hydrolytic removal of sulphate groups from the acid mucins in tissues is therefore always likely to be accompanied by their eventual elution from the section.

Experimental. Section 9. Sulphate Groups

1. Metachromasia with thiazine dyes (v, after Pearse, 1960 and Kramer and Windrum, 1954).

Method

1. Take sections to 30% alcohol.

2. Stain in 0.02% solutions of toluidine blue or azure A in 30% alcohol for 20 mins.

3. Rinse once each in 30% alcohol and water.

4. Mount in water, examine in visible light and, if necessary, photograph at once.

Result

Acid mucopolysaccharides exhibit red or pink γ- metachromasia, while nucleic acids and nucleoproteins are stained blue (orthochromasia) or purple (β- metachromasia).

2. The coriphosphine-thiazol yellow method (f).

Method

1. Take sections to water.

2. Stain in 0.1% coriphosphine (pH 5) for 10 mins.

3. Rinse twice in water.

4. Counterstain in 0.001% thiazol yellow for 5 mins.

5. Wash once in water.

6. Mount in water and examine for fluorescence.

Result

Acid mucopolysaccharides exhibit an orange or reddish-brown fluorescence which is clearly distinguishable from the yellow fluorescence of nuclei and the blue fluorescence of proteins and RNA.

3. Selective basophilia (v or f).

Only acid mucopolysaccharides show any distinctive staining towards basic dyes when nucleic acids are selectively removed by lithium aluminium hydride in hot dioxane as described in exp. sec. 9.15. If 0.1% coriphosphine (pH 5) is used for staining, the sulphated acid mucopolysaccharides exhibit a brown fluorescence which gradually disappears on prolonged exposure to ultra-violet light. The basophilic background fluoresces light green. A few nuclei fluoresce a weak yellow-brown colour.

Sites containing acid mucopolysaccharides are visibly stained red (γ- metachromasia) by azure A. There is no other basophilic staining.

The Selective Extraction of nucleic acids

Chemical methods

4. Hydrochloric acid.

(a) 2-4 hours incubation of sections in N hydrochloric acid at 37° (after Dempsey, Singer and Wislocki, 1950), extracted RNA, DNA and acid mucopolysaccharides simultaneously as shown by the decreasing basophilia of the tissue towards toluidine blue.

A negative PA - SH reaction (exp.sec.12.14) showed that neutral mucopolysaccharides were also extracted.

(b) When sections were incubated in N hydrochloric acid at 60° for 5 min. (Feulgen hydrolysis), subsequent staining with coriphosphine indicated a loss of RNA and soluble proteins. The staining of acid mucopolysaccharides appeared unaltered, although the nuclear (DNA) basophilia was reduced but never abolished.

Both variations of this method are unsatisfactory for general use.

5. Perchloric acid (after Erickson, Sax and Ogur, 1949).

Preliminary experiments were carried out by treating sections with 5% perchloric acid at 60° for 5 - 40 mins. The sections were then washed in dilute (1%) sodium carbonate solution, rinsed in water, 30% alcohol and stained in toluidine blue.

RNA was always removed, and there was some reduction in the intensity of nuclear staining for acid incubation times up to 20 mins. The staining of the acid mucopolysaccharides appeared constant. After 30 and 40 mins. incubation, the staining due to acid mucopolysaccharides disappeared, although a very weak DNA staining still remained.

This method was only useful for removing RNA.

6. Method for the extraction of RNA.

1. Bring sections to water.

2. Treat with 5% perchloric acid at (a) 60° for 5-8 mins. or (b) at 5° for 18-24 hours.

3. Wash once in dilute sodium carbonate solution.

4. Wash twice in distilled water, and stain.

7. Trichloracetic acid (after Schneider, 1945).

Sections brought to water were treated with 4% trichloracetic acid at 60° or 90° for 60-90 mins. RNA, DNA and acid mucopolysaccharides were removed simultaneously. This method was of little diagnostic value.

8. Enzyme digestions.

Because of the high cost of crystalline enzyme preparations, sections that were intended for enzymic digestions were enclosed in a shallow perspex ring adfixed to the glass slide with the minimum amount of paraffin wax. Sufficient enzyme solution was pipetted onto the section to fill the perspex-cell completely. A cover slip was placed on top of the cell so there were no air bubbles in the cell chamber. 2 ml. of enzyme solution was sufficient for about five separate tissue sections.

9. Ribonuclease (RNase) for the extraction of RNA.

Crystalline RNase (purchased from L. Light & Co., Colnbrook) which had been extracted from bovine pancreas by McDonald's (1949) modification of Kunitz's (1940) method was used. It had been re-crystallised five

times, the final recrystallisation being from dilute alcohol. The sample had an activity of not less than 40 Kunitz units/mg (Kunitz, 1946) and had no protease activity.

Method

(1) Bring sections to water.

(2) Incubate in RNase solution (20-40 Kunitz units/ml. in glass-distilled water of pH 6-7) for 1 hour at 37°.

(3) Wash in running water for 1-2 mins.

(4) Rinse in distilled water and stain.

Result

The material, which is stained blue by toluidine blue (v) in control sections, but is absent after RNase treatment, is considered to be RNA.

10. Deoxyribosenuclease (DNase) for the extraction of DNA.

Crystalline DNase I was purchased from Serovac Laboratories (Pty) Ltd., Colnbrook. It had been extracted from bovine pancreas by a modification of the method of Kunitz (1950). The sample used had a specific activity of 0.48 units/mg (= 4,320 dornase units/mg) as measured by the method of Kunitz (1950).

Method (a)

(i) Trial experiments were carried out with 0.1% (4,320 dornase units/ml) and 0.4% (17,280 dornase units/ml) of the enzyme in 0.003M solutions of magnesium sulphate

adjusted to pH 6.8 - 7.0, and used at 37° for 60-90 mins.

(ii) Sections were also digested in enzyme solutions containing 0.5% and 1.0% cetyl pyridinium chloride in addition to magnesium sulphate.

Control sections were digested in enzyme solutions containing no magnesium salt activator in both (i) and (ii) concurrently.

The effectiveness of the digestion was tested by the subsequent chromasia of the nuclei towards toluidine blue and by the intensity of the Feulgen hydrolysis - salicyloyl hydrazide reaction (exp. sec. 8.4).

Results

None of the variations given above selectively removed (nuclear) DNA from sections which also contained RNA and acid mucopolysaccharides. Misleading results were obtained with sections incubated for an inadequate period; partially depolymerized DNA exhibited the same red ɣ- metachromasia as the acid mucopolysaccharides.

Method (b) (after Pearse, 1960)

(1) Bring sections to water.

(2) Digest with 0.1% DNase (4,000 dornase units/ml) dissolved in M/10 phosphate buffer (ph 7.6) containing 0.003M magnesium sulphate for 90 mins. at 37°.

(3) Wash twice in distilled water, once in 30% alcohol, and stain.

Result.

The nuclear staining in sections fixed in alcohol or glycol ether is abolished. Nearly all the RNA is removed. The ✓- metachromatic staining of the acid mucopolysaccharides is hardly impaired.

11. Iron mordant techniques (f. Modification of Hicks and Matthaei's (1958) method).

Method.

(1) Bring sections to water.

(2) Mordant in 4% ferric alum at pH 1.8 for 5-10 mins.

(3) Wash in running water for 1 min.

(4) Rinse in distilled water.

(5) Stain in 0.1% solution of coriphosphine at pH 5 for 5-10 mins.

(6) Rinse briefly in two changes of water.

(7) Examine for fluorescence as a water mount.

Result

The acid mucopolysaccharides exhibit a brown-red fluorescence which can be distinguished from the dull yellowish-brown fluorescence of the nuclei. Most of the cytoplasmic fluorescence is quenched. Elastin, however, fluoresces a light green colour which is not always apparent in stained, unmordanted sections.

12. <u>The colloidal ferric iron - ferrocyanide method</u> (v. After Hale, 1946).

<u>Preparation of the colloidal iron solution</u> (Mowry's (1958) modification of Müller's (1955) preparation).

2.2 ml 30% ferric chloride is slowly added to 125 ml boiling, distilled water which is stirred until the solution turns a dark-red colour. The cooled "stock" solution is used for making up the following working solution. The pH is adjusted to the appropriate value with x ml. hydrochloric acid.

The working solution: x ml. concentrated hydrochloric acid
(20-x) distilled water
20 ml. Iron "stock" solution.

<u>Method</u>

(1) Bring sections to water.

(2) Immerse in colloidal iron solution of the appropriate pH for 10 mins.

(3) Wash with water.

(4) Immerse in acid ferrocyanide solution (equal parts of 2% potassium ferrocyanide solution and 2% hydrochloric acid) for 10 mins.

(5) Wash with water and, if appropriate, counter-stain nuclei with 1% neutral red or 0.1% coriphosphine.

(6) Dehydrate through the alcohols, clear in xylene and mount in DPX.

Result

Nearly all the sites of acid mucopolysaccharides are stained blue. Some nuclei and cytoplasmic basophils may also be stained.

Aluminium mordant techniques

13. Heath's Method (1961, 1962).

An alum-dye reagent was prepared by dissolving 0.03% nuclear fast red or coriphosphine in boiling 5% aluminium sulphate solution.

Sections were taken to water, stained in the reagent solution for 5-30 mins. at room temperature, washed in water and examined for fluorescence as water mounts.

There was little difference in the fluorescence appearance of sections stained with the alum-dye reagent and those stained in dye solutions from which alum had been omitted.

14. Experiments with colloidal aluminium.

Colloidal aluminium was prepared as follows (Sen, 1926):0.88 ammonia was added dropwise to a 5% solution of aluminium chloride. The colloidal precipitate was washed by decantation with several changes of distilled water, and finally dissolved in dilute acetic acid.

Sections of guinea pig gut were immersed in the aluminium colloid solution for 2 hours, washed with water, and finally treated with 0.1% Solochrome Black solution.

No specific fluorescence was subsequently observed in any site.

15. Selective reduction.

Method

1. Take sections to methanol.

2. Methylate in methanolic 2% thionyl chloride for 3-4 hours.

3. Wash once each in methyl alcohol and dioxan.

4. Incubate in a fresh 1% suspension of lithium aluminium hydride (LAH) in dry dioxan (stored over barium oxide) for 24-48 hours at 60°.

5. Wash in 70% alcohol acidified with dilute hydrochloric acid to destroy excess LAH and to dissolve precipitated aluminium hydroxide.

6. Rinse in alcohol.

7. Saponify in 1% potassium hydroxide in 80% alcohol for 20 mins.

8. Wash twice in 70% alcohol and take to (a) 30% alcohol or (b) water.

9. Stain in (a) toluidine blue (v) or (b) 0.1% coriphosphine (f) for 20 mins.

Result

All the phosphate groups of the nucleic acids are removed; only the acid mucopolysaccharides are stained with toluidine blue. There is very little loss of acid

mucopolysaccharides from the section during the methylation or reduction stages. The structure of the section is well preserved.

The acid mucopolysaccharides exhibit a brown fluorescence when stained with coriphosphine which contrasts with the green or even non-fluorescent protein background. A few nuclei are sometimes made evident by a very weak yellow-brown fluorescence.

This method is used to infer the presence of sulphate groups.

16. Reductive methylation

Method

1. Bring sections to absolute methanol.

2. Methylate in a 2% methanolic solution of thionyl chloride for 6-12 hours, depending on the nature and fixation of the tissue.

3. Wash once each in methanol and alcohol.

4. Saponify in 1% potassium hydroxide in 80% alcohol for 20 mins.

5. Rinse in alcohol, and take to 30% alcohol or water.

6. Stain in toluidine blue (v) or coriphosphine (f).

Results

Normally only the acid mucopolysaccharide are distinctively stained. Variation of the methylation time

leads to variable extraction of both nucleic acids and acid mucopolysaccharides. Prolonged treatment (for 24 hours) abolishes practically all the metachromasia towards toluidine blue. The cytological structure is invariably distorted.

17. Attempted removal of O-sulphate groups.

An acetylative desulphation reagent was prepared by mixing pre-cooled acetic anhydride and concentrated sulphuric acid (S.G.1.82). A 50:50 mixture was very viscous but became fluid again when excess acetic anhydride was added. Sections of guinea pig gut were dewaxed, dried thoroughly and immersed in 10% and 33% solutions of sulphuric acid in acetic anhydride at -20° for periods up to 24 hours. This was followed by washes in acetic anhydride, water and 30% alcohol and finally by staining in toluidine blue. Very little change in the metachromatic staining was ever observed, indicating that the gastric acid mucopolysaccharides were not desulphated.

Section 10

Uronic Acids

1. Introduction

There are no histochemical tests specifically designed for uronic acid groups. It is therefore difficult to devise fluorescence histochemical tests when there is no established comparison. However, some incidental observations made by Spicer (1960) suggested that the uronic acid groups of acid mucopolysaccharides may react specifically with alcian dyes at about pH 2. The specificity has now been confirmed by investigating the mechanism of the uptake of this dye by tissue sections.

The only means of detecting uronic acids *in vitro* is by Dische's test. This involves degrading the acid with concentrated sulphuric acid to an intermediate which can form a coloured complex with carbazole. It has been found that the intermediate is itself highly fluorescent *in situ*.

2. The affinity for alcian dyes.

1. Introduction

The visible uptake of alcian blue or alcian green has been used as the diagnostic test for acid mucopolysaccharides against which the performance of fluorescence techniques could be gauged The alcian dyes have also been used for blocking acid mucopolysaccharides when staining sections specifically for nucleic acids with coriphosphine (sec. 8.1). It will be suggested

later that alcian dyes react with the uronic acid rather than with the sulphate group of acid mucopolysaccharides.

Phthalocyanine dyes of the alcian type were first introduced into histochemistry by Steedman (1950) for the visible demonstration of acid mucopolysaccharides. In his method, nuclei also stained but this can be avoided by lowering the pH below 2.8 (Wagner and Shapiro, 1959) with acetic acid (Lison, 1953; Lowry, 1956) or hydrochloric acid (Bugibe et al, 1959) and keeping the staining times short. In fresh tissues, however, some of the nuclei stain even if the time of staining is kept to a minimum. Vialli (1951) and Wagner and Shapiro (1957) have reported that alcian blue parallels metachromatic staining, but this is not universally true (Spicer, 1960). Moreover, alcian blue has a relatively poor affinity for some mucins in situ such as heparin in most cells (Spicer, 1960). Another phthalocyanine dye, "Astrablau", although not yet commercially available from Bayers' is more reliable, being taken up equally well by all acid mucopolysaccharides (Gedigk, 1956; Bloom and Kelly, 1960). Even after staining for 18 hours, there is very little uptake of this dye by other basophilic elements. The structure of the dye has not yet been revealed by the manufacturers (Harms, 1963) so that its reactive centres are still unknown.

Spicer's detailed investigations (1960) into the histochemical affinity of various sulphated and non-sulphated acid

mucopolysaccharides led him to conclude that alcian blue reacts primarily with uronic acid groups. The reactivity of alcian blue towards sulphate free mucins (e.g. hyaluronic acid) is blocked by methylating the tissues beforehand, but is restored again after demethylation. However, many sulphated mucins retained or even increased their affinity for alcian blue after methylation with hot methanolic hydrochloric acid. This was interpreted as being due to a removal of the sulphate group during the methylation process which made the uronic acid groups more accessible for reaction with alcian blue. Using a milder method of methylation (sec. 11.4) which is unlikely to remove O - sulphates, I have been able to confirm Spicer's observations for sulphated gastric mucins. On the other hand, cartilage in the rabbit epiphysial plate (containing mostly chondroitin sulphate) took up no alcian blue following methylation but regained it after demethylation (with alcoholic potash), contrary to Spicer's results with tracheal hyaline cartilage, chondrocyte cytoplasm and lacunar borders taken from various rodents.

Some types of sulphate can also react with alcian blue provided they are not present in high concentration; for example, neutral polysaccharides that have been fully sulphated with cold, concentrated sulphuric acid (Moore and Schoenberg, 1957) lack reactivity towards alcian blue, but if they are methylated and/or desulphated again with warm methanolic

hydrochloric acid, their affinity for the dye is restored (Spicer, 1960). However, Moore and Schoenberg's technique may also lead to the oxidation of the primary (C_6) hydroyxyl groups of glycogen to uronic acids if concentrated sulphuric acid can react in the same way as other mineral acids (e.g. nitric acid) on sugars. Cold concentrated sulphuric acid preferentially Sulphates the primary hydroxyl groups in vitro (Guiseley and Ruoff, 1961) and this may be followed by hydrolysis and oxidation (sec. 13.2). This may explain the capricious affinity for alcian blue of glycogen in tissue sections that have been treated with conventional acidic sulphating reagents. I have never been able to induce any uptake of alcian blue by glycogen in liver sections treated in this way. My experience confirms that of others who have found that glycogen cannot be sulphated with such reagents (as shown by the absence of any additional γ- metachromatic staining towards azure A). Prolonged immersion of sections in strong acids would hydrolyse any O - sulphate groups that may have been initially formed and as well as the glycosidic linkages between each glucose unit. This would lead eventually to the complete extraction of glycogen and the absence of alcian blue affinity.

2. <u>The specificity of the alcian dye reaction of uronic acids</u>

The absolute affinity of alcian blue for uronic acids appears to be vitiated by its negative reaction with simple hexuronic acids <u>in vitro</u> (Zugibe, 1963) but this cannot be

used as conclusive evidence as it is well known that hexuronic acids readily undergo conversion to their lactones and decarboxylation in acid conditions (Hirsch, 1952; Stutzand Deuel, 1958). This conversion could prevail in the acid solutions of alcian blue.

Treatment of tissues with acetic anhydride in pyridine decreases their capacity to combine with the dye (Rizzoli, 1955). As this procedure primarily converts C-terminal carboxyl groups to ketones provided they are adjacent to amine groups (sec. 7.2), Rizzoli's observation might be considered favourable to Spicer's theory of specificity for uronic (or carboxylic) acids.

More direct evidence has been obtained by observing the alcian blue affinity of the sulphated gastric mucins in guinea pig gut which have been methylated and reduced with lithium aluminium hydride in hot dioxane (sec. 9.9). Such mucins retain their full metachromatic azurophilia following saponification (Fig. 39) but lost nearly all their affinity for alcian blue, unlike the control, methylated sections. It is believed that the O-sulphate groups are left intact by this technique, but that uronic acids (or their methyl esters) are reduced to primary alcohol groups. However the latter could not be demonstrated by sulphating the unsaponified section with DMF-sulphur trioxide complex (sec. 13.7) and staining in azure A (Fig. 52). There was <u>no</u> staining in the acid mucopoly-

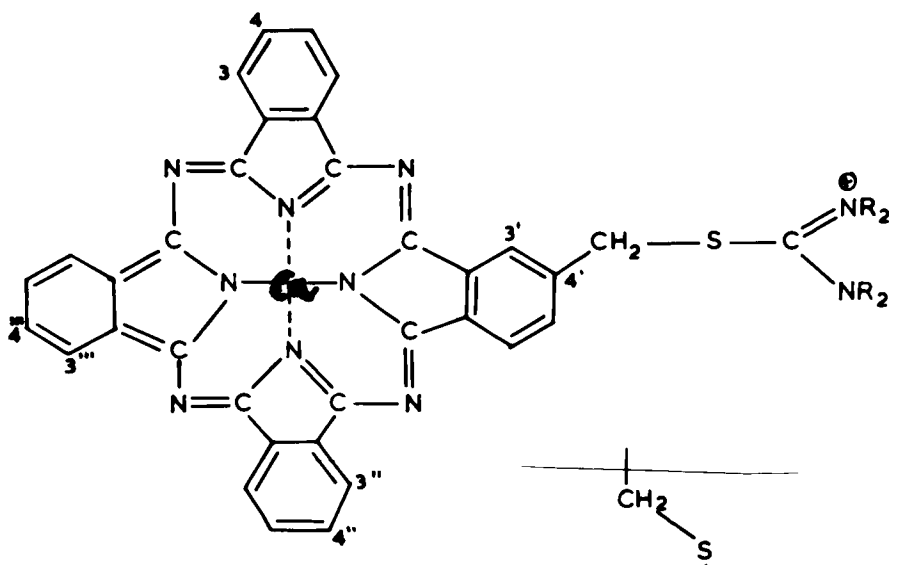
saccharide sites, although the staining was very intense in the muscle where there is a high proportion of (reduced) protein carboxyl groups (secs. 9.1/3 and 10.4/1).

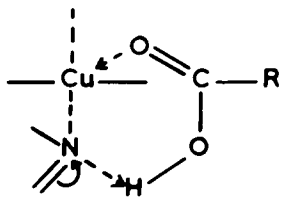
The precise mechanism whereby alcian dyes combine with uronic acid groups remains obscure. The greater resistance to decolourisation by acid and alkali in sections stained with alcian blue as compared with the facile extraction of thiazine dyes suggests that the former is not held by a salt type linkage. The ability of polyuronic acids to stain at pH levels where they are largely unionised even more conclusively argues against a salt-type of combination with the dye.

Spicer (1960) believes an amide bond is the most compatible with the known properties of the complex. The comparative effectiveness of acid methanol in reversing staining was considered consistent with a transesterification with an amide group. As alcian blue is a tetra-alkylated thiouronium derivative(I) of chloromethylated phthalocyanine (Haddock, 1948), amide formation cannot take place unless the amine group is spontaneously dealkylated or the sample of dye contains an incompletely alkylated derivative.

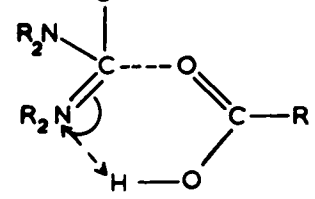
A more likely mode of combination is through hydrogen bonding of the unionised carboxyl group with the copper atom and phthalocyanine nitrogen atoms: a stable six-membered ring structure (II) can be formed. Alternatively, bonding may occur at the thiouronium moiety to form the structure (III). The hydrogen bonding theory seems more likely than the amide bond

ALCIAN BLUE





II



III

theory especially as it is thought that other dyes are adsorbed onto the carboxyl and hydroxyl groups of fabrics through such hydrogen bond forces (Venkataraman, 1952).

Goldstein (1961) has put forward a quite different theory. He deduced empirically that there is an inverse relationship between the ionic weight of a basic dye and the density of its stainable substrate. The least dense substrates (in terms of dry weight per unit area, and not charge density; Goldstein, 1963), such as acid mucopolysaccharides, can take up dye cations whose ionic weight excedes say a 1000 (e.g. alcian blue, molecular weight = 1342), whereas the denser nuclei and cytoplasmic RNA are better stained by dyes whose cationic weight is less than 280.

3. <u>The concentrated sulphuric acid test.</u>

Uronic acids, including acid mucopolysaccharides, are usually detected <u>in vitro</u>, by warming them quickly with concentrated sulphuric acid to 60° and coupling the degradation products with carbazole to form a purple coloured complex. (Dische, 1947; 1950). The hexosamine part of acid mucopolysaccharides is not responsible for the reaction (Helbert and Brown, 1957).

Both hexoses and uronic acids give a violet colouration with hot 50% sulphuric acid <u>in vitro</u> before the addition of carbazole, but on dilution with water, the colour of the hexose solution is enhanced, while uronic acids become colourless

under the same conditions. This distinction has now been applied as a histochemical test: sections were taken through increasing strengths of sulphuric acid, immersed in 50% sulphuric acid at 75° for 1 - 2 minutes and mounted in 50% sulphuric acid at room temperature. A purple colouration appeared in sites known to contain uronic acids. The intensity of the colour developed at 60° was much less, although Dische (1950) claims that a more selective degradation takes place in vitro at this temperature. When the sections were washed with 30% sulphuric acid, the purple colour disappeared.

If the sections treated with hot sulphuric acid were taken down to water and examined for fluorescence, an intense blue colour was observed in sites containing uronic acids (Fig.53). This is an extremely sensitive test.

The chemical nature of the fluorescent product is unknown. It may be related to the blue fluorescent product observed by Vanzetti (1933) when carbohydrates are treated with concentrated sulphuric acid, diluted with water and allowed to stand for a few days. Undoubtedly the first degradation product is 5 - carboxyfurfural (Stutz and Deuel, 1950; Nenkom and Hui, 1959), but it is not known whether it, or a hydrated form, can fluoresce. The fluorescent product in situ exhibited a slightly more intense fluorescence after coupling with salicyloyl hydrazide but as aldehydo - salicyloyl hydrazones have the same fluorescence colour as the original degradation product itself,

Fig. 52. Guinea pig gut. Fixed in glycol ether.

Methanolic thionyl chloride, 4 hrs. : lithium aluminium hydride + dioxan, 60°, 23 hrs.: dimethylformamide - sulphur trioxide complex: azure A. Transmitted white light, water mount, X 165.

There is no staining in sites known to contain acid mucopolysaccharides. The muscle exhibits a strong γ-metachromatic azurophilia.

Fig. 53. Mouse salivary gland. Fixed in neutral calcium-formalin.

50% sulphuric acid, 60°, 2 mins. Dry mount, X 180.

An intense blue fluorescence (of a deeper tint than that shown in the photomicrograph) is emitted from the chromidial regions which are known to contain uronic acid groups.

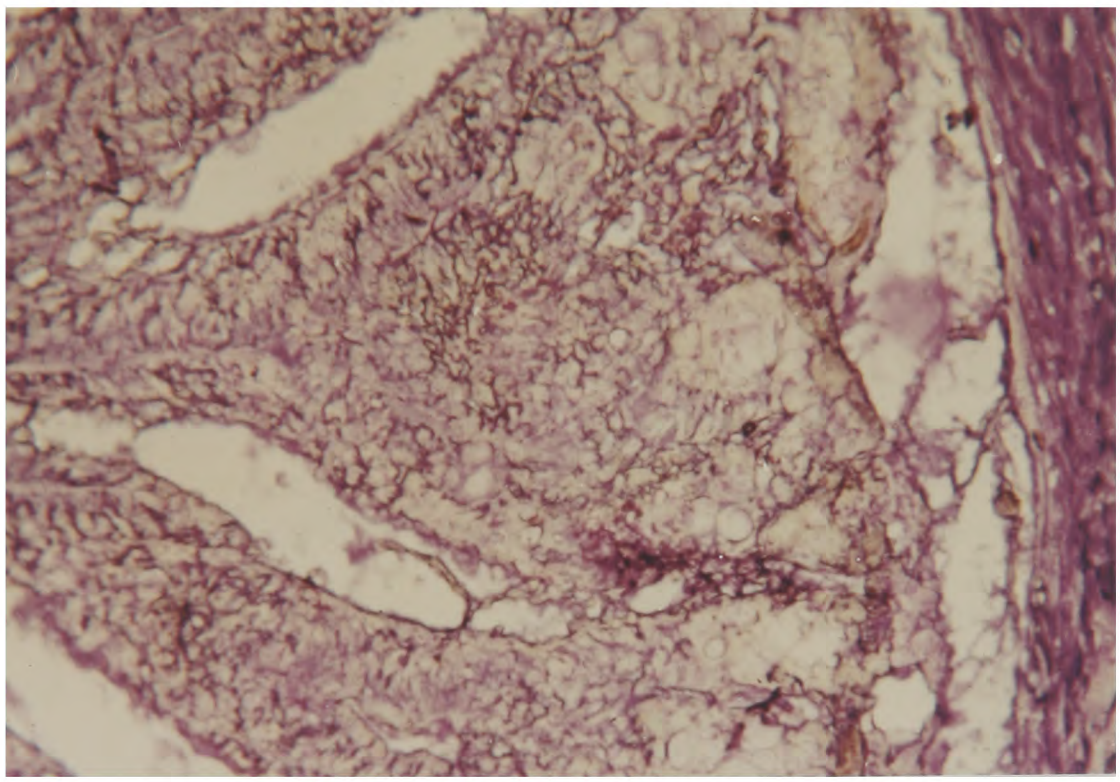


Fig. 52

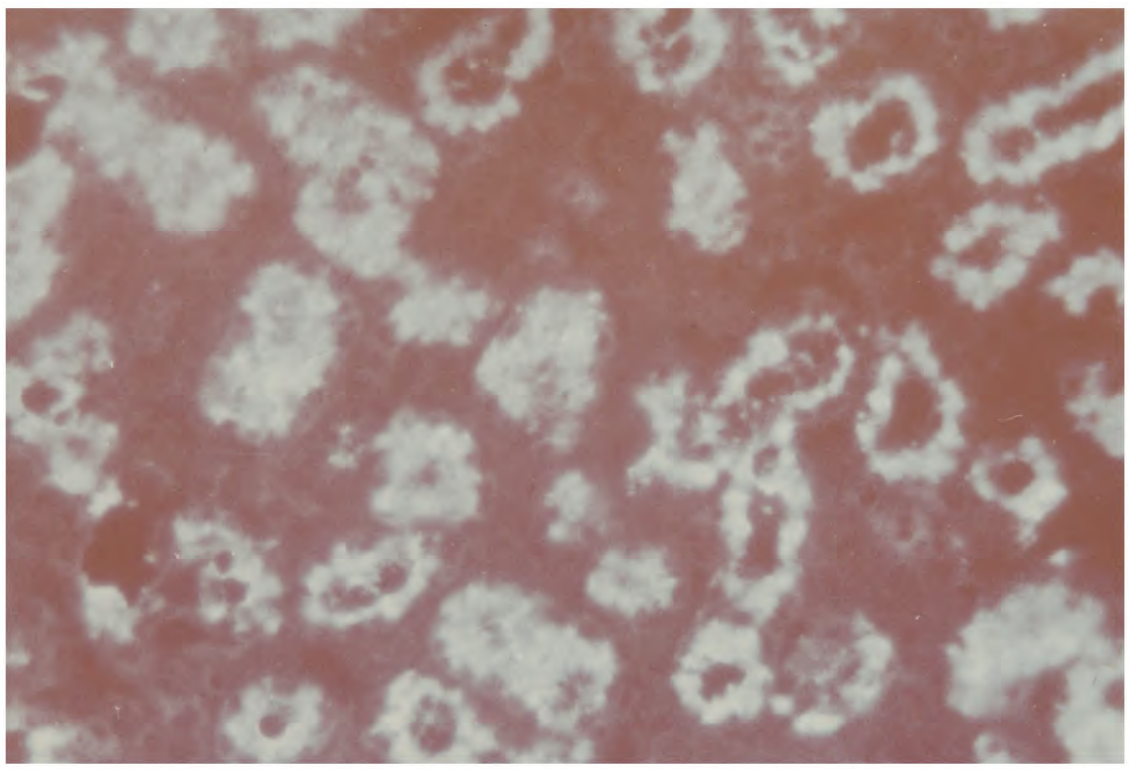


Fig. 53

it cannot be inferred that the latter is an aldehyde. If it is not, as was intimated by a negative reaction with acridine yellow - SO_2 or acriflavine - SO_2 (sec. 12.7) it might be a hydrated furfuraldehyde or even 2,5 - furandicarboxylic acid formed by further oxidation (Yoden and Tollens, 1901).

4. Other reactions of uronic acids

Two other reactions of uronic acids (as their methyl esters) which are known to take place *in vitro* were tried without success on acid mucopolysaccharides *in situ*. Methylated sections of guinea pig gut were used as test material.

(1) Reduction with complex metal hydrides

The carboxyl groups of the amino acid components of proteins can often be reduced *in vitro* to alcohols by LAH (Fromageot *etal*, 1950; 1952), particularly if they are first methylated (Jolles and Fromageot, 1952). Hexuronic acids are reduced similarly (Chander *etal*, 1951; Hardegger *etal*, 1952). The milder reducing agent, sodium borohydride, does not reduce unmethylated carboxylic acids (Chaikin and Brown, 1949) and has been used specifically for reducing the methyl esters of hexuronic acids (Adams, 1952) and of degraded acid mucopolysaccharides (Weissmann and Meyer, 1952; Danishefsky *etal*, 1960; Wolfrom and Juliano, 1960) from which the sulphate groups have been previously removed.

The ability of both reducing agents to reduce carboxyl groups in tissue sections was therefore investigated. Attempts

were made to detect the resulting primary alcohol groups by their δ- metachromatic azurophilia following their sulphation with the DMF - sulphur trioxide complex (exp. sec. 13.7). The decrease in the number of residual carboxyl and uronic acid groups was followed by the changes in azurophilia or affinity for alcian blue.

LAH in hot dioxane has little effect on unmethylated sections (exp. sec. 9.15, stages 3 - 9): the azurophilia was hardly altered afterwards, indicating very little reduction of protein carboxyl or mucin uronic acid groups. The phosphate radicals of nucleic acids were also unaffected, unlike their methyl esters (sec. 9.9) which are labile.

Methylated sections behaved quite differently. The sites containing acid mucins subsequently took up very little alcian blue which means that, if this dye is specific for uronic acids, the uronic acid groups and their esters have been reduced. However, the primary alcohols which should be produced in this process could not be detected; there was no azurophilic staining in the acid mucin sites following sulphation (Fig. 52). The metachromatic azurophilia in the acid mucin sites of reduced sections, after saponification proves that acid mucopolysaccharides were not eluted from the section during this experiment. It is quite probable that the original O - sulphate groups interfere with the attack of the sulphating reagent at their neighbouring, engendered primary hydroxyl groups.

The protein sites, particularly those in muscle which contain a relatively high proportion of terminal carboxyl groups (sec. 9.1/3), showed very strong δ- metachromasia towards azure A after reduction and sulphation. This intense reaction may have arisen either from the reduction of carboxyl groups or the sulphation of protein amine groups. As the intensity of the induced metachromasia is very much less in unreduced sections, the additional chromasia in the muscle after reduction and sulphation must therefore be due to the reduction of carboxyl groups.

Cold sodium borohydride (sec. 3.14/41) had no action on methylated sections, and was not investigated further.

(2) Condensation with hydrazines.

Esters of carboxylic acids condense with phenylhydrazine *in vitro* in the presence of phosphoric acid, but not if hydrochloric acid, sulphuric acid or their salts are present (Jones *etal*, 1953). It is a base-acid catalysed reaction in which H_3PO_4 is the acid and $H_2PO_4^-$ is the base. No reaction takes place when water is absent or is present in a concentration greater than 15M.

$$R\cdot CO\cdot OR' + Ph\cdot NH\cdot NH_2 \longrightarrow R\cdot CO\cdot NH\cdot NH\cdot Ph + R'OH$$

No visible colouration could be seen in methylated test sections treated with mixtures of phenylhydrazine and phosphoric

acid of varying proportions, nor could any fluorescence be induced with salicyloyl hydrazide, with or without post-treatment in zinc acetate solutions.

Experimental Section 19

Uronic Acids

1. Alcian dyes (v, after Steedman, 1950, and Lowry, 1956)

Method

(1) Take sections to water.

(2) Stain in a filtered 0.1% solution of Alcian Blue (or Alcian Green) in 10% acetic acid for 10 mins.

(3) Wash through alcohols to xylene, and mount in DPX. Examine by visible light.

Result

Acid mucopolysaccharides are stained blue by Alcian Blue and yellowish - green by Alcian Green. No other basophilic sites are stained.

If the stained sections are treated with 1% potassium hydroxide in 80% alcohol for 20 minutes, the alcian blue complex is converted to the stable, bright blue pigment, Monastral Blue.

2% concentrated sulphuric acid method (v and f)

Method

(1) Take dewaxed sections to water.

(2) Immerse in 10% and 30% sulphuric acid for 1 minute each.

(3) Immerse in 50% sulphuric acid for 3 minutes.

(4) Immerse in 50% sulphuric acid at 70 - 75° for 1 - 2 minutes.

(5) Wash carefully in 50% sulphuric acid and examine by transmitted white light as a mount in 50% sulphuric acid. If necessary, this mount is photographed.

(6) Remove cover slip and wash in 30% sulphuric acid. Any change or disappearance of the purple colourations observed in stage (5) is noted.

(7) Wash in 10% sulphuric acid.

(8) Wash in water and examine for fluorescence as a water mount.

Result

Sites that are coloured purple after stage (5) but which disappear after stage (6) contain uronic acids. An intense blue fluorescence is formed in stage (8).

Section 11

Methylation techniques

1. Introduction

One of the differences between conventional and fluorescence histochemistry is the necessity in the latter to block pre-existing tissue basophils before staining engendered groupings with basic dyes. For example, pseudo-Schiff reagents (sec. 13.7) can only be used for the demonstration of aldehydes in Feulgen - hydrolysed nuclei if the pre-existing phosphate basophils in the same site are methylated beforehand; otherwise they would also be coloured by the basic dye in the reagent.

The ability to form the methyl esters of anionic groups in tissue sections is also useful for studies of the mechanism of histochemical reactions. The investigation into the specificity of alcian blue for uronic acid groups for example, necessarily includeda methylation sequence (sec. 10.2/2).

Organic chemists use methyl halides, usually in the presence of alkali to replace acidic hydrogen atoms with methyl groups <u>in vitro</u>. Unfortunately alkali would distort the structure of tissue sections and cause extensive hydrolysis of proteins. Therefore milder acidicand neutral methylating reagents were investigated for use in histochemistry.

2. Methanolic hydrochloric acid

Most histochemists use the hot methanolic solutions of hydrochloric acid introduced by Fisher and Lillie (1954) for abolishing tissue basophilia. This reagent was originally devised for the specific esterification of carboxyl groups of proteins in vitro by Fraenkel - Conrat and Olcott (1945). When tissues are treated with this reagent at room temperature or at 37°, the process is sometimes referred to as "mild methylation". If the methylation is carried out at 60° or above, it is called "drastic methylation".

The γ- metachromasia (exp. sec. 9.1) of intestinal mucin, cartilage and mast cell granules is inhibited after 2 hours methylation at 58° or 48 hours at room temperature. The cytoplasmic basophilia is destroyed after 4 hours at 58° or 48 hours at room temperature. Pancreatic acinar cell cytoplasm required 8 hours drastic methylation or more than 9 days mild methylation. The nuclear and nucleolar basophilia required 14 days treatment at room temperature or 24 hours at 58°. By contrast, there is very little inhibition of basophilic staining after prolonged methylation at 3°.

The primary amine groups of protein residues are converted to secondary and tertiary amines when tissue are methylated at 60°, but not at room temperature (Terner and

Clarke, 1960).

Fisher and Lillie (1954) implied that the abolition of the PAS reaction of colonic mucin, mixed tumor stroma and cartilage by drastic methylation for 96 hours was due to the esterification of hydroxyl groups. It is now believed that hydroxyl groups are not esterified at either temperature, and that the abolition of the PAS reaction is due to the extraction of mucopolysaccharides from the section.(sec. 3.7). This fact has been utilised in devising a specific method for the detection of low concentrations of thiol groups in tissue sections (sec. 4.3).

Fisher and Lillie's reagent has two other disadvantages as a methylating agent in histochemistry: firstly, some basophilic materials, particularly RNA and acid mucopolysaccharides, may be extracted from the section instead of being methylated under the conditions of time and temperature recommended by them.

Secondly, some sulphate groups, may be hydrolysed off from acid mucopolysaccharides (sec. 9.15).

3. [illegible].

Peters (1955) claimed that an ethereal solution of diazomethane would methylate tissue carboxyl groups selectively after 4 hours treatment at 18°, provided the sections were "hydrated" beforehand with 75% alcohol. This claim for

specificity does not agree with the reactions of diazomethane *in vitro*. Rutherford, Patterson and Harris (1940) found that phenolic groups (of tyrosine) could be methylated with this reagent, although it had no effect on the amine or hydroxyl groups of serine. Glycine does not react in dry ether, but on adding small amounts of water or methanol, betaine is formed vigorously (Biltz and Laetzold, 1922). The hydroxyl groups of carbohydrates can also be methylated by diazomethane in the presence of methanol (Kuhn and Baer, 1953). Sulphated mono and polysaccharides are easily methylated *in vitro* by an ethereal solution of diazomethane (Holt, 1962).

The wide range of reactivity of diazomethane *in vitro* made it promising as a histochemical reagent, particularly as it could be used at room temperature and so would avoid the complications associated with the use of Fisher and Millie's reagent at higher temperatures.

In my hands, a fresh 0.5% ethereal solution of diazomethane blocked the basophilia of proteins, RNA and DNA within 16 hours and that of the sulphated acid mucopolysaccharides after 18 hours. The hydroxyl groups of neutral mucopolysaccharides were unaffected after 24 hours treatment as shown by an unchanged periodic acid-salicyloyl hydrazide reaction (sec. 12.19). Some RNA was extracted after 24 hours as shown

by the reduction of cytoplasmic azurophilia after saponification (sec. 11.5).

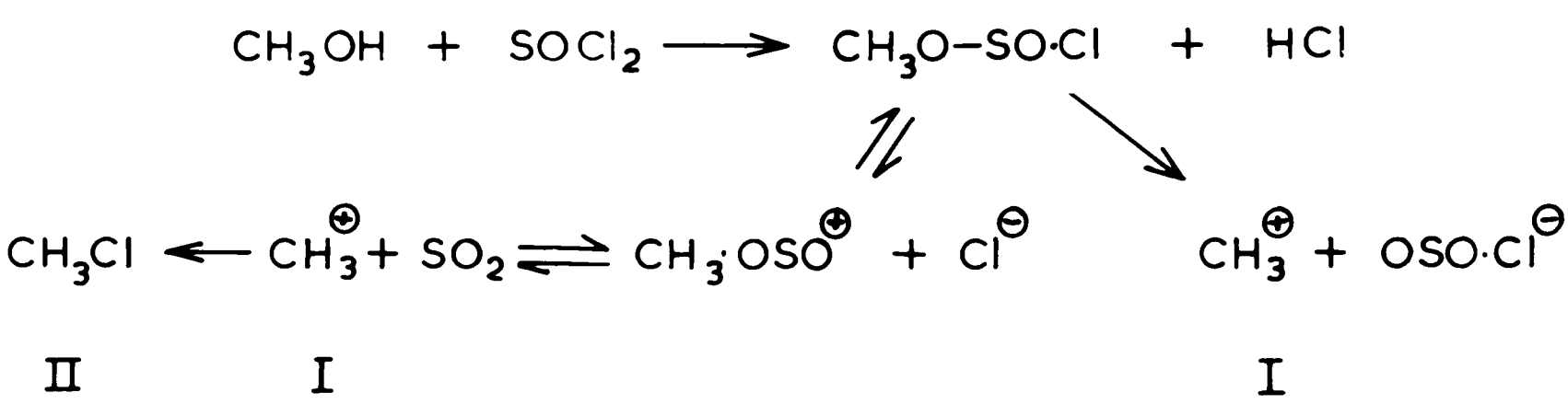
Although diazomethane can be successfully used for methylating tissue basophils, its preparation is inconvenient. A fresh solution must be prepared before each experiment as it loses its activity after about 24 hours. As each preparation of diazomethane entails a distillation, it is unlikely to come into general use in histochemistry. For this reason, the chemically similar ethyl diazoacetic acid was investigated as a histochemical reagent. It can be prepared by the action of nitrous acid on an aqueous solution of glycine ethyl ester hydrochloride at 0 - 2° (Womack and Nelson, 1944). Ether extraction of the reaction mixture gives a product suitable for use without distillation. Unfortunately, this ethereal solution had no effect on the basophilia of various test tissues after 24 hours treatment.

4. Methanolic thionyl chloride.

Bremner and Huber (1953) and Bello (1956) claimed that thionyl chloride in methanol will methylate the carboxyl groups of proteins specifically in vitro, but would leave amine and phenolic groups intact. As this reagent was used at room temperature, it seemed worthwhile investigating for the methylation of tissue basophils as it would not have the disadvantages associated with drastic methylation at higher

temperatures.

Thionyl chloride probably reacts with methyl alcohol to form intermediates I and II (Lewis and Boozer, 1952; Boozer and Lewis, 1953) capable of methylating anionic groups in tissues rapidly.



In my experiments, a 2% solution of thionyl chloride in methyl alcohol completely abolished the basophilia of all the tissues examined so far towards fluorescent dyes within 4 - 6 hours. The cytoplasmic basophilia due to protein carboxyl groups and RNA was usually abolished after 30 minutes treatment. A further 2 - 3 hours treatment was required before the last traces of basophilic staining in the nuclear sites were abolished. The basophilia of both unsulphated and sulphated acid mucopolysaccharides was abolished after 4 hours methylation. Exceptionally the dense deposits of chondroition sulphates in thick (50μ) decalcified section of epiphysial plate required about 6 hours

treatment for the abolition of their azurophilia.

After 6 - 8 hours immersion in the methylation mixture, the phosphate groups began to be removed from their parent nucleic acids(perhaps by the sulphur dioxide in the mixture), the process being complete after about 24 hours. This selective reduction process was followed by the decreased azurophilia after sections had been saponified in alcoholic potash.

Acid mucopolysaccharides were extracted much more slowly; the extraction was neglible in sections of guinea pig gut left in the methylation reagent for about 10 - 12 hours, but it was practically complete after about 24 - 30 hours. This property would appear, at first sight, to be useful for the selective basophilic staining of acid mucopolysaccharides. Unfortunately there are two disadvantages which prevented the general application of this technique. First, the structure of the tissues became distorted. Second, the optimal time of methylation required for the maximum reduction of nucleic acids and the minimal extraction of acid mucopolysaccharides varied from tissue to tissue. Further discussion of this point, together with some illustrative photomicrographs, can be found in sec. 9.9.

The hydroxyl groups of neutral mucopolysaccharides were unaffected by four hours methylation. Therefore the

can be oxidised with periodic acid to "dialdehydes" which can be demonstratedspecifically with pseudo-Schiff reagents (sec. 12.7).

The phenolic groups of tyrosine residues were also unaffected. The sites, giving a positive fluorescent nitroso-naphthol reaction (sec. 6.2) for phenols and a negative NEM - SH/SN reaction for thiols (sec. 4.2), gave a positive DNFB reaction at pH 5.2 (sec. 6.3) both before and after methylation. This was contrary to what was expected from Rutherford et al's (1940) work cited earlier. However, as Corti (1949) found that the methylation of tyrosine with methyl sulphate and alkali gave only N - methyl tyrosine leaving the phenolic group intact, the chemical properties of methanolic thionyl chloride and diazomethane were not strictly comparable.

Contrary to the results obtained in vitro, methanolic thionyl chloride rapidly methylated the primary amine groups of proteins in tissue sections. The chloramine T - salicyloyl hydrazide reaction (sec. 3.8), the hydrogen peroxide - tungstate (sec. 3.15) and hypochlorite deamination (sec. 3.14) reactions were all negative on sections which had been methylated for 30 - 60 minutes. However, their König - Sassi reaction (sec. 3.3) was still positive. Their acidophilic reaction towards the Solochrome Black - alum complex

(sec. 3.10) was even stronger. The last two reactions indicate that strong cationic poly - (2^{o} and/or 3^{o}) methylamines were formed. The formation of such groups would explain the abolition of the sulphation reaction of neighbouring hydroxyl groups of neutral mucopolysaccharides (sec. 13.8). Presumably the bulky polymethylamine groups sterically hinder the approach of the comparatively large sulphur trioxide complex to the hydroxyl groups. The same objection does not always apply to the sulphation (of thiol groups) with the smaller sulphuryl chloride molecule (sec. 14.3).

Since the thiol groups of cysteine were readily methylated *in vitro* by methyl bromide (Blackburn *et al*, 1944; Lewis, 1948), it was expected that thiol groups *in situ* would be methylated by the species II or I in methanolic thionyl chloride mixtures. However, in practice, the thiol groups retained their full histochemical reactivity. One application of this property is described in sec. 12.7/7.

Geyer (1962) has recently investigated the use of 4% methanolic thionyl chloride in histochemistry. However, he has limited his published observations only to its effect on tissue basophilia when this mixture is used at room temperature and at 58^{o}. The objections raised against Fisher and Lillie's methanolic hydrochloric acid reagent would also be applicable to Geyer's reagent.

5. The reversal of the methylation blockage.

The methyl groups can be hydroylsed off basophilic groups by saponifying the methylated sections in 1% potassium hydroxide in 80% alcohol for 20 minutes (Spicer and Lillie, 1959). The alcoholic potash reagent loses some of its potency after storage for about one week at room temperature. An unmethylated control section should be taken through the saponification process at the same time as the test sections. This enables a check to be made on what might appear to be anomalous basophilic staining in certain tissue sites of saponified test sections. The muscle of guinea pig gut, for example, exhibits variable streaky deep purple colourations when stained in axure A after saponification (Fig.39). The reasons for the additional basophilia are not known, but the hydrolysis of peptide and N-acetyl linkages may be partly responsible. Some O-sulphate groups of acid mucopolysaccharides may also be removed.

Experimental Section 11 Methylation techniques.

The effect on tissue constituents of the following methylation procedures was tested by the intensity of the azurophilia (i) before and (ii) after saponification (sec.11.5) and the (iii) PA - SH and (iv) Alcian Blue reactions.

1. Methanolic hydrochloric acid (after Fisher and Lillie, 1954)

Sections were taken to methanol and immersed in N/10 methanolic hydrochloric acid at 60° for 2 - 24 hours.

There was a progressive and parallel decrease in the intensity and extent of reactions (i) - (iv) when carried out on guinea pig gut which had been fixed in glycol ether. After about 6 hours treatment, all the reactions were negative.

Most basophilic material was therefore extracted from the sections rather than methylated <u>in situ</u>

2. <u>Diazomethane</u>

The reagent was prepared just before use by the method of de Boer and Backer (1954). 2g tolylsulphonyl methyl nitrosamide was dissolved in 12.5 ml diethyl ether and slowly added to 0.5g potassium hydroxide dissolved in 2.5 ml 95% alcohol and 1 ml water. This mixture was warmed on a water bath (70 - 75°) and distilled into 20 - 30 ml ether. Ether was added to the distilling mixture until the distillate no longer appeared yellow. The final concentration of the ethereal diazomethane solution was about 0.5%.

Dewaxed, dried sections were immersed in the ethereal solution for periods up to 24 hours. At least 10 hours treatment was required before the basophilia was abolished. The acid mucopolysaccharides were more resistant to methylation than the nuclei. No distortion of the tissue structure was observed.

3. <u>Diazoacetate</u>.

1 g ethyl aminoacetate hydrochloride dissolved in 30 ml cold water was added to 1 g sodium nitrite in 20 ml and 2 N hydrochloric acid and left for 30 mins. at 0 - 5°. The

diazoacetate was then extracted with 3 x 15 ml portions of ether.

Dewaxed, dried sections were immersed in the ethereal solution for 24 hours at 5°. The nuclear basophilia was hardly altered. The staining of the acid mucopolysaccharides was reduced by about a half. Cytoplasmic (RNA) staining was abolished.

4. Methanolic thionyl chloride.

The reagent was prepared by slowly adding 1 ml thionyl chloride dropwise to 50 ml methanol, and left to "age" overnight. A violent spitting exothermic reaction took place when the thionyl chloride first came into contact with the methanol.

Tissue sections were taken to methanol and immersed in the reagent for periods up to 24 hours.

For the straight-forward methylation of tissue basophilic materials without any secondary extraction or degradation, 4 hours treatment usually sufficed. This did not produce any change in the PA - SH reaction (exp. sec. 12.14).

Most tissues lost both RNA and DNA when methylated for more than 6 hours. This led to considerable distortion.

Section 12.

Vic - Glycols

1. Introduction

The unique property of free vicinal glycols is their rapid oxidation to "dialdehydes" by periodic acid at room temperature in the pH range 3 - 5 provided their stereochemical configurations are favourable. Other groupings are also susceptible to oxidation by periodic acid, but they usually require more drastic conditions before any significant reaction takes place.

The engendered "dialdehydes" can be condensed with numerous homo- and heterocyclic amine compounds, sometimes in the presence of sulphurous acid, to form visibly coloured or fluorescent conjugates. When Schiff's solution is used for this purpose, the reaction is referred to as the periodic acid - Schiff (PAS) reaction.

2. The periodic acid reaction

Periodic acid was first employed by Malaprade (1928, 1934) for the chemical estimation of glycols and later introduced into polysaccharide chemistry by Jackson and Hudson (1937). Its use in histochemistry was first described by McManus (1946) for the demonstration of mucin, but later Lillie (1947 a, b) and Hotchkins (1948) elaborated this method into a histochemical one for the detection of a variety of polysaccharides in tissues.

Other reagents (lead tetra-acetate, sodium bismuthate) have been used for the cleavage of 1, 2 - glycols but they seem to have little advantage over periodic acid in histochemical practice (Pearse, 1960). On the contrary, the fact that most of them contain heavy metals capable of quenching fluorescence militates against their use in fluorescence histochemistry. Another particular advantage of periodic acid, which renders it immeasurably superior to other oxidising agents, is that it does not oxidise the engendered aldehydes further to carboxylic acids.

The optimal pH for the histochemical oxidation of 1,2 - glycols in carbohydrates is about 5. This avoids any complications due to the oxidation of amino-sugars and α- amino acids; usually their protonated form resists oxidation, so that their most rapid reactions occur at a pH near neutrality (7.5). This has been confirmed for simple amino-alcohols (Fleury et al, 1949) ephedrine (Wickstrom, 1950), serine and threonine (Shin and Nicolet, 1941). On the other hand, the carbon dioxide liberated during the oxidation of D - glucosamine was ten times greater at pH 1.2 than at pH 5 (Cantley and Hough, 1960). In this investigation, the pH of the periodic acid solutions were adjusted to a value within the range 3 - 5 with dilute sodium hydroxide. Buffered solutions were not used, as it is known that phosphate buffers cause erroneous

results (Lindstedt, 1945; Greville and Northcote, 1952).

3. The reactions of periodic acid with functional groups other than vic - glycols.

Periodic acid can oxidise numerous compounds which do not contain vic - glycols. Unless the reaction conditions are carefully controlled, such compounds detract from the specificity of the reaction for vic - glycols. The following groups or compounds, other than 1,2 - glycols in carbohydrates, are oxidised by periodic acid.

(1) α - Hydroxyamino acids.

It has already been pointed out that this class of compounds is only oxidised in highly acid or neutral media. However, in view of the extreme sensitivity of the fluorescent methods described later for the detection of aldehydes, it is necessary to consider the interference due to the oxidation of the α- amino acid components of proteins even though it may only occur to a small extent. Lhotka (1953) has also stressed the possible interference of this reaction in interpreting the PAS reaction for carbohydrates, particularly as oxidation of compounds containing 2 - hydroxylamine (e.g. serine) is almost instantaneous (Nicolet and Shinn, 1939).

Serine, threonine and hydroxylysine are oxidised in vitro to various products including formaldehyde or acetaldehyde (Nicolet and Shinn, 1939, 1941). The diffusibility of these

degradation products is probably very high and it is unlikely that they will remain in tissue sections during the staining stage of a histochemical procedure, even supposing that these amino acid components are susceptible to attack in proteins. When Glegg et al, (1952) carried out in vitro tests on proteins containing these amino acids, they obtained negative reactions in all except serum globulin, serum albumin and thryoglobulin, These results were interpreted as being due to the infrequent occurrence of serine and threonine at the ends of protein chains.

Arakawa (1957) found that neutral periodic acid oxidised many non-hydroxy amino-acids at 60° after 24 hours, but the oxidiation was negligible or very slow (Nicolet and Shinn, 1939; Fleury et al. 1949) at room temperature. Therefore, the possible interference due to the oxidation of serine and threonine can be excluded together with those of most other amino acids which have only about 0.01% of their reactivity.

(2) Phenols

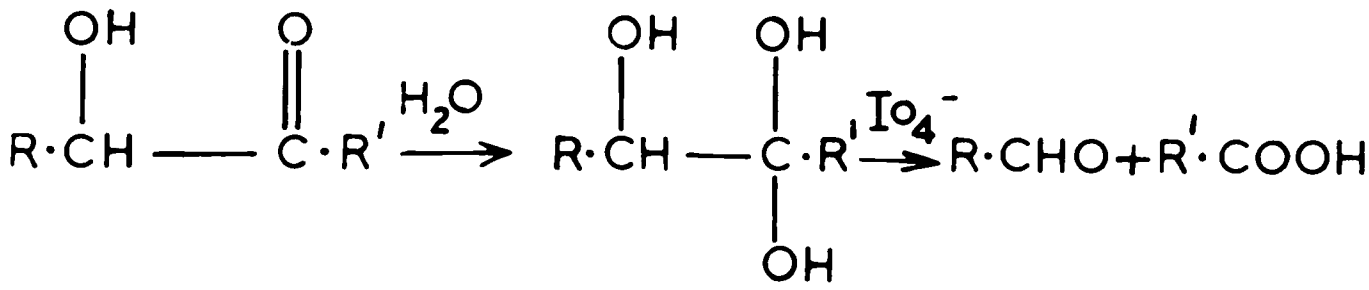
Dennington and Ritter (1947) observed that many ortho-diphenols comsumed appreciably more periodic acid at room temperature after a few hours than did monohydric or meta- or para- diphenols. This reaction would apply to few phenols occuring in tissues (e.g. tyrosine), but it is possible that the positive PAS reaction in materials giving a positive chromaffin reaction (Lillie, 1950) may be due to the oxidation

of the catechol component of adrenalin (Lison, 1953). Lillie (1950) interpreted his reaction, however, as being due to the production of an aldehyde from the hydroxyl methylamino group of adrenalin or one of its precursors, 3,4 - dihydroxy serine. If Lison's (1953) interpretation is correct, noradrenalin would also give a positive periodate reaction (Hale, 1957).

Before carrying out the periodic acid reaction on fresh-frozen tissues, they should be tested for catecholamines by examining them for an intense green fluorescence after fixing them briefly in formalin (Falck, 1951, 1955a, b) under controlled conditions (Falck, 1962).

(3) Ketones

In the presence of water, α - hydroxy- ketones (or ketols) are oxidised (Fleury and Lange, 1932) since they tend to be hydrated to give pseudoglycols (Malaprade, 1934).



The positive PAS reaction of the adrenal "fuscin" was attributed to the presence of a steroid containing the α-ketol group by Lillie (1950). Corticosterone and dehydrocorticosterone are oxidised by periodate to aldehydes (Jackson, 1944),

and it is possible thatthey may be responsible for the positive PAS reaction in the adrenal. However, they may also give a positive reaction without periodate oxidation as the result of the condensation of their ketone groups with Schiff's reagent. The histochemical proof for the reactions of this class of compounds is difficult: the aldehydes produced by the oxidation of co-existing lipids overshadows possible reaction due to the ketosteroids (cp. sec. 15).

Periodic acid will also oxidise ∝- diketones and ∝- ketonic aldehydes (Clutterbuck and Reuter, 1935), but it is necessary to have a hydroxyl group present if an aldehyde is to be produced.

Although it is unlikely that ∝- hydroxyacids (e.g. glycolic acid) and ∝- ketoacids (e.g. glyoxylic acid formed in the rapid periodate oxidation of tartaric acid; Fleury and Bon-Vernatets, 1936) will be present as metabolic intermediates in substantial quantities in fixed tissues, their interference in the specificity of the vic - glycol reaction should be borne in mind (Fleury et al., 1953, 1954). Fortunately, however, they are only slowly oxidised by periodic acid (Fleury et al., 1945; Sprinsom and Chargaff, 1946).

(4) Acetal phosphatides.

One group of saturated lipids which give positive PAS reactions, although they do not contain vic - glycols, are

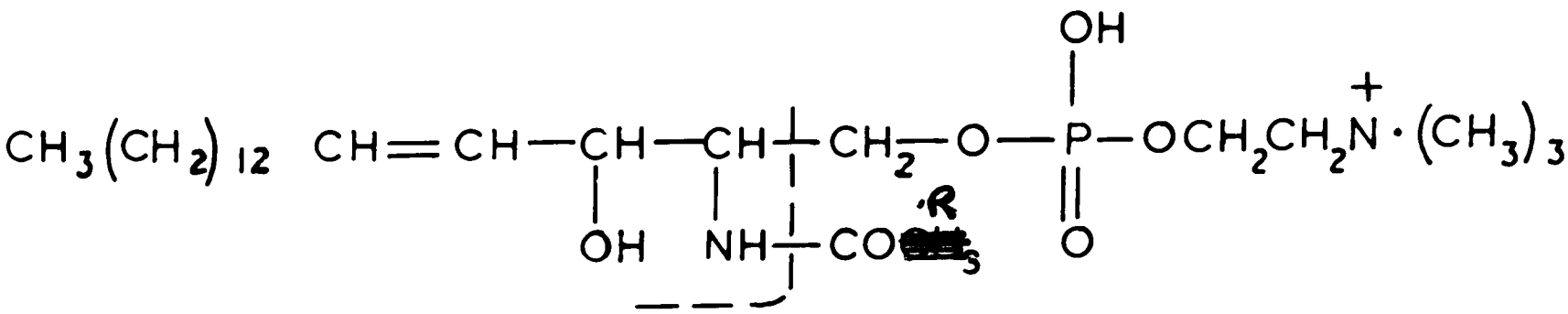
the acetal phosphatides or plasmalogens (Hack, 1949). Brief immersion of fresh-frozen tissue sections in dilute mineral acids or even the acid Schiff's reagent is sufficient to hydrolyse the acetal linkages and to expose the higher fatter aldehydes or plasmals (Feulgen and Voit, 1924). Presumably periodic acid acts in the same way.

The periodic acid reaction can be made specific for vic - glycols in the presence of plasmalogens by releasing and blocking the aldehydes of the latter beforehand. Mercuric chloride is usually used for this purpose (Hayes, 1949; Cain, 1949).

(5) Sphingomyelin

In general, the N - acylated vic - aminohydroxyl grouping is stable towards periodic acid; the N - acyl-derivatives of serine, for example, react only very slowly (Posternack and Pollaczek, 1941). In any case, vic - glycols are preferentially oxidised (Niemann and Hays, 1940). However, the lipid sphingomyelin which contains this grouping gives a strong PAS reaction in vitro (Hack, 1949) although it lacks vic - glycol groups.

The structure of the unsaturated lipid sphingomyelin was finally established by Marinetti's group (Rouser et al., 1953).



Sphingomyelin

Previously Carter et al., (1947, 1951) had shown that the sphingosine part (to the left of the dotted line of the sphingomyelin structure written above) gave a positive PAS reaction as also did its dihydro and N - acetyl derivatives. The ethylinic bond cannot be responsible for the positive reaction which must be due to the cleavage of the N - acetyl amino - hydroxyl group.

Fortunately, however, Kolman (1950, 1956) found the intensity of PAS staining due to sphingomyelin depended on the strength and duration of oxidation; if a 1% solution of periodic acid was used for less than 10 minutes, practically no staining occurred. This can still interfere with the specificity of the periodic acid reaction for vic - glycols if sensitive fluorescent reagents are used to show up the engendered aldehydes. If sphingomyelin is suspected to be present, as can be shown by its specific fluorescence (sec. 15.2) with protoporphyrin (Kosaki et al., 1957), it should

be extracted with hot methanol and chloroform (Keilig, 1944) before carrying out the periodate oxidation.

(6) Unsaturated lipids

Wolman (1950, 1956) found that unsaturated lipids were PAS positive while saturated lipids were not. This forms the basis of the pseudo-plasmal or Feulgen - Verne reaction (Cain, 1949) and is due to the breaking of ethylinic linkages of unsaturated fatty acids to form the corresponding aldehydes. Peracids, in general, possess this property of cleaving olefinic bonds although periodic acid is probably not as efficient as others in this respect. To eliminate their interference in the periodic acid reaction for vic - glycols, they should be oxidised beforehand with performic acid and their engendered aldehydes condensed with aniline (secs. 12.22 and 15.3).

Alternatively, unsaturated lipids can be prevented from being reactive towards periodic acid by brominating their olefinic bonds beforehand (Lillie, 1954).

(7) Thiols and disulphides (proteins)

The amino acids cystine, cysteine and methionine all form insoluble products in the presence of periodic acid, implying oxidation at their sulphuratoms (Nicolet and Shinn, 1935). Cystine and cysteine are even attacked when incorporated into proteins; egg albumin that had been precipitated by

periodic acid *in vitro* lost all its cystine and cysteine content, although its methionine component remained intact (Desnuelle, Antonion and Cusel, 1947). Even simple organic sulphides (Bonner and Brisko, 1951) and thiosugars (OKui, 1955) are oxidised to at least the sulphone stage.

The interference of cystine and cysteine in histochemical oxidations was first implied by Dempsey, Singer and Wislocki, (1950). They found that prolonged immersion in periodic acid increased the basophilic staining normally present in the keratinised portions of epidermis and hair. A strong basophilia was also induced in purified fibrin, endothelium and the cells of the pancreatic islets and acini. Although prolonged hydrolysis in warm hydrochloric acid abolished the basophilia attributable to the phosphate groups of nuclear and cytoplasmic proteins, and to the sulphate groups of acid mucopolysaccharides, it did not remove the strong basophilia induced by periodate oxidation nor that normally occuring in the cornifying regions of the hair shaft or epidermis.

Knowing that organic mercaptan and disulphide compounds can be oxidised by peracids through various intermediate stages to sulphinic and sulphonic acids (Sutur, 1944), Dempsey *et al*. (1950). suggested that the induced basophilia in tissue sections may be due to the oxidation of cystine thiol and cysteine disulphide groups to their corresponding

sulphonic acids. Their hypothesis received further support from Lillie, Bangle and Fisher (1954) who found that oxidation of Keratinous structures by peracetic acid produced an insoluble, strongly acidic substance capable of taking up basic dyes even from a solution of pH 1.2. The oxidation product strongly resisted methylation, unlike the basophils of nuclei, mast cell granules and cartilage. Keratinous structures were also rendered basophilic by two hours treatment in periodic acid at 60°, but with less intensity.

The preceding observations applied only to oxidations in hot periodic acid for a comparatively long time. It could be argued that there would be very little protein oxidation during the brief oxidation carried out at room temperature when periodic acid is used for the histochemical oxidation of polysaccharides. This supposition appears plausible by reason of the negligible change in staining towards methylene blue or toluidine blue when suitable tissues are examined in the visible light microscope. However, when tissues are oxidised in periodic acid and stained in fluorescent pseudo-Schiff reagents (sec.12.7), this hypothesis is immediately vitiated. A detailed discussion of the evidence for the interference of thiol groups in the periodic acid reaction for vic-glycols in fluorescence histochemistry is given in sec. 12.7/7.

4. Compounds containing vic-glycols in tissues

The following classes of compounds containing vic-

glycols can be oxidised *in situ* by periodic acid:

(1) *Polysaccharides and neutral mucopolysaccharides.*

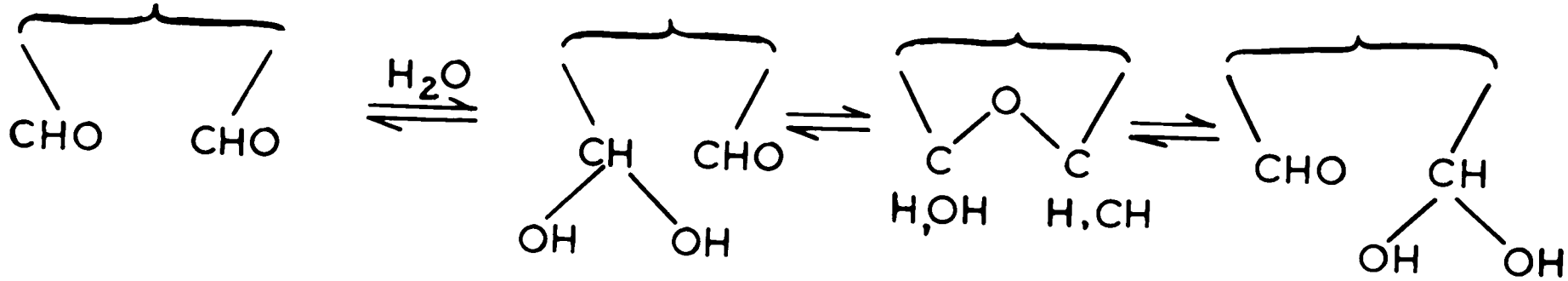
As far as is known, all members of this group are oxidised to their corresponding "dialdehydes" by periodic acid within 5-10 minutes even though most of their *vic*-glycols may be initially in the *trans*-configuration.

On the other hand, not all engendered "dialdehydes" react with Schiff's solution or with pseudo-Schiff reagents (sec.12.7). Not only is Schiff's solution relatively insensitive to some aldehydes owing to variable rates of colour formation (Sawicki, 1962), but it is now known that it will not react with engendered "dialdehydes" (I) that have undergone hydration (II and IV) and internal cyclisation to hemialdals, III (Guthrie and Honeyman, 1959).

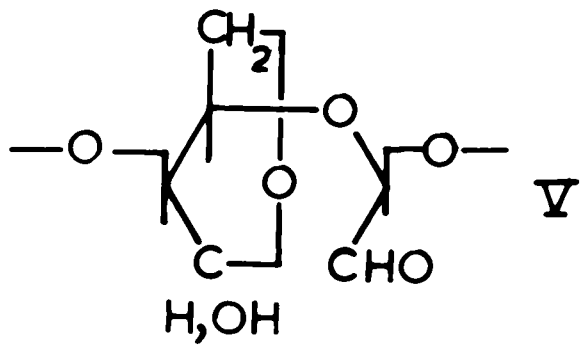
If the oxidised polysaccharide has free primary hydroxyl groups (as in glycogen), hemiacetals (V) may also be formed.

The hydration of periodate-engendered "dialdehydes" was first suspected by numerous workers about 10 years ago (Rowen *et al.*,1951; Hurd *et al.*, 1953; Barry and Mitchell, 1953) and has been studied in detail by Smith's group (Cadotte *et al.*,1957; Goldstein *et al.*, 1958) and by Guthrie and Honeyman (1958, 1959) simultaneously.

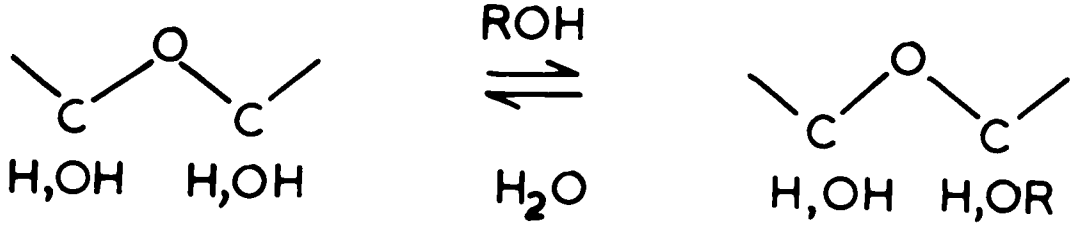
The system has been extended to include the formation of the hemialdal monoglycerides (VI) by the reaction of the



I II III IV

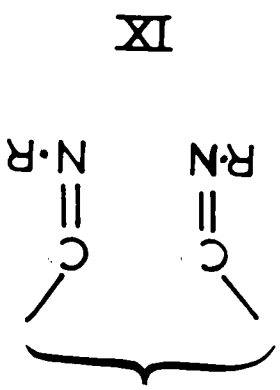


V

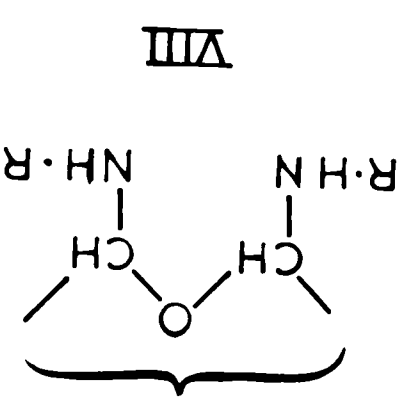


III VI

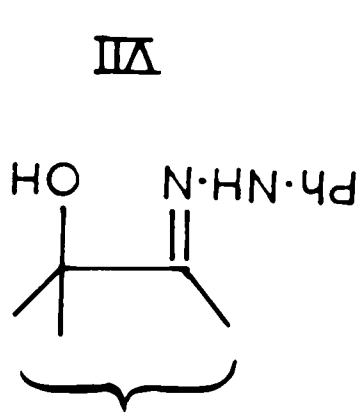
XI



VIII



VII



hemialdal with alcohols (Guthrie and Honeyman, 1958).

Hemiacetal formation could occur on one aldehyde group, followed by ring closure, to give eight isomeric monoglycosides. This type of reaction could occur when alcoholic solutions of periodic acid are used for the histochemical oxidation of dextran (Mowry, 1952; Mowry and Millican, 1952). It may account partly for the difficulty in obtaining a good Schiff staining for dextran oxidised in this way.

The possibilities for hemiacetal and hemialdal formation in the polysaccharides found in tissues are numerous: the internal links can be intermolecular as well as intramolecular (Guthrie, 1961).

Although not all mucin "dialdehydes" may react with Schiff's solution because of hemialdal formation, they all condense with hydrazines. If they are in the hemialdal form, derivatives of the type (VII) are produced (Guthrie, 1960).

However, the structures of the conjugates with the aromatic and aliphatic amines that are used for blocking the engendered aldehydes in confirmatory tests (sec. 12.22) are of the types VIII and IX respectively (Guthrie et al., 1959).

For these reasons, it is now proposed that only hydrazine-type reagents, and not Schiff-type reagents, can be reliably used for demonstrating the "dialdehydes" formed by the periodate oxidation of vic-glycols in histochemistry.

Salicyloyl hydrazide has been successfully used for this purpose as a fluorescent histochemical reagent (sec. 12.19).

Theoretically it should be possible to distinguish those mucin "dialdehydes" that form hemialdals from those that do not: formazans can be produced from the latter (Mester and Major, 1955), but not from the former (Guthrie and Honeyman, 1959). Unfortunately the histochemical investigations carried out to prove this point were equivocal and no clear-cut example has been found of hemialdal formation (sec. 12.23).

The most widely distributed neutral polysaccharide in tissues is glycogen; its presence can be confirmed by showing that the periodic acid reaction on sections previously incubated with diasatase (Lillie and Greco, 1947) is negative.

(2) Muco- and glycoproteins (sialic acid).

The chemistry of this group of compounds has not been studied extensively (Stacey and Barker, 1962) and so it is not yet possible to identify particular substances in tissue sites. However, as a group, they often give intense PAS colourations which have been correlated by Leblond *et al.*, (1957) with their hexose content.

On the other hand, most reviewers of the histochemistry of glycoproteins (Pearse, 1960; Hale, 1952; McManus, 1961) have not emphasised the important role played by sialic acid residues in the PAS reaction, probably because much of the

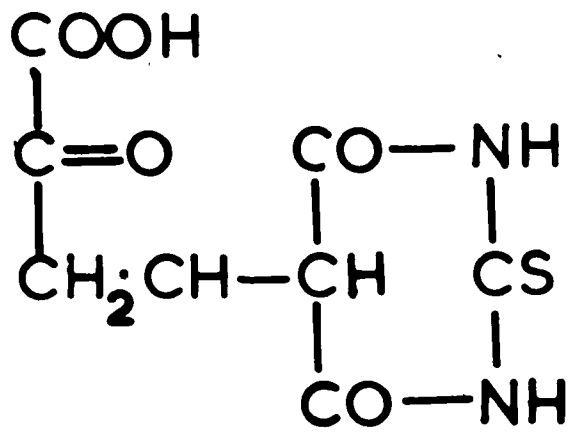
chemical literature is not explicit about this point. Montreuil and Biserte (1957) claim that sialic acid is responsible for most of the PAS coloration of glycoproteins, while the sugars and aminozugars contribute very little. Although ovosomucoid (or Winzler's α_1- glycoprotein) contains about 10% neuraminic acid (Odin and Werner, 1952), the PAS colouration is about 10 to 100 times the intensity obtained for glycogen (Jeanloz 1957).

No extensive studies seem to have been made of the oxidation of protein-bound sialic acids by periodic acid to elucidate the site of attack, although it seems certain that the sialic acid fraction is responsible for the PAS reaction of glycoproteins: *in vitro* tests on sialic acid itself gave a PAS sensitivity about ten times greater than that of glucose (Bjornezjo, 1957).

As ketosidically bound N-acetylneuraminic acid (Fig.4) consumes two moles of periodate (Klenk *et al.*, 1956), the three contiguous hydroxyl groups on the erythro-chain (C_7 - C_9) are probably responsible for the PAS reaction of sialoproteins. That the tris-hydroxyl group is involved is also indicated by the ready hydrolytic removal of the sialic acid residues from the protein *in situ* showing that they occupy a terminal position (Montreuil *et al.*, 1958).

It might be argued that periodic acid attacks sialic acid residues at the N-acetylaminohydroxyl linkage

(Cp. sec. 12.3/5). This possibility is ruled out by Warren's work (1959, 1960) which has been substantiated by my histochemical experiments (exp. sec. 12.17). Warren's test depends on the oxidation of sialic acid by strong acid solutions of periodic acid to an intermediate β - formylpyruvic acid (Weissbach and Hurwitz, 1959), which can then be condensed selectively with 2 - thiobarbituric acid to form a pinkish-red fluorescent chromogen. The probable structure of the chromogen (shown below) is presumably comparable to that of the compound formed between furfural and thiobarbituric acid (Fox and Plaisance, 1916).



Warren's test works with free, but not with bound sialic acid. As sialic acid is usually attached to its protein fragment by an osidic linkage at C_2 (Fig. 4), periodic acid must attack the N-acetylhydroxyl bonds of

free sialic acid in the severe conditions imposed in Warren's test. This can be shown to proceed in two stages: the N-acetyl group is first hydrolysed off (by phosphoric acid) and exposes a vic-aminohydroxyl group which is then slowly attacked by periodate ions (secs. 1.2 and 12.3/1).

The stringent conditions required for this test eliminate the possibility that N-acetylhydroxyl groups interfere with the specificity of the mild periodic reaction for vic-glycols.

It is just possible that the aldehyde produced by periodic acid from sialoprotein is of the furfuraldehyde type which has originated from the uronic acid group. Concentrated oxidising agents (sulphuric acid) can bring this about at high temperatures (sec. 10.3) but it is unlikely that dilute periodic acid can do this at room temperature.

(3) Lipids

Lipids, other than the acetal phosphatides, sphingomyelin, and unsaturated lipids, can also be PAS-positive (Hack, 1949), but this is usually due to their carbohydrate components which possess vic-glycols. The gangliosides, cerebrosides and inositol phosphatides come within this category.

Undoubtedly the intense reactions of cerebrosides, such as Kerasin (Morrison and Hack, 1949), and ganglio-

sides (Bo:och, 1958) are due to their high hexose and neuraminic acid content.

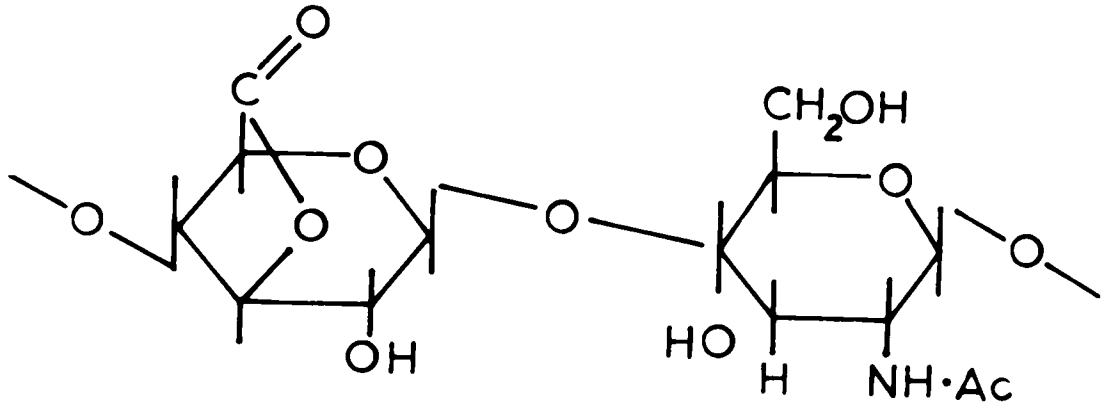
(4) Acid mucopolysaccharides.

The vic-glycols in acid mucopolysaccharides are believed to be left intact in situ after 10 minutes oxidation in periodic acid, despite earlier reports to the contrary (Pearse, 1953). This would appear to nullify the claim that the periodic acid reaction is specific for all vic-glycols. However, most acid mocopolysaccharides consume periodic acid in vitro, although some time may be required before oxidation is complete. Chondroitin sulphates, for example, require up to 60 hours before their oxidation is fully accomplished (Wolfrom et al., 1959). As a general working rule, Bobbitt (1956) has stated that up to 48 hours should be allowed for "selective" oxidations as distinct from the so-called "over-oxidations" whose rate of reaction may not level off during a period of days or weeks.

It will now be suggested that vic-glycols of acid mucopolysaccharides in situ can be demonstrated after 24 hours oxidation in periodic acid provided the engendered aldehydes are condensed with a hydrazine type reagent and not with a Schiff-type reagent. The latter would also show up oxidised proteins containing sulphur in the same sites.

(i) Hyaluronic acid

From its postulated structure (Meyer, 1952), hyaluronic acid should be attacked by periodic acid between C_2 and C_3 of its glucoronic acid moiety. However, it only consumes about 0.1 - 0.2 mole or less of periodic acid per repeating unit (Meyer and Fellig, 1950; Meyer et al., 1951; Blix, 1951; Jeanloz and Porchielli, 1951). One explanation for this failure is that the acid conditions of the PAS test would favour the formation of a 3:6-lactone ring in the glucoronic acid residues thus rendering them immune to attack (Stacey and Barker, 1962).



This hypothesis was tested without success on the hyaluronic acid "band" in cock's comb. After immersion in periodic acid for about 20 minutes, the sections were treated with salicyloyl hydrazide (sec.12.19) which might have condensed with the carbonyl group of the lactone. As expected, no fluorescent conjugate (of aldehyde) was formed, but neither was any fluorescence produced when the sections were subsequently treated with zinc acetate solu-

tions. Salicyloyl hydrazide derivates of ketones (and lactones?) fluoresce an intense blue colour when complexed with zinc salts (sec. 7.2/3). However, a fairly strong reaction was obtained when tissue sections of cock's comb were oxidised in periodic acid for 24 hours and condensed with salicyloyl hydrazide. Controls which had been oxidised first for 10 minutes in periodic acid and then treated with aniline solution before carrying out the prolonged oxidation showed the same result: the positive result cannot be due to neutral mucopolysaccharides and must be attributed to the vic-glycols of its hyaluronic acid content. An attempt was made to prevent lactone formation by methylating the uronic acid residues before periodate oxidation with methanolic thionyl chloride. A negative result was obtained subsequently for brief periodate oxidations, indicating that lactone formation is not necessarily responsible for the inefficient oxidation.

On the other hand, sites of high hyaluronic acid content in tissues are generally reported to be PAS positive (Stoughton and Wells, 1950). Its intensity is hardly diminished by a prior digestion in hyaluronidase, although its metachromasia towards toluidine blue is diminished. Braden (1955) suggested that native hyaluronic acid has an associated neutral mucopolysaccharide which is responsible for the positive PAS reaction. The positive

reaction could also be attributed to the oxidation of sulphur-containing proteins (sec.12.7/7) to basophilic entities which would give a positive colouration with Schiff's solution. This explanation is supported by Glegg et. al.'s (1952) observation that sulphur-free preparations of hyaluronic acid gave no reaction in vitro, unlike undegraded preparations containing sulphur which did (Davies, 1952; Braden, 1955).

A similar explanation can be offered for the differential staining of mixed mucins obtained by Spicer (1961) in tissue sections that had been oxidised in periodic acid, condensed in phenylhydrazine and stained in Schiff's solution. He believes that the apparent PAS negativity of acid mucopolysaccharides in situ is not due to an inherant lack of reactivity towards periodic acid, but is due to a preferential uptake of Schiff's reagent by the engendered aldehydes of neutral mucopolysaccharides. If the latter are condensed with phenylhydrazine between the periodic acid oxidation and the Schiff staining stages, the periodate-reactive acid epithelial mucins are conspicuously and selectively coloured against an unstained background. Spicer's interpretation may be correct, although there is no a priori reason for supposing that phenylhydrazine has a selectively affinity for the aldehydes of neutral but not acid mucopolysaccharides. On the

contrary, the ability of phenythydrazine to condense with both reactive and "unreactive" aldehydes (hemiacetals) tends to detract from Spicer's hypothesis, especially when it is remembered that it is Schiff's solution which has the selective affinity for aldehydes.

(ii) Heparin

Although heparin is rapidly oxidised by periodic acid *in vitro* at room temperature (Wolfrom *et al.*, 1950), its histochemical demonstration is uncertain as shown by a variable PAS reaction in mast cells (Lillie, 1950). In general, those mast cells which were strongly metachromatic towards toluidine blue were often PAS-negative; other cells which werePAS-positive were orthochromatic. These differences have been attributed to the degree of sulphation on the heparin molecule (Jorpes and Gardell 1948; Jorpes *et al.*, 1948). However, part of the difference may be due to the presence of some occluding substance which protects heparin from attack in mast cells; the low uptake of alcian blue (sec. 10.2) by mast cells (Spicer, 1960) is a further example of the anomalous behaviour of heparin *in situ*.

The presence in tissues of heparins of different degrees of sulphation has been confirmed by Meyer *et al.*, (1956). The monosulphate, now known as heparitin sulphate (Meyer, 1956), is probably responsible for most of the PAS reactivity in heparin-rich sites, and may also be the PAS-

positive acid mucopolysaccharide in gastric mucins (Smith et al., 1952). If the latter assumption is correct, this would explain the lack of extra reactivity of the acid mucopolysaccharides in the guinea-pig gut following prolonged oxidation in periodic acid; when gut sections are oxidised in periodic acid for 10 minutes and then treated with acidified aniline to block the dialdehydes initially formed, no PA-SH (sec.21.19) fluorescence was found after a further 24 hours oxidation in periodic acid. Presumably all the vic-glycols are destroyed in the initial oxidation (cp. Wolfrom et al., 1950).

Further indications that the acid mucopolysaccharide in guinea-pig gut is heparin or heparitin sulphate is the increase in the extent of the PA-SH fluorescence following "drastic" methylation which removes, rather than methylates, sulphate groups; these two acid mucopolysaccharides are the only common ones in which the number of vic-glycols would be increased after O-desulphation (sec.9.15).

(iii) Chondroitin Sulphates

According to Glegg et al., (1953b) and Braden (1955), chondroitin sulphate A gives only a weak PAS reaction, if it reacts at all. Jorpes et al.,(1948b) and Dziewiatowski (1951) reported negative in vitro spot-tests. As with hyaluronic acid, Braden (1955) suggests that the strong PAS reactivity in areas of known chondroitin sulphate content is due to an associated neutral mucopolysaccharide.

The most probable explanation for the negative PAS-reactivity of chondroitin sulphate is due to inadequate oxidation times. Even *in vitro*, chondroitin sulphate requires up to 60 hours treatment before oxidation is complete (Wolfrom *et al.*, 1952; Smith and Gallop, 1953). If tissues are oxidised for 24 hours, the *vic*-glycols of chondroitin sulphate can be cleaved to give a moderately intense fluorescent conjugate with salicyloyl hydrazide. Fig. 54 shows the appearance of the cartilage band in the tibia of the rabbit. The interference from neutral polysaccharides was avoided by a preliminary oxidation in periodic acid for 10 minutes which was followed by a blocking of the engendered aldehydes with aniline before carrying out the prolonged oxidation. Another control section which had been treated with aniline following brief oxidation in periodic acid was kept for 24 hours in hydrochloric acid of the same pH as the periodic acid. There was no uptake of salicyloyl hydrazide by the control, thus showing that the positive results of the 'test' sections were not due to an acid-catalysed reversal of the aniline blocking. Enzyme digestions were used to show that the acid mucopolysaccharide in this site was either chondroitin sulphate A or C (Bradbury and Stoward, 1963).

(5) RNA

The sugar moiety of RNA sometimes gives a positive periodic reaction *in vitro* (Allen, 1951) thus showing that

Fig. 54. Rabbit tibia. Fixed in formalin-cetyl pyridinium chloride.

Periodic acid, 24 hrs. : salicyloyl hydrazide. DPX mount, X 180.

The blue fluorescent sites contain acid mucopolysaccharides whose glycol groups have been cleaved by prolonged oxidation in periodic acid.

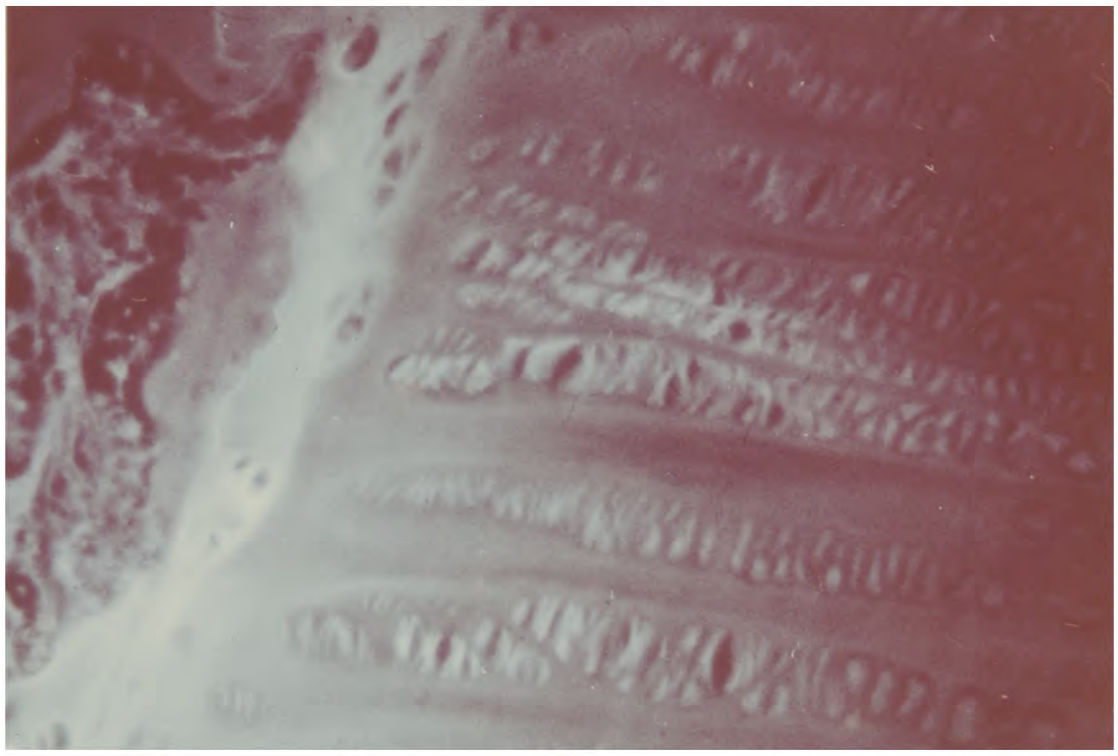


Fig. 54

the hydroxyl groups at C_2 and C_3 are no longer esterified. However reports that the PAS colouration of cytoplasmic and nuclear material in the liver, spleen and lymph nodes is due to such desterified RNA must be treated with some caution as no specific evidence has been put forward for this hypothesis (Ihnuma and Saka, 1952). On the contrary, engendered dialdehydes have never been detected in sites of RNA of sections which have been oxidised in periodic acid and treated with salicyloyl hydrazide (sec.12.19); no fluorescence was produced in such sites. One explanation for the coloration by Schiff's reagent is that initially colourless basic fuchsin ions are adsorbed onto the negatively charged RNA by a salt-like linkage (sec. 12.6/1) which are later converted to the coloured quininoid form either by aerial oxidation or during the washing stages (sec. 12.6/2).

5. The mechanism of glycol cleavage by periodic acid.

Vicinal glycols in the cis position are oxidised much faster than those in the trans configuration (Criegge, 1934). The relative rate of reaction has been correlated with the strain associated with the formation of a cyclic complex in which the C-O bonds of the glycols are rotated to become nearly coplanar (Honeyman and Shaw, 1959). The conformation of the hydroxyl groups is also important: cyclisation to form the initial complex takes place much more easily with equatorial-axial substituents than with two equatorial groups. Forcing two axial bonds more

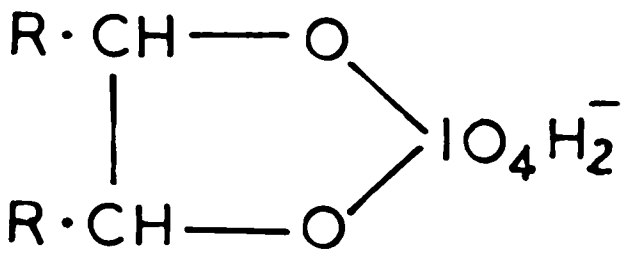
nearly into the same plane requires an even greater amount of energy. In some compounds of this type, such as the altroside derivatives, the energy barrier is so high that the intermediate complex cannot be formed and consequently the glycol groups remain intact.

The exact course of the reaction has not yet been elucidated, but it is well-established that it proceeds by way of the singly-charged anionic complex of the diol with periodate as originally proposed by Criegge et al., (1933; 1934). The complex (I) is readily dehydrated to the species (II) which can decompose unimolecularly into the final reaction products (R.CHO and IO_3^-) by a process analogous to an internal rearrangement (Buist and Bunton, 1954; Nevell, 1959).

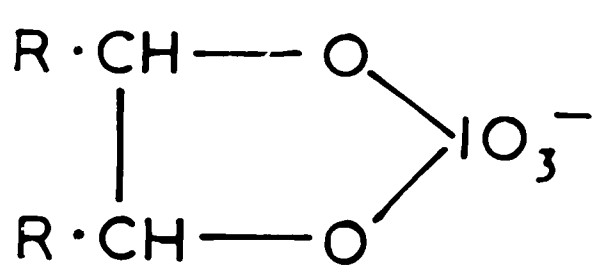
Apart from the kinetic evidence, further proof for the existence of the cyclic intermediates was provided by Heidt, Gladding and Purves' (1945) calculations; when the calculated interatomic distances of II were combined with certain steric and oxidising potential considerations, it was successfully predicted that sodium bismuthate and trivalent silver ions would also specifically oxidise 1,2-glycols. These reactions have since been studied (Rigby, 1950; Greenspan and Woodburn, 1954).

No oxidation takes place with cis-cis 1:2:3 tr'ols. Instead a triester (III) or (IV) is formed by non-oxidative

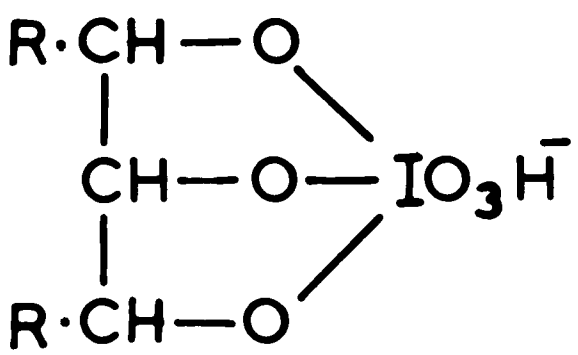
consumption of iodate which is incapable of decomposing directly by electronic rearrangement (Barker and Shaw, 1957; 1959). Ester formation can be avoided by using a high enough pH. Therefore, failure of a compound to undergo periodate oxidation cannot be regarded as positive proof that it contains no 1,2-glycol structures. If the glycol is securely locked in a <u>trans</u> position, oxidation has been shown not to occur (Dimler, 1952). In addition, even <u>cis</u>-glycols may be attacked very slowly if neighbouring groups shield the glycols from the approach of the iodate ion. This last effect is the probable explanation for the perceptibly slow cleavage of glycols of acid mucopolysaccharides <u>in situ</u>, (sec.12.4/4).



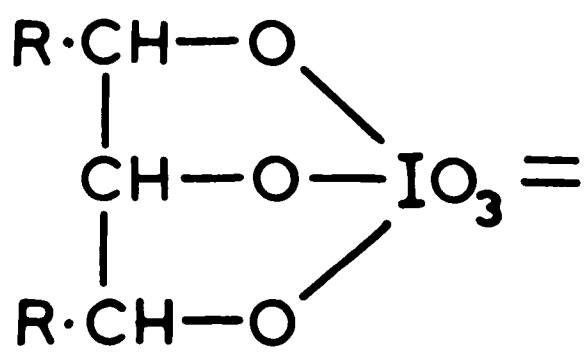
I



II



III



IV

Fluorochemical techniques for the demonstration of engendered dialdehydes.

6. Schiff's reagent.

(1) Introduction

Colourless Schiff's solution is the standard reagent for visualising engendered aldehydes in conventional histochemistry and has been used throughout this work as a check on the efficacy of other reagents: It forms a red coloured product with aldehydes.

Schiff's solution consists essentially of an acidified solution of basic fuchsin saturated with sulphur dioxide. The sulphurous acid formed by the reaction of sulphur dioxide and the aqueous solvent converts the coloured dye to its colourless leuco form. The excess coloured fuchsin is usually removed by shaking the solution with activated charcoal.

A purple fluorescence in tissue sections processed through the PAS technique was first reported without comment by Kasten (1958a). The purple fluorescence is characteristic of PAS positive sites, but it does not always contrast sharply with the remainder of the section which fluoresces blue. The blue fluorescence is presumably due to the adsorption of the colourless fuchsin complex onto basophilic sites (protein carboxyl groups, etc.).

In view of the widespread use of Schiff's reagent in histochemistry, it is useful to consider the various

factors which influence the intensity and extent of the final colouration. This is particularly important here because it is suggested that Schiff's solution may give different results from those obtained with the fluorescent reagents considered later; Schiff's reagent may give positive colourations with groups other than engendered aldehydes, but may not react with all the "dialdehydes" produced from some mucins.

(2) The effect of a reducing rinse.

The intensity and extent of visible PAS staining depends on the washing of tissue sections after both the periodate oxidation and the Schiff staining stages. The use of a reducing rinse (acidified thio_sulphate) was first suggested by Hotchkiss (1948) for removing excess periodate or iodate remaining in the tissues after treatment with periodic acid, because they can restore the colour to Schiff's solution. McManus (1948b) objected to its use on the grounds that it decreases the number of aldehydes available for colouring by the Schiff reagent.

In this investigation three types of rinse have been used:

(a) A brief rinse in water to remove the bulk of excess periodic acid was followed by a wash for one minute in dilute sulphurous acid, which reduced the periodate remaining on the section. The preliminary aqueous wash is essential as otherwise excessive amounts of iodine

which are initially formed in the sulphurous acid rinse would destroy many of the engendered aldehydes (Bonner and Drisko, 1951).

The sulphurous acid rinse seems very satisfactory on theoretical grounds if the ideas advanced in sec. 12.6/4 about the mechanism of aldehyde - Schiff staining are correct.

(b) When reagents other than Schiff's solution were being tried for visualising aldehydes, test sections were rinsed first in a dilute solution of ethylene glycol followed by a wash in water.

(c) Two rinses in water for about 30 seconds each were found quite satisfactory for routine work with reagents (e.g. salicyloyl hydrazide, sec.12.19) other than pseudo - Schiff solutions (sec. 12.7).

Both Hotchkiss (1948) and McManus (1948) employed sulphite washes after the PAS reaction as also did Lillie (1947) in his version of the reaction. This should remove the excess adsorbed leuco-complex from non-aldehyde sites which would become coloured subsequently by auto-oxidative processes. Most sections, in fact, show a pale-red colouration in non-mucin areas. Examination of PAS - stained sections by fluorescence microscopy confirms the fact that excess Schiff solution is adsorbed and there is no guarantee that it will not become visibly coloured at

some later stage: this detracts from the absolute reliability of Schiff's reagent for aldehydes.

McManus (1948a) noticed that by washing sections in running water for 5-10 minutes after the last sulphite bath, the final colour was considerably enhanced. No explanation for this has been put forward; water probably accelerates the breakdown of intermediate aldehyde-Schiff complexes to their coloured form (sec. 12.6/4). However, there is a danger that the more soluble mucopolysaccharide complexes might be extracted by running water: consistent results have been obtained in this investigation with two or three rinses in distilled water, each of 1-2 minutes duration.

(3) Lack of specificity.

Using the technique detailed in exp. sec. 12.1, weak but significant staining was often seen in sites believed not to contain neutral mucopolysaccharides. The visible pink PAS staining of the muscle in guinea pig gut is shown in Fig. 55. It is possible that the positive reaction in this example is due to glycogen or adylenic acid (Pearse, 1960), but the periodic acid - salicyloyl hydrazide (sec. 12.19) fluorescence in this area is absent.

In some tissues, particularly in gastric mucin areas, the PAS positive entities show very little discrete structure. The gastric mucin sites of rabbit stomach, whose fluorescent appearance is shown in Fig.57, appear to be

Fig. 55. Guinea pig gut. Fixed in glycol ether.

Periodic acid - Schiff's reagent. Transmitted white light, DPX mount, X 66.

The goblet cells and some gastric secretion sites are stained The muscle is weakly stained.

Fig. 56. Guinea pig gut. Fixed in glycol ether.

Periodic acid - Schiff's reagent. Transmitted white light, DPX mount, X 165.

Fewer gastric secretion sites are stained than in the corresponding salicyloyl hydrazide reaction (Fig.)

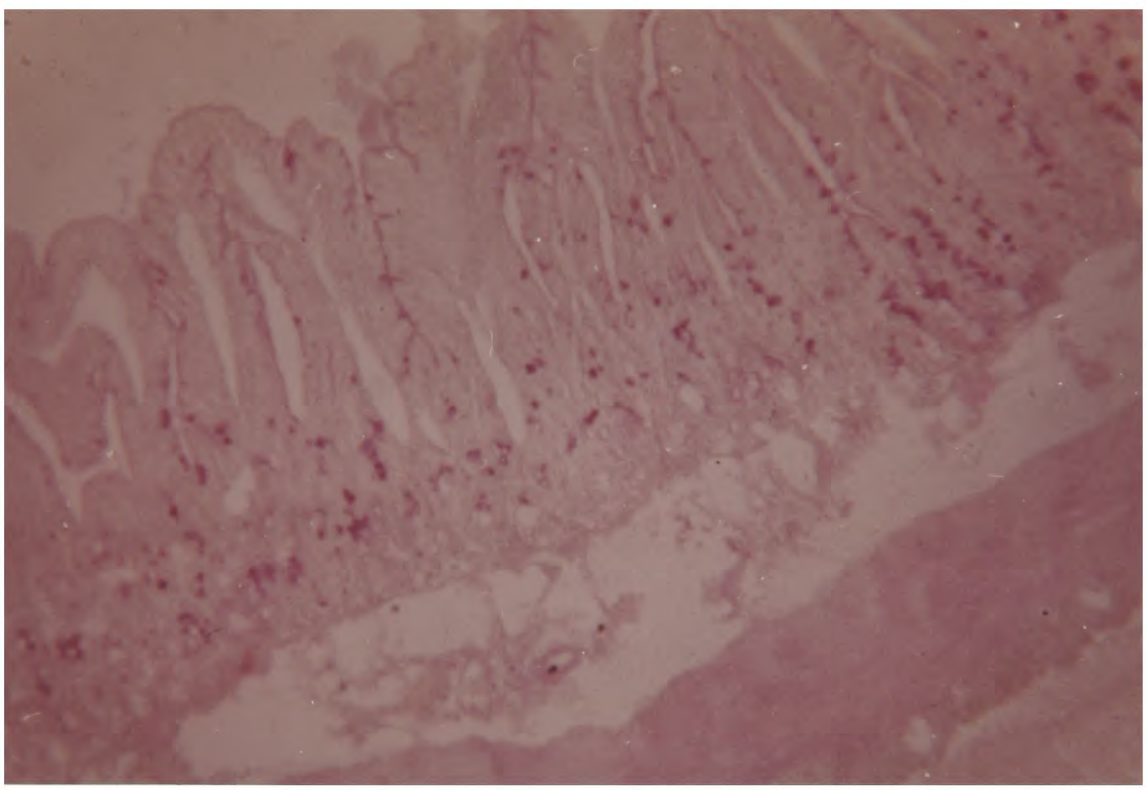


Fig. 55

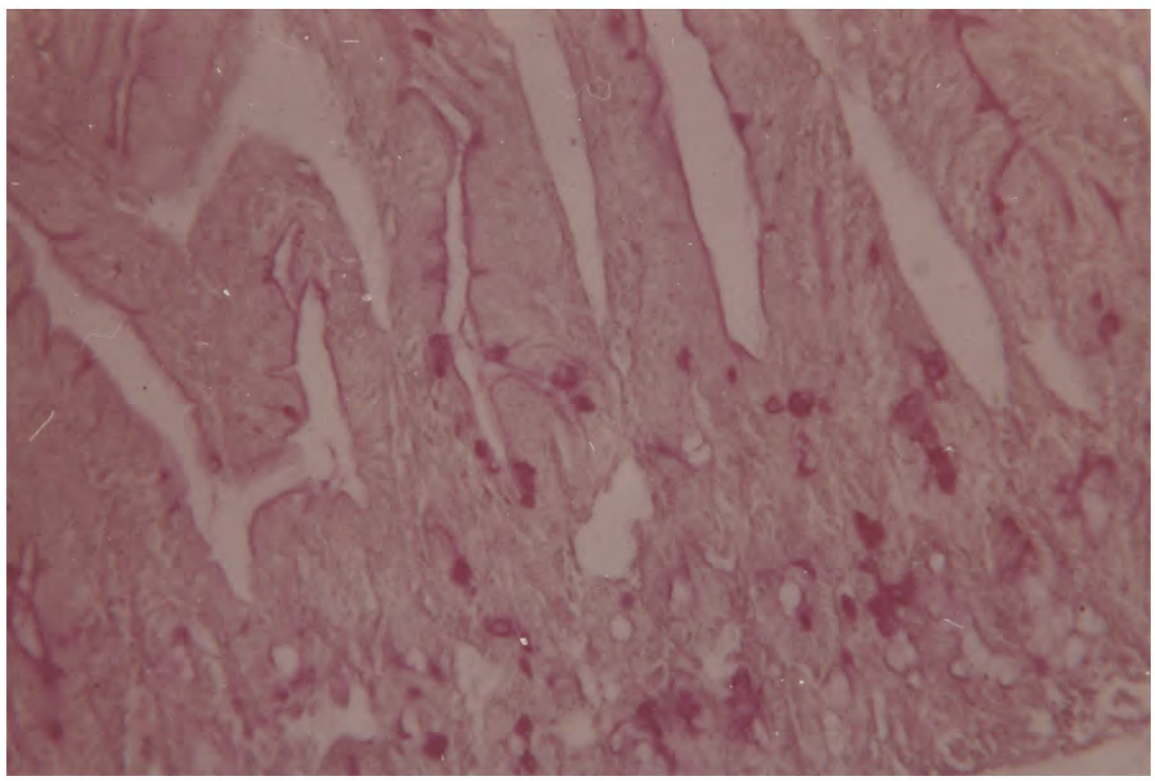


Fig. 56

Fig. 57. Rabbit stomach. Fixed in alcohol.

Periodic acid : Schiff's reagent. DPX mount, X 180.

Sites containing oxidised neutral mucopolysaccharides exhibit a purple fluorescence whose distribution is less discrete than that indicated by the salicyloyl hydrazide reaction (e.g. Fig. 58). Basophilic sites emit a blue fluorescence.

Fig. 58. Guinea pig gut. Fixed in glycol ether.

Periodic acid : salicyloyl hydrazide : Solochrome Black + alum complex. DPX mount, X 180.

Sites containing dialdehydes engendered from neutral mucopolysaccharides emit a deep blue fluorescence. Acidophilic proteins fluoresce a brick-red colour.

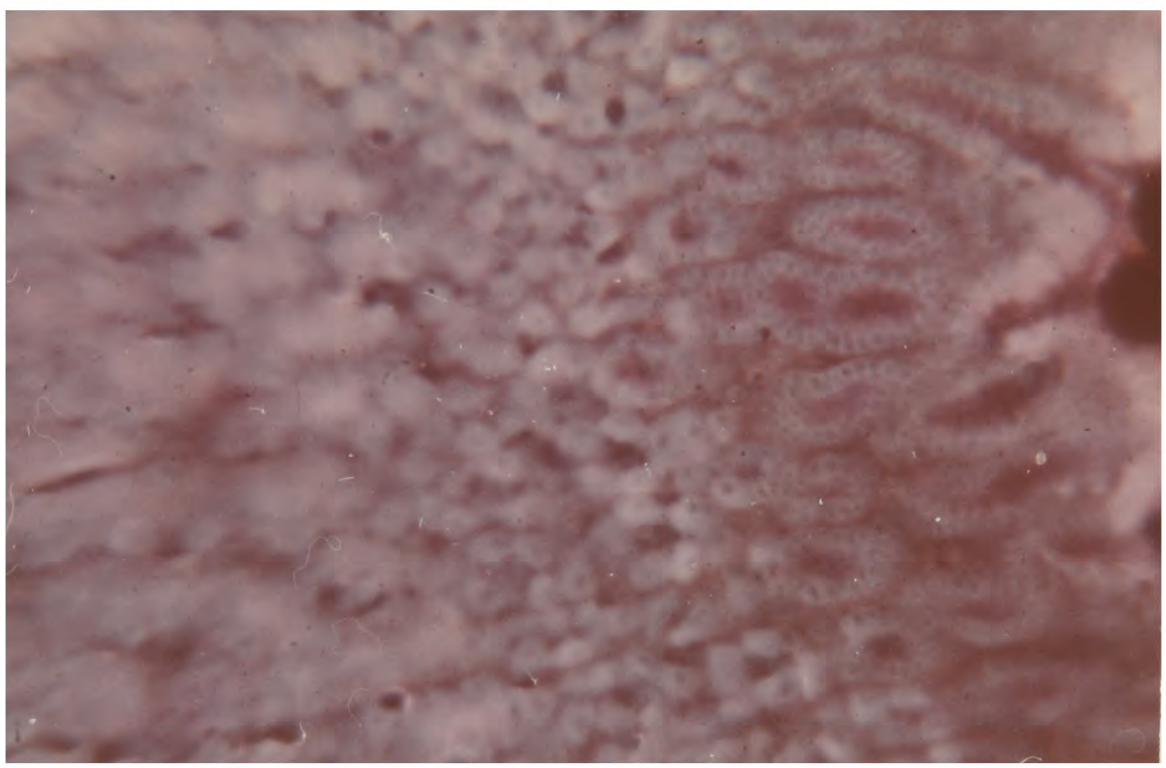


Fig. 57

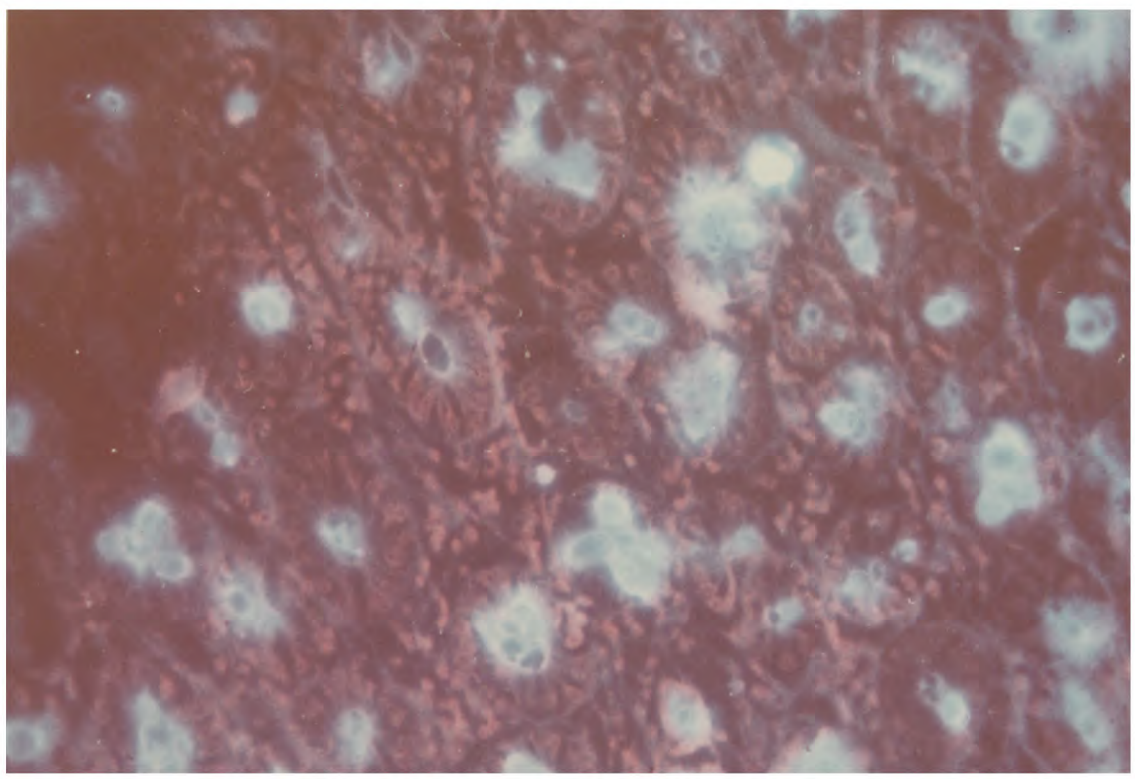


Fig. 58

completely filled with PAS positive materials. However, periodate-oxidised sections which have been treated with hydrazine-type reagents usually exhibit a more discrete distrubution of neutral mucopolysaccharides in the gastric secretion areas (e.g. Fig. 58).

A detailed comparison of Figs. 51, 57 and 58 confirms the hypothesis that hydrazide-type reagents condense with more periodic acid reactive substances than do Schiff-type reagents; removal of some of the engendered "dialdehydes" due to hemiacetal formation is probably responsible for the incomplete uptake of Schiff reagents.

The disadvantage of Schiff's solution for the histochemical visualisation of engendered aldehydes for both visible and fluorescence microscopy can be summarised as follows:

(1) Excess Schiff solution can be adsorbed (followed by auto-oxidation) onto basophilic sites that are unreactive towards periodic acid.

(2) Excess Schiff solution can be co-adsorbed onto PAS positive sites, so that a critical determination of the distribution of periodic acid-reactive substances is difficult.

(3) Schiff reagent does not react with all engendered aldehydes. This may be due to the relative insensitivity of Schiff's reagent or to the internal condensation of the

dialdehydes into a non-reactive form.

(4) Although PAS-stained materials have a specific violet fluorescence, its intensity and contrast are relatively poor.

(4) The Mechanism of the Schiff reaction.

One promising approach to the development of new fluorochemical techniques for demonstrating aldehydes in situ hinges on the understanding of the Schiff reaction itself. Three different mechanisms have been proposed:

(1) The formation of a Schiff base (Shoesmith, Sosson and Hetherington, 1927).

This theory does not involve the intervention of sulphur dioxide in the formation of the coloured complex. The amino groups on the fuchsin molecule ($R.NH_2$) condense directly with aldehyde groups ($R^1.CHO$).

$$R\cdot NH_2 + R'\cdot CHO \longrightarrow R\cdot N{=}CH\cdot R' + H_2O$$

This reaction is a general one for aldehydes and amines, but there is very little specific evidence that this reaction takes place with fuchsin. DeLamater (1948) and Arzac (1950) claim that the aldehydes exposed by the acid hydrolysis of DNA can react with the amino groups of basic fuchsin to give red stained nuclei. However the

Schiff base which is formed does not withstand alcoholic dehydration in sharp contrast to the extreme stability of the aldehyde - fuchsin/sulphurous acid complex.

(ii) The formation of an aminosulphinic acid (Wieland and Scheuing, 1921).

$$R\cdot NH_2 + SO_2 \longrightarrow R\cdot NH - SO_2H$$

$$R\cdot NH-SO_2H + CHO\cdot R' \longrightarrow R\cdot NH\cdot SO_2\cdot CH\begin{matrix}R'\\OH\end{matrix}$$

This mechanism was favoured by Kasten (1960) in his extensive review in which he formulated a comprehensive scheme that took into account the numerous coloured products found in practice.

(iii) The formation of a sulphonic acid (Rumpf, 1935)

$$R\cdot NH_2 + SO_2 + CHO\cdot R' \xrightarrow{H_2O} R\cdot NH\cdot CHR'\cdot SO_3H$$

Rumpf found no evidence for the existence of aminosulphinic acids and concluded that they played no part in the Schiff reaction. However he was able to isolate α-aminoalkyl sulphonic acids. As his work was based on simpler aromatic amines than fuchsin, an extrapolation of

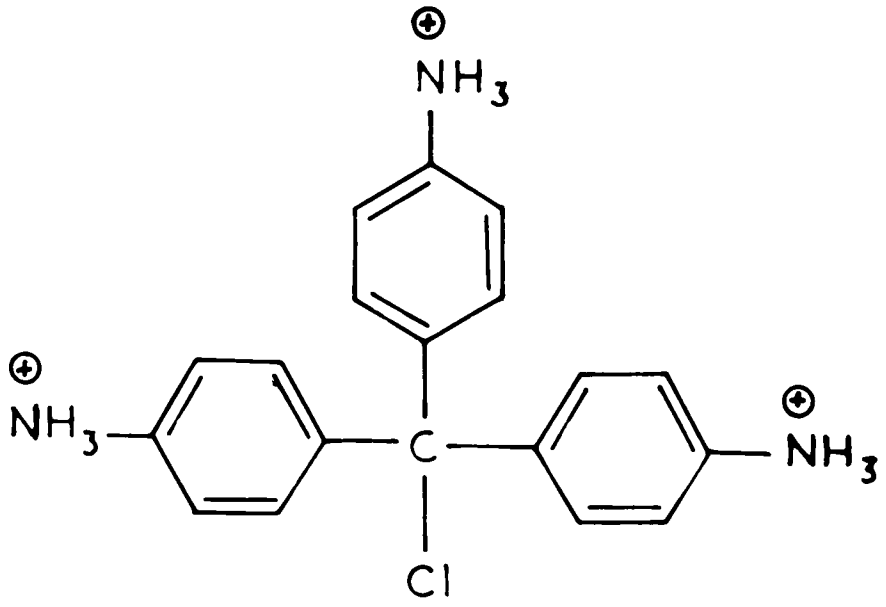
his mechanism to the Schiff reagent may not be justifiable.

Later Hörmann, Grasman and Fries (1958) studied the kinetics of the Schiff reaction in detail with formaldehyde and acetaldehyde, together with the periodate-oxidation products of serine, methylglucoside and starch. Their study supported the idea that a sulphonic acid group was attached to the central carbon atom and that two amine groups of fuchsin combine with aldehyde and sulphurous acid in two steps by a Mannich-type condensation. The formation of this condensation product is followed by the release of an $-SO_3H$ group from the central carbon atom of the colourless molecule which reverts to the coloured quinonoid-type structure. In the case of formaldehyde, the amount of coloured dye complex is proportional to the square of the aldehyde concentration. But with the polyaldehydes of periodate-oxidised starch, hyaluronic acid (Jorpes, Wermer and Aberg, 1948) and glycogen (Hooghwinkel and Smits, 1957), there is a linear relation. A similar relationship was found for DNA-aldehydes in the Feulgen reaction <u>in vitro</u> (Widström, 1928; Caspersson, 1932; Lessler, 1951) and in stained nuclei (Swift, 1955). The situation is complicated by Sloane-Stanley and Bowler's report (1962) that long-chain aldehydes react with fuchsin in the Schiff complex in the molecular proportion of three to one.

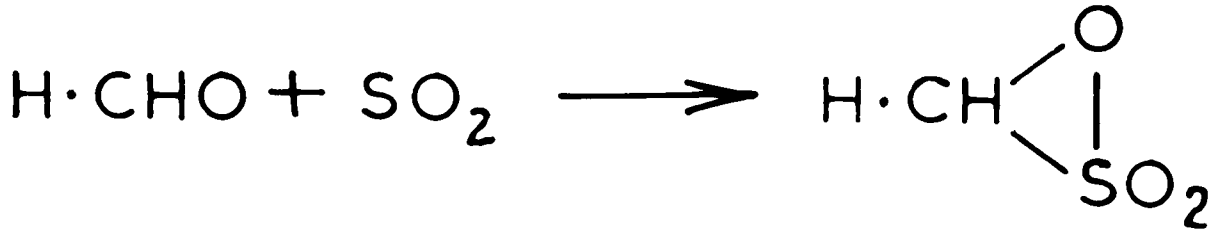
Later work by Nauman, West, Tron and Gaeke (1960)

has clarified the details of Rumpf's mechanism but differs considerably from Kasten's (1958a, 1960) interpretation of Hörmann et al.'s scheme. Changes in the ultraviolet and visible absorption spectra indicate that when hydrochloric acid is added to coloured pararosaniline, the decolorisation occurs because of the formation of a trianilium chloromethane (I) which has an absorption spectrum similar to that of either toluene or triphenylmethane, both of which are colourless. Formaldehyde has no effect on this reaction but combines with sulphur dioxide as proposed by Rumpf (1935). The compound II or III thus formed then combines with the amine salt to give a sulphonic acid IV which is the purple compound. The new coloured product is consistent with the spectra obtained in sulphonic acid compounds synthesised by direct procedures. The colour appears in the Schiff reaction because the existing acid concentration is not great enough to give the colourless anilinium-like salt of the sulphonic acid. Naumann et.al. (1960) found that both aniline and pararosaniline sulphonic acids require appreciably higher (10 M) hydrogen ion concentrations for the formation of the colourless anilinium-like salts than do the unsubstituted amines.

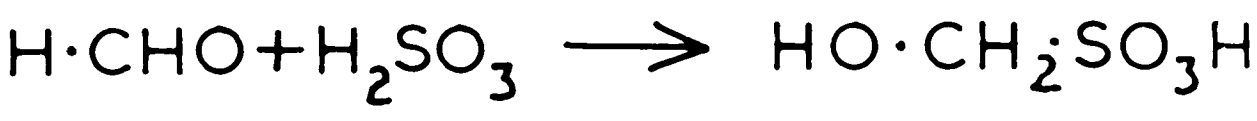
Another difference between this scheme and the others is the role played by sulphur dioxide at the central carbon atom. Naumann et.al. (1960) found that changes in



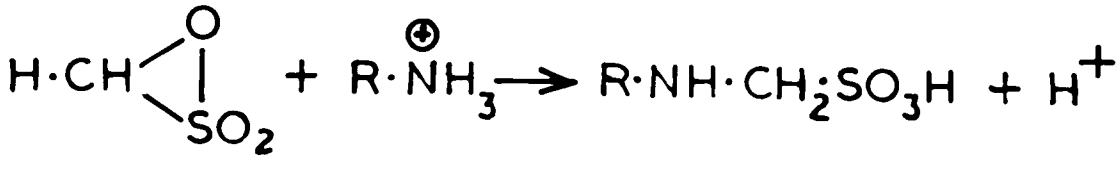
I



II

or 

III



IV

absorption spectra were consistent only with the addition of sulphur dioxide at the central carbon atom of rosaniline only at a pH above 6. This compound is sensitive to oxidation and reverts to pararosaniline.

A new scheme (Fig. 59) will now be put forward in which the concepts used by chemists for the interpretation of reactions in terms of electronic mechanisms will be invoked.

As the electronegativity of oxygen is much greater than that of sulphur, a dipole will be formed in the S=O bond of sulphur dioxide. Similarly a dipole is formed in the carboxyl bond of aldehydes. Consequently when sulphur dioxide reacts with aldehydes in solution, there is an interaction between the electron-dense oxygen atoms of the sulphur dioxide molecule and the electrophilic carbon centre of the aldehyde (R.CHO). The transient intermediate is then "neutralised" with the equivalent of one molecule of water to form the sulphonic acid, V. The sulphonic acid can react with the anilinium leuco-form of fuchsin in two ways.

The sulphonic acid itself may dissociate and then form a simple salt (VI)with fuchsin at its electrophilic nitrogen centre. Fluorescent dyes probably react by this route; this point will be discussed later.

It is feasible that V undergoes condensation with

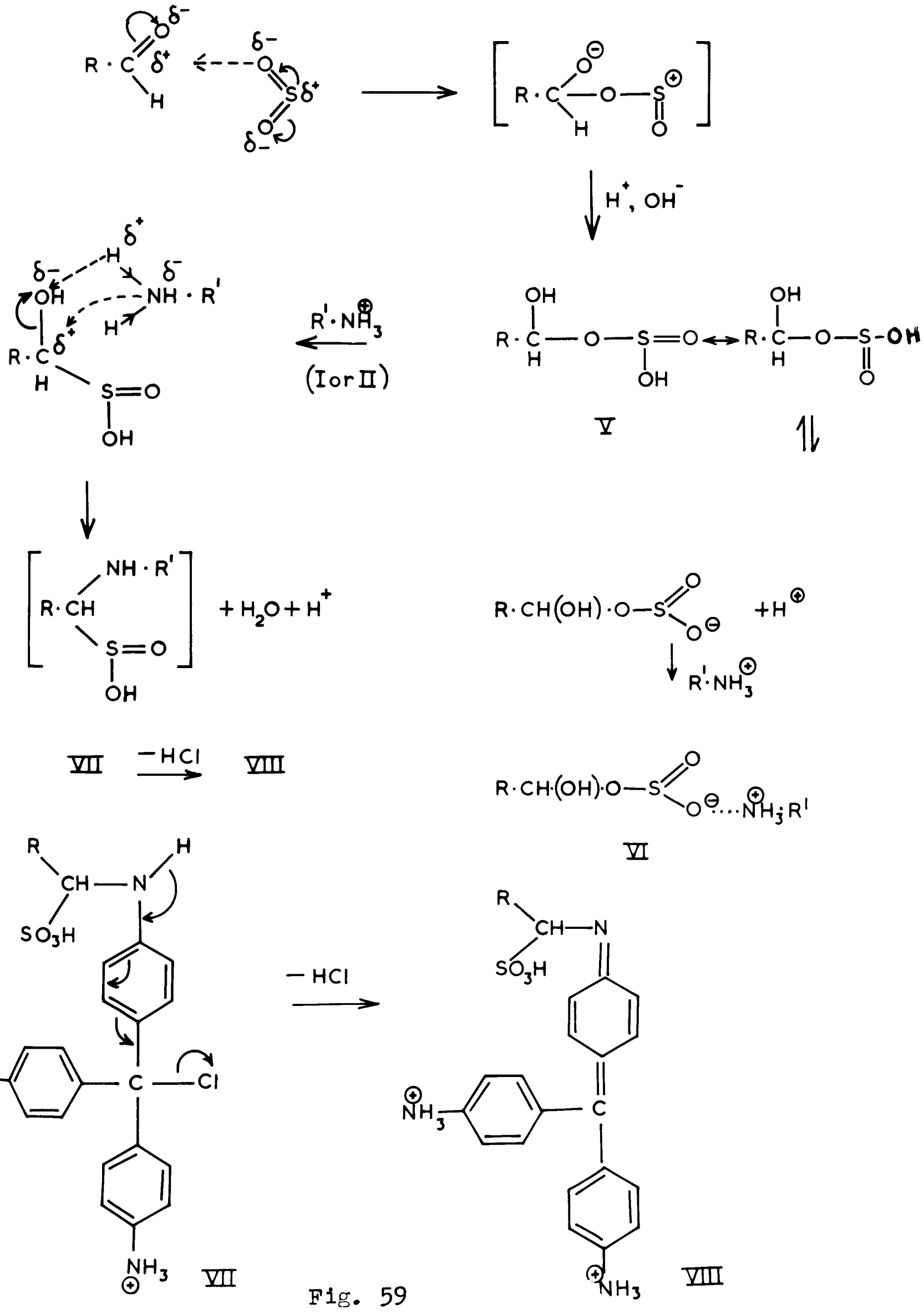

R·CH(OH)·O—S(=O)(=O)O⊖ +H⊕
R'·NH3⊕
R·CH(OH)·O—S(=O)(=O)O⊖···⊕NH3R'
VI
VII
−HCl
VIII
−HCl
NH3⊕
VII
VIII

Fig. 59

fuchsin to form the first coloured complex (VIII) through the unstable intermediate (VII). It is obviously possible for two to three aldehydes to be accomodated on one fuchsin molecule in the final complex: this is exactly what has been found in practice. The second and third aldehydes are probably attached to their respective nitrogen atoms in the form shown in VII.

The mechanism just proposed differs considerably from Kasten's (1954a) modification of Hörmann et al.'s (1958) scheme. However, my scheme was partly foreshadowed by Damionovitch (1910) who thought that an aldehyde bisulphite addition product was formed first which then reacted with an aromatic amine. Experimental evidence in support of this hypothesis has been provided recently by Ingles (1959) who found that the bisulphite addition compounds of reducing sugars reacted with aromatic amines to form stable addition products. Conversely Wieland and Scheuing (1921) claimed that the addition products did not give a colour with Schiff's reagent.

7. Pseudo-Schiff reagents

(1) Introduction.

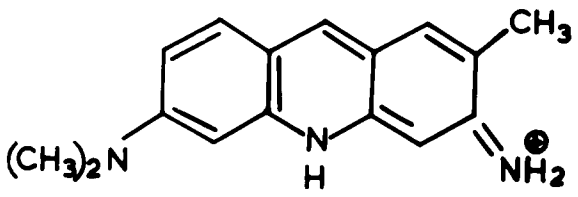
Dyes other than basic fuchsin were first introduced into histochemistry by Ostergren (1948) as potential Schiff-type reagents for the cytochemical detection of aldehydes. Later van Duijn (1956) and Ornstein et. al. (1957) used

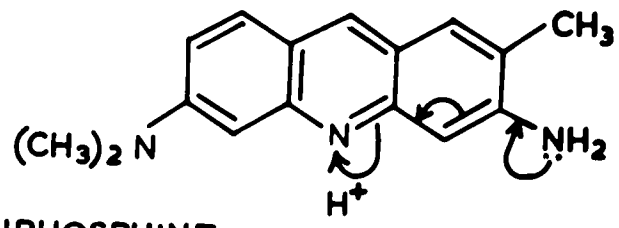
thionin and acriflavine respectively, but their investigations were confined to visible light histochemistry.

Kasten (1958a,b; 1959) devised techniques specifically for fluorescence histochemistry by using solutions of fluorescent dyes saturated with sulphur dioxide. As most basic fluorescent dyes possessed primary amine groups (Fig. 60), Kasten suggested that they might react with engendered aldehydes by the same mechanism proposed for the aminotriphenylmethane dyes constituting the active ingredient of Schiff's reagent. His hypothesis was supported by experiments with tissue sections previously oxidised with periodic acid or hydralysed in hot hydrochloric acid and subsequently stained in his dye solutions. Comparison with stained but otherwise untreated control sections showed specific staining of neutral mucopolysaccharides and nuclei (DNA) respectively.

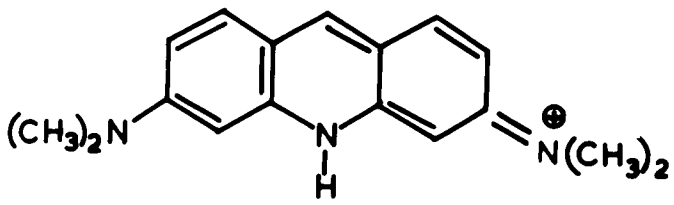
Kasten (1958a) preferred to call his reagents Schiff-type solutions and to identify the particular reagent by naming the dye followed by the word Schiff (or SO_2). Reasons will be put forward later for believing that Kasten's solutions do <u>not</u> react with aldehydes in the same way as Schiff's reagent, and therefore to continue calling his solutions Schiff-type reagents would be erroneous. In this thesis, they will be called pseudo-Schiff reagents, but a particular reagent will still be identified by the name of

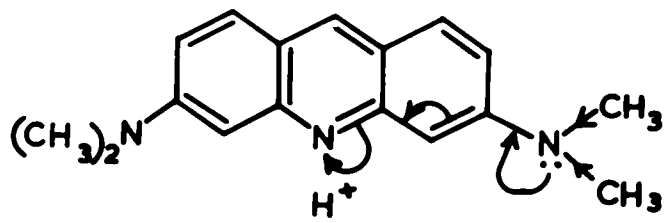
BASIC FLUORESCENT DYES



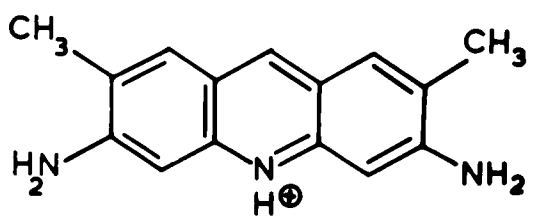


CORIPHOSPHINE

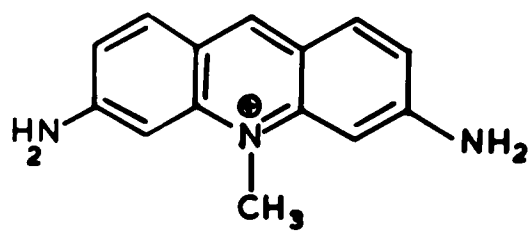




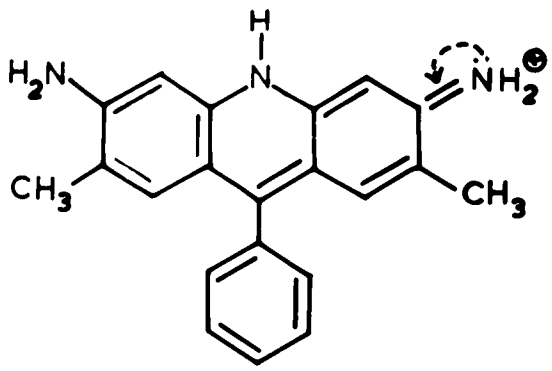
ACRIDINE ORANGE



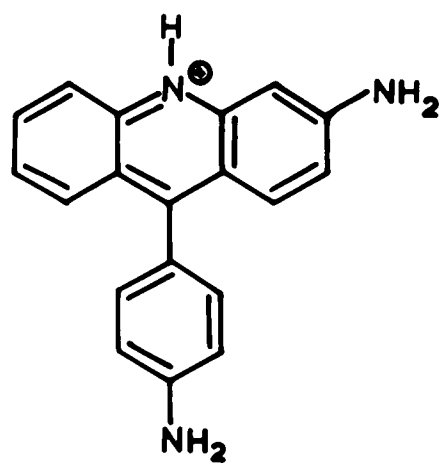
ACRIDINE YELLOW



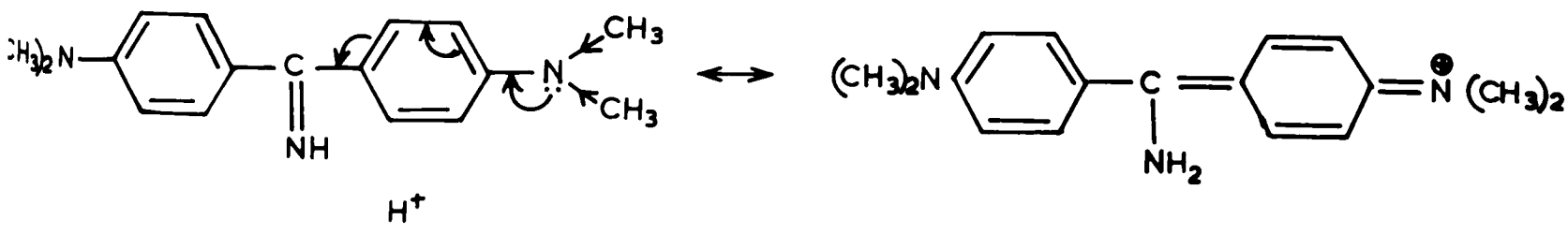
ACRIFLAVINE



BENZOFLAVINE



PHOSPHINE



AURAMINE O

Fig. 60

the dye followed by the symbol SO_2.

In my hands, several practical difficulties and theoretical objections have been encountered with Kasten's techniques.

(2) Overstaining.

When the relatively strong 0.1% and 0.5% solutions of aminoacridine dyes recommended by Kasten were used for staining sections treated with periodic acid or hot hydrochloric acid, it was often difficult in practice to discern the additional fluorescence of reactive sites, in comparison with stained control sections (e.g. Figs. 61, 65). This was particularly noticeable when potentially polyfluorochromatic dyes such as coriphosphine were used. The overstaining produced by these strong solutions obliterated any additional staining when sections were rinsed in water and examined for fluorescence as water mounts.

In a later review, Kasten (1960) included one important practical detail not stressed in his earlier papers; after staining sections in Schiff-type reagents, he dehydrated them though the alcohols before mounting them in a synthetic medium from xylol. However, it is questionable whether the extent and actual colour of the final fluorescent appearance is strictly invariable or reliable: sections stained in 0.1% coriphosphine - SO_2 followed by progressive dehydration to 95% alcohol showed discrete staining but the

Fig. 61. Guinea pig gut. Fixed in glycol ether.

Periodic acid - 0.1% coriphosphine-80 .
2

Water mount, X 180.

The additional red fluorescence attributable to oxidised neutral mucopolysaccharides is difficult to distinguish from the fluorescence of the nuclei (and acid mucopolysaccharides). The additional fluorescence appears to be more extensive than the specific red fluorescence in Fig. 62. The reddish-brown fluorescence which should be present in the sites of acid mucins was destroyed while this photomicrograph was being taken.

Fig. 62. Guinea pig gut. Fixed in glycol ether.

Methanolic thionyl chloride, 4 hrs. : periodic acid : 0.1% coriphosphine-80 . Water mount X 180.
2

Only a few of the sites containing enhanced dialdehydes emit a specific red fluorescence. The intensity of the blue fluorescence of the remainder of the tissue is much stronger than that indicated in this photomicrograph.

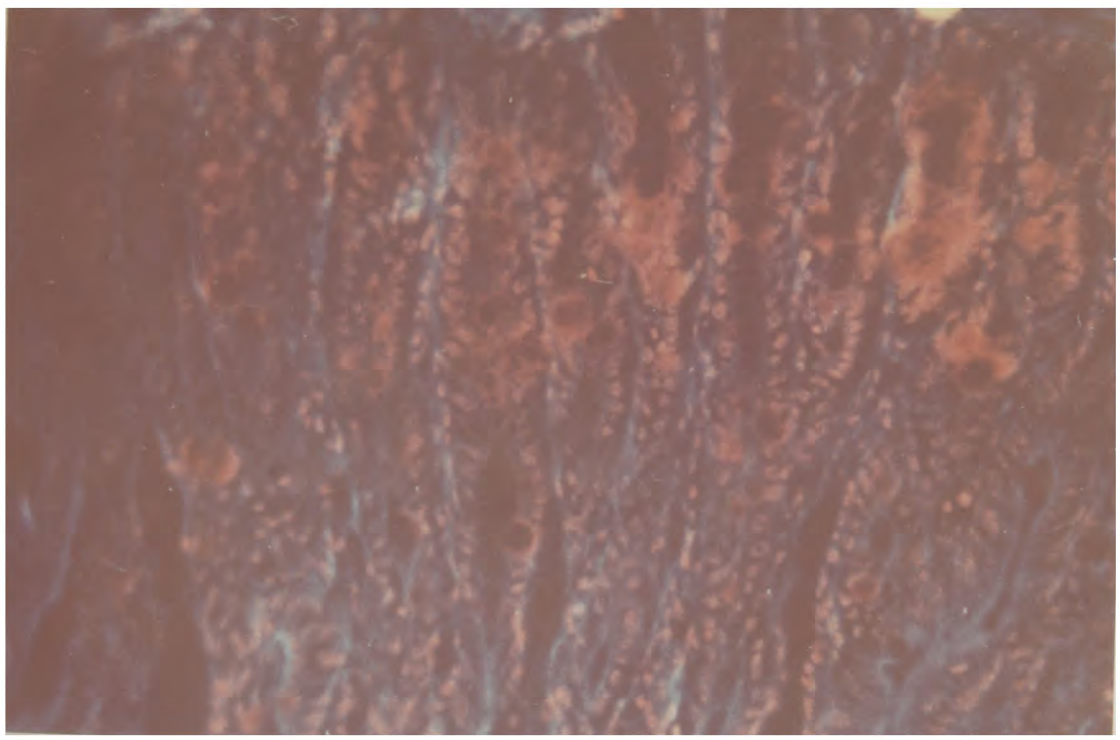


Fig. 61

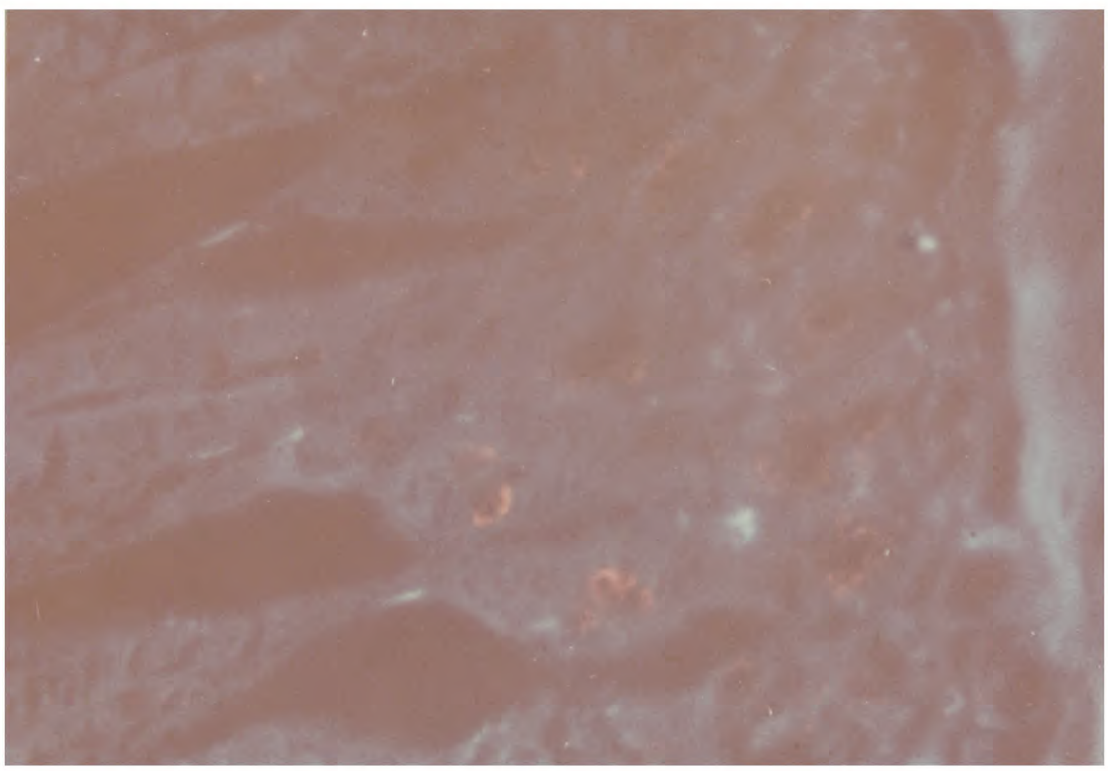


Fig. 62

Fig. 63. Guinea pig gut. Fixed in glycol ether.

Methanolic thionyl chloride, 4 hrs. : periodic acid : 0.1% acridine yellow-SO_2. Water mount, X 180.

Only a few of the sites containing engendered dialdehydes emit a specific yellow-green fluorescence. The intensity of the blue fluorescence of the remainder of the tissue is much stronger than that indicated in the photomicrograph.

Fig. 64. Rabbit embryonic umbilical cord. Fixed in glycol ether.

Periodic acid : sulphurous acid : 0.01% acridine orange. Dry mount, X 180.

Material which is oxidised by periodic acid emits a bright red fluorescence. The red fluorescence is not produced if the intermediate wash in sulphurous acid is omitted.

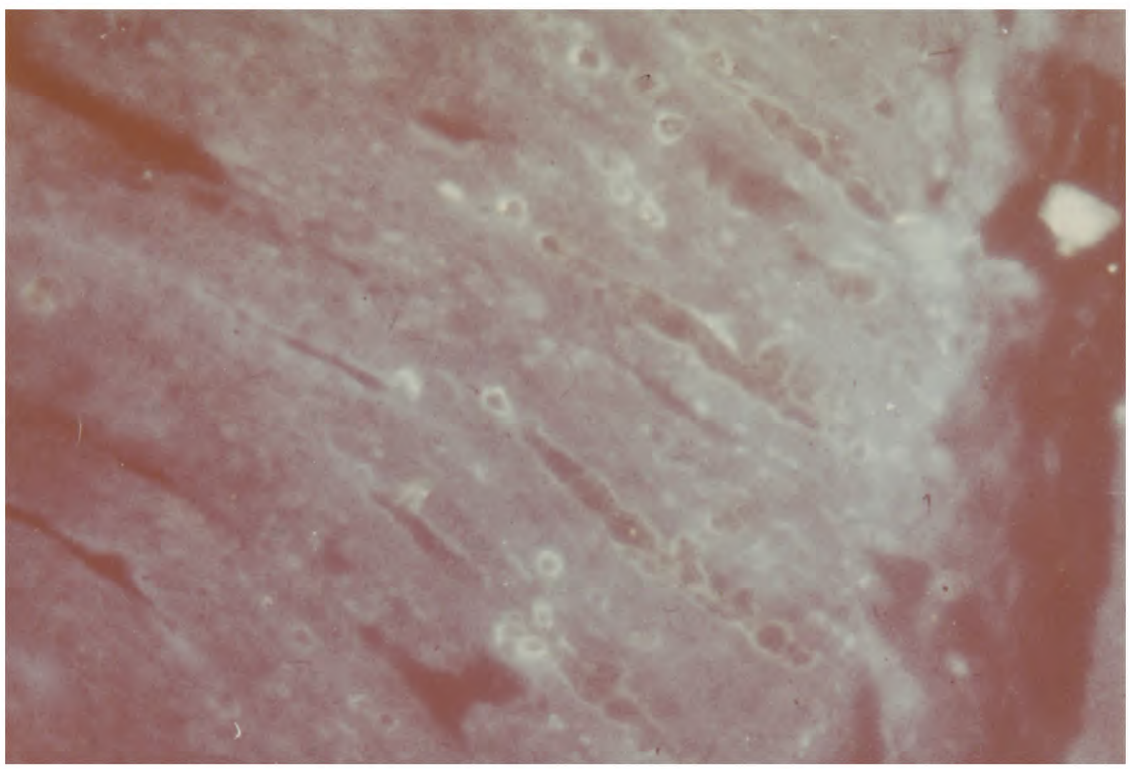


Fig. 65

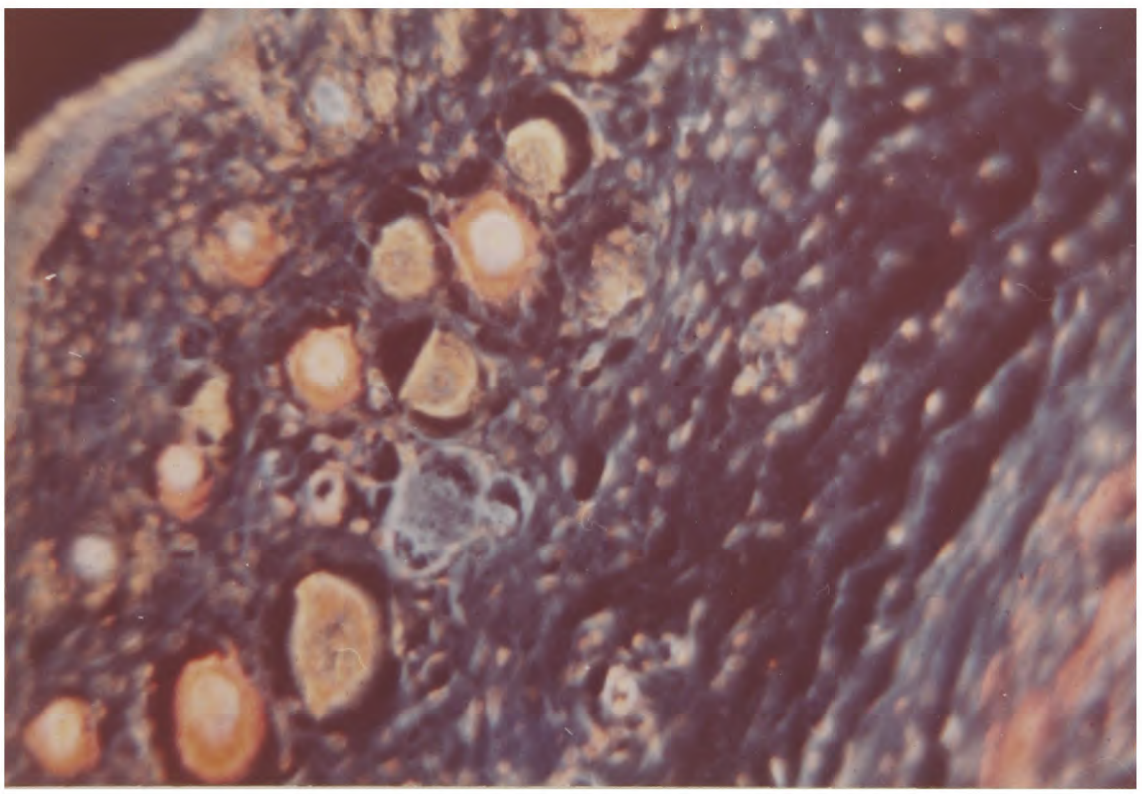


Fig. 65 Guinea pig gut. Fixed in glycol ether.

Periodic acid : 0.1% benzoflavine-SO_2.

Water mount, X 180.

The sites containing <u>vic-</u> glycols are stained indistinguishably, if at all. Cp. Fig. 66.

Fig. 66. Guinea pig gut. Fixed in glycol ether.

Methanolic thionyl chloride, 4 hrs.: periodic acid : 0.1% benzoflavine-SO_2. Water mount, X 180.

The greenish-yellow fluorescence of sites containing <u>vic-</u>glycols is clearly discernible. The protoplasmic proteins fluoresce a light-blue colour.

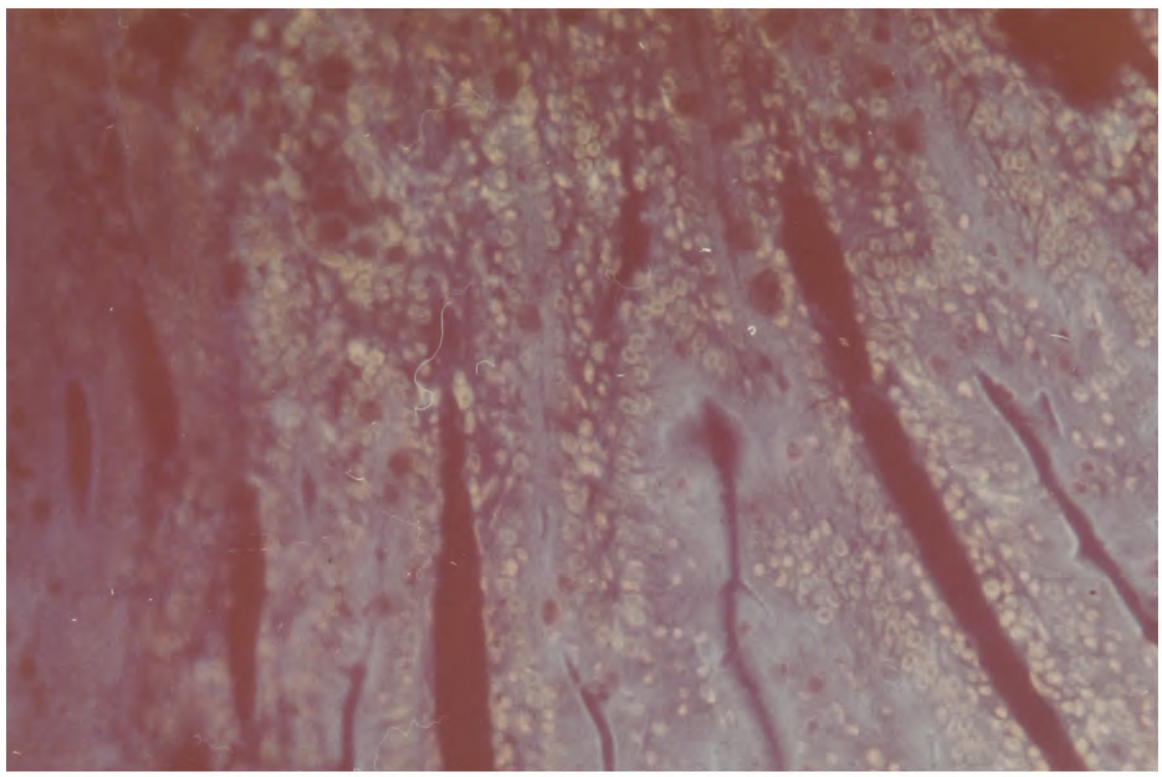


Fig. 65

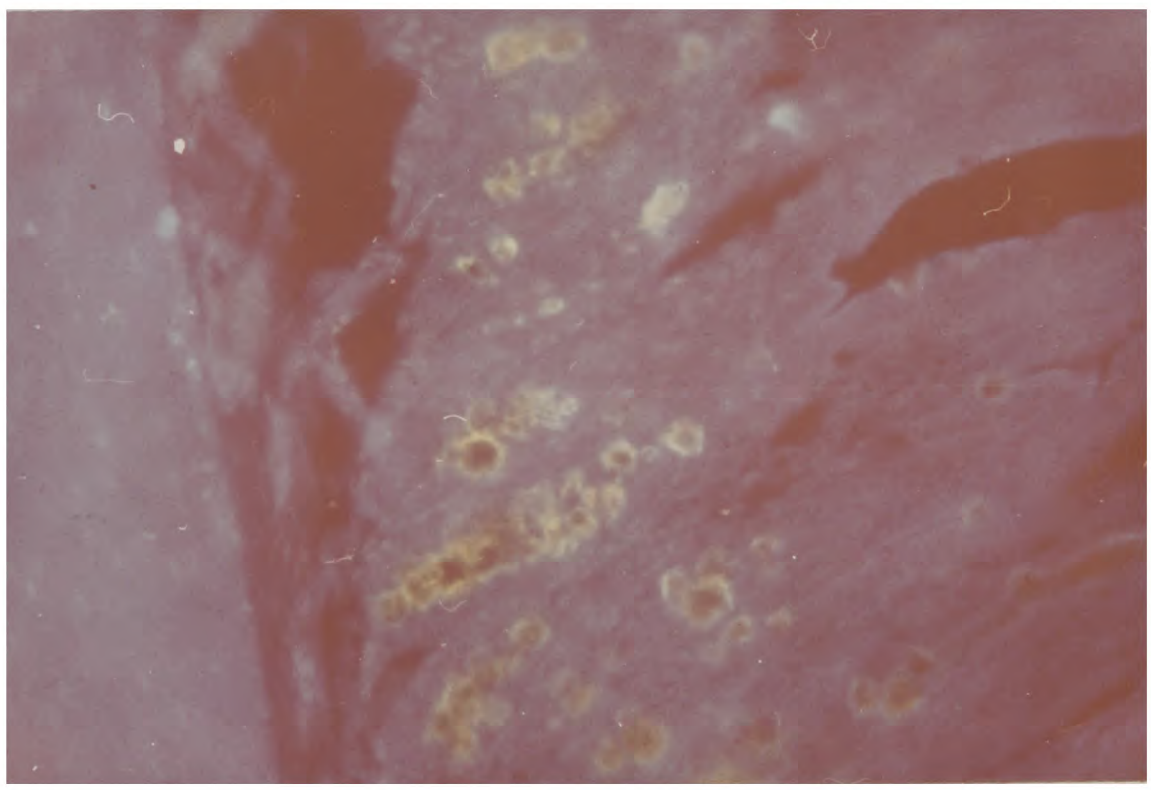


Fig. 66

final appearance was not absolutely reproducable as the critical examination of serial sections processed in exactly the same way showed. A more reliable pattern of staining could be obtained by using 0.01% solutions of coriphosphine - SO_2 if the stained sections were examined for fluorescence as water mounts.

On the other hand 0.1% solutions of other dyes, such as acridine yellow - SO_2 (Fig. 67) or benzoflavine - SO_2 (Fig. 66), stained sections discretely and specifically after oxidation in periodic acid. However the dye was extracted completely <u>from the periodic acid-reactive sites</u> when the stained sections were dehydrated in alcohol. In this respect, the aldehyde-dye/SO_2 complexes resemble basic dyes (e.g. toluidine blue) adsorbed onto anionic sites (e.g. acid mucopolysaccharides) by simple electrostatic forces.

When 0.01% solutions of acridine yellow - SO_2 or benzoflavine -SO_2 were used for staining sections previously oxidised in periodic acid, only a non-specific intense blue fluorescence could be elicited from such sections mounted in water without any intermediate dehydration. There was no additional fluorescence in sites known to contain neutral mucopolysaccharides.

For these reasons, differentiation by alcohol or by other organic liquids having a fairly wide solvent action is to be deprecated, and has been avoided in this investigation

whenever basic dyes were used as fluorchromes; sections which had been stained in solutions of these dyes were always rinsed briefly in water, and examined for fluorescence as water mounts.

Relatively specific differentiation has been reported with lesser-known solvents, such as triethylphosphate in the methyl green-pyronin test for nucleic acids (Rosa, 1950), but their use in fluorescence histochemistry has not yet been explored.

Another approach to the practical problem of removing the excess dye is to wash the overstained sections with an aqueous or other relatively inert solvent containing a solute which is chemically similar to the particular tissue substrate under investigation. The solute forms a soluble complex with the excess dye that is loosely held onto the section by secondary adsorption forces. The dye which remains on the tissue substrate after the washing process is firmly bound by ionic forces. The use of phosphate buffers for "differentiating" sections stained for nucleic acid-phosphates with acridine orange (von Bertalanffy, 1961) is an example of this approach; in my experience, a strictly standardised technique has to be used if consistent results are to be obtained.

(3) <u>Non-specific staining</u>.

Even when discrete staining can be observed, its

specificity is often open to doubt: the dye -SO_2 complex may be taken up by groups other than aldehydes which co-exist in the same site.

The staining of nuclei after a Feulgen acid hydrolysis illustrates this difficulty. The dye - SO_2 complex may react with the exposed aldehyde from the deoxyribose moiety of DNA, but at the same time the basic dye itself can combine with the acidic phosphate groups of DNA. Unless one of these groups is blocked before staining, it is impossible to infer which is the reactive centre. However, it is very difficult to design a procedure for blocking one group without removing the other group simultaneously, or at least impeding its accessibility to test reagents. Thiosemicarbazido solutions (Pearse, 1960) are often used to block DNA aldehydes which then take up no pseudo-Schiff dyes (Kasten, 1958a). However the high acidity of the thiosemicarbazide solution (used for ¾-3 hours at 60°) is sufficient to complete the hydrolysis and extraction of the remainder of the DNA molecule, so that its use cannot be justified for demonstrating the specificity of pseudo-Schiff solutions for (DNA) aldehydes. In this connection it is interesting to note that exposed DNA-aldehydes which have been blocked with hydroxylamine still show a marked affinity for pseudo-Schiff reagents (Kasten, 1958a). Undoubtedly the milder conditions of blocking when buffered solutions are

used at room temperature (Danielli 1949) would not remove remaining phosphate groups. It is unlikely that the hydroxylamine blockage is reversed by the acidity of the pseudo-Schiff reagent or is incomplete (Staple, 1958) and therefore the uptake of the dye - SO_2 complex is due partly to the residual phosphate groups. This explanation is contrary to Kasten's (1958a) interpretation.

The same sort of difficulty has been encountered with tissues in which the PA-reactive sites are in close proximity to acid mucopolysaccharides and nuclei. It is very difficult to distinguish the additional fluorescence due to the oxidised neutral mucopolysaccharides in guinea pig gut, for example, that has been oxidised in periodic acid and stained in dilute (0.01%) coriphosphine -SO_2 (Figs.33 and 61). The basophilic nuclei and acid mucopolysaccharides form practically the same coloured fluorescent complexes.

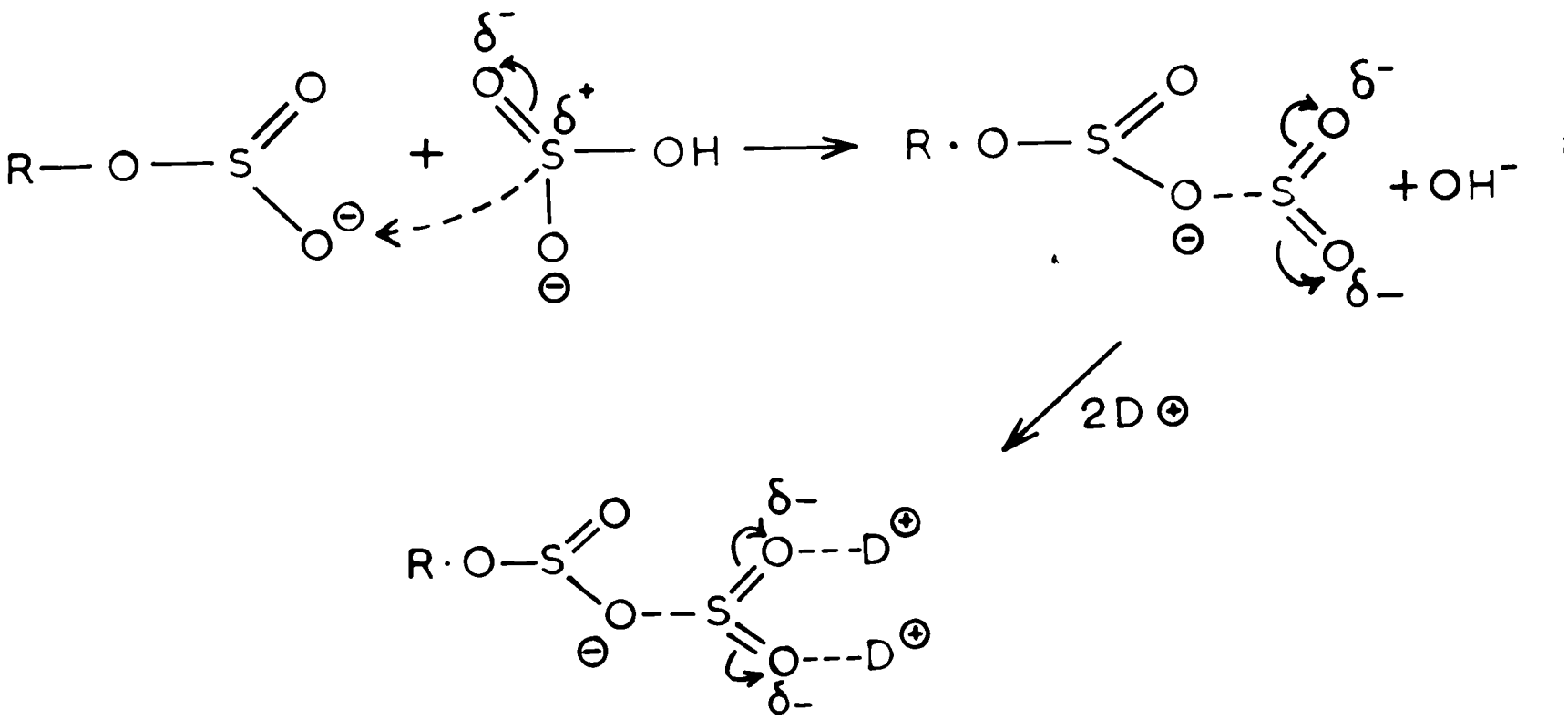
(4) Anomalous staining.

The interpretation of stained control test sections such as Fig. 36 is even more complicated than might at first appear; when control sections are stained in coriphosphine - SO_2, their distribution of fluorescence colours are more like those of oxidised test sections than, as might be expected, those of control sections stained in acidified coriphosphine solution containing no dissolved sulphur dioxide. Both nuclei and acid mucopolysaccharides apparently take up more

dye from solutions containing sulphur dioxide, judging by their increased resistance to alcohol extraction and the shifts towards the red of the nuclear fluorescence. The latter is presumably due to a close stacking (and uptake) of dye molecules onto the DNA chain.

The intensity and colour of the fluorescence of acid mucopolysaccharides changes even more noticeably after a brief (Feulgen) acid hydrolysis; they look like periodate-oxidised neutral mucopolysaccharides!

The reasons why basophilic sites take up more dye from pseudo-Schiff solutions are not known. Possibly sulphur dioxide is adsorbed at its electrophilic sulphur atom onto the negatively charged phosphate or sulphate groups of nucleic acids and acid mucopolysaccharides respectively (see diagram). This would produce an intermediate complex having two centres of comparatively high electron density on the two oxygen atoms of the sulphur dioxide moiety. The basic dyes (D+) could then form salt-like linkages at each of these oxygen atoms. This would explain the increased fluorescence intensity: presumably the fluorescent dye molecules are further away from the mucin sulphate groups and are less likely to be quenched. This hypothesis will be invoked again in sec. 12.10 for explaining the mechanism of the reaction between pseudo-Schiff reagents and engendered dialdehydes.


R — O — S
O
O⊖
+
δ⁻
O
δ⁺
S — OH
O
⊖
R · O — S
O
O
δ⁻
O
S
+OH⁻
O
⊖
O
δ−
2D⊕
R · O — S
O
O
⊖
δ−
O
S
O
δ−
O---D⊕
O---D⊕

(5) Quenching

In sections in which neutral mucopolysaccharides co-exist in practically the same sites as acid mucopolysaccharides, specific staining of engendered aldehydes is often indeterminate and weak ~~=~~ following oxidation ~~oxidation~~ in periodic acid, and staining in pseudo-Schiff solutions other than coriphosphine $-SO_2$ or acridine orange-SO_2.

Fig. 65 shows this effect on oxidised guinea pig gut stained in 0.1% benzoflavine-SO_2. Comparison with a stained control section shows that the periodic acid-reactive neutral mucopolysaccharides could easily be missed unless it was known, from previous experience, where they might be found. It can also be seen that the acid mucopolysaccharides themselves are not fluorescent; with the exceptions of coriphosphine and acridine orange, no fluorescent dyes form fluorescent conjugates with acid mucopolysaccharides although it is believed dye is actually taken up (Sec.93). Presumably the electron density on the mucin sulphate groups is sufficiently high to localise or distort the π-electron system of the dyes sufficiently to "quench" their potential fluorescence.

The "dialdehydes" formed through the oxidation of the neutral mucopolysaccharides probably take up the pseudo-Schiff complex but much of the potential fluorescence is quenched by the neighbouring sulphate groups of the acid

mucopolysaccharides. If the quenching properties of the latter are destroyed, the former exhibit their normal fluorescence. This can be achieved by a mild methylation of the acid mucopolysaccharides (sec.11.4) which abolishes their basophilia. Fig. 66 shows the effect of this treatment: the extent of the yellowish-green fluorescence compares favourably with the distribution of the fluorescence obtained in oxidised sections which have been treated with salicyloyl hydrazide (Fig.51).

(6) Incomplete reactions

Many of the difficulties involving the unspecific staining of basophilic sites which have just been described can be avoided by abolishing the general basophilia before carrying out oxidations in periodic acid. This can be conveniently done by methylating sections beforehand in methanolic thionyl chloride for 3-4 hours at room temperature (sec.11.4). When methylated sections are oxidised in periodic acid and stained in pseudo-Schiff reagents, all the observed fluorescence should be attributable to engendered aldehydes. Unfortunately, some pseudo-Schiff reagents do not stain as extensively as others. 0.1% Coriphosphine-SO_2 and acridine-yellow-SO_2 stain very few of the engendered aldehydes produced by oxidation of methylated guinea pig gut (Figs. 62 and 63), whereas 0.1% benzoflavine-SO_2 stains almost all of them (Fig. 64). The insensitivity of the first two reagents

may be due to their polynuclear structures being unable to penetrate through the network of bulky methylated sulphate groups surrounding the mucin dialdehydes. Benzoflavine, on the other hand, possesses a comparatively small p-anilino grouping which may interact preferentially with the engendered aldehydes leaving the "fluorescent" heterocyclic moiety outside the "sphere" of the tissue substrate groups.

The failure of the pseudo-Schiff reagents to stain all the engendered aldehydes may be due in part to hemi-acetal formation.

On the other hand, other tissues can be made to stain normally with these reagents. Acridine-yellow-SO_2, for example, stains oxidised glycogen in guinea pig liver sections very well (Fig.67), if they are stained for at least one hour. The 20 minutes staining recommended by Vasten (1958a) has never been found sufficient.

Even in methylated liver sections (Fig.68), the orange-yellow fluorescence of the glycogen dialdehyde complex is surrounded by an intense <u>blue</u> cytoplasmic fluorescence. It might be thought that this is due to autofluorescence or to a reversal of the blockage of the cytoplasmic protein carboxyl groups, but this is unlikely as the intensity is much stronger than the normal staining of unmethylated proteins.

Some demethylation by periodic acid may take

Fig. 67. Guinea pig liver. Fixed in glycol ether.

Periodic acid : 0.1% acridine yellow-$3G_2$, pH 3, 90 mins. Water mount, X 180.

The nuclei and protoplasmic proteins fluoresce green whereas the sites containing vic-glycols emit yellowish-orange fluorescence.

Fig. 68. Guinea pig liver. Fixed in glycol ether.

Methanolic thionyl chloride, 4 hrs.: Periodic acid : sulphurous acid wash: 0.1% acridine yellow-$3G_2$, pH 3, 60 mins. Water mount, X 180.

Sites containing vic-glycols emit a yellowish-orange fluorescence. The blue fluorescence of the surrounding protoplasmic proteins is not apparent in the photomicrograph.



Fig. 67

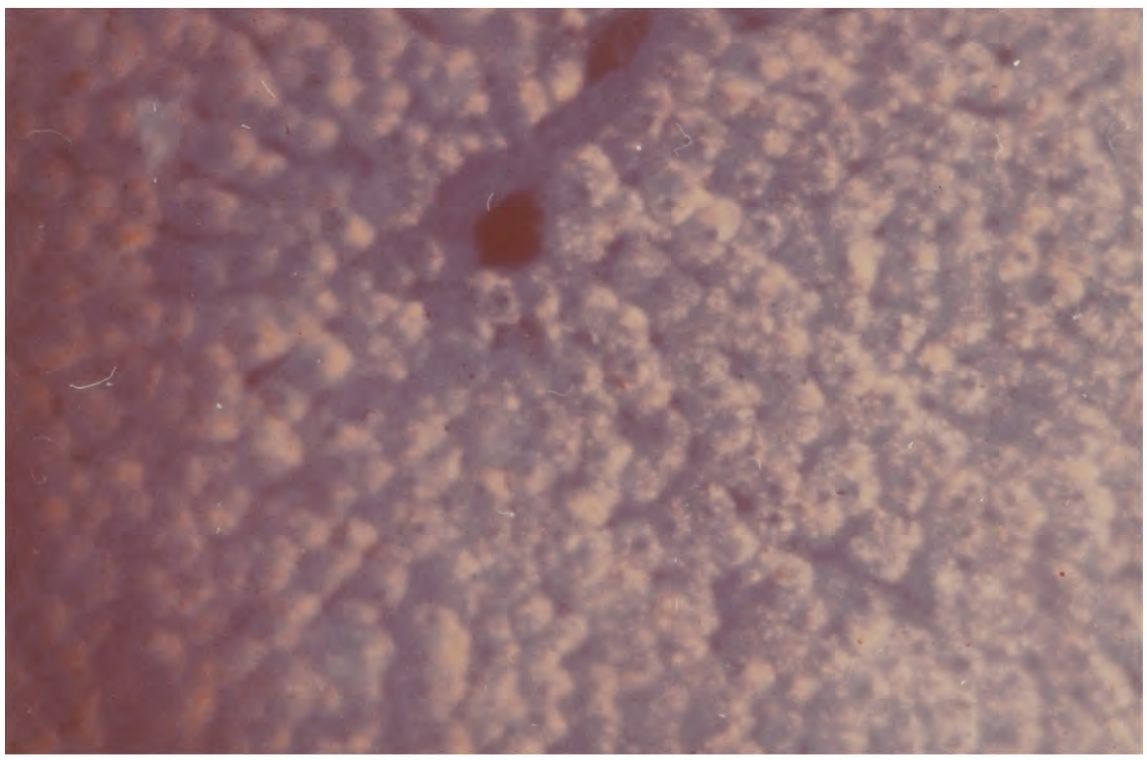


Fig. 68

place, as it is well known that other oxidising agents, such as potassium permanganate, can do this (Fisher and Lillie, 1954). The extraneous fluorescence is more likely to be due to another factor which will now be considered.

(7) The interference from tissue proteins containing sulphur.

It has already been pointed out that periodic acid oxidises tissue proteins containing sulphur (cystine and cysteine) to basophilic sulphinic or sulphonic acids (sec. 5.2). In the normal histochemical test for vic-glycols, Schiff's reagent takes longer to form a coloured complex with these acids than with aldehydes (Pearse, 1951). A noticeable colouration is only developed when all the proteins are oxidised with very strong peracids (e.g. performic acid) and little is formed with periodic acid even though the tissue basophilia may be markedly increased.

The undue sensitivity of fluorescence methods is illustrated here by the interference of sulphur proteins, particularly in tissues where they co-exist in exactly the same sites as neutral mucins containing vic-glycols (e.g. glycoproteins), which renders the periodic acid-pseudo Schiff reaction hopelessly unspecific as a general test for vic-glycols. Sections of artery taken from ovine umbilical cord (fixed in glycol ether) were chosen to

investigate this difficulty in detail. They gave strong PA-SH (sec.12.19) and PA-pseudo Schiff reactions which would normally have been taken as indications of neutral mucopolysaccharides. Interference from tissue proteins would not have been considered as the DDD reaction for tissue thiols (sec.4.1) was weak (but positive) over the whole section. Since this reaction is probably the most sensitive test for tissue thiols (Pearse, 1960), most histochemists would consider the weak staining obtainable on this tissue as being negative.

The following tests were designed to show that most of the fluorescence obtained in the periodic acid-pseudo Schiff reaction on this tissue were due to tissue protein thiols (and disulphides).

(a) Sections were subjected to "drastic" methylation (18 hours at 60° or 90°, sec.11.2) before and after oxidation in periodic acid. Subsequent washing in sulphurous acid followed by staining in 0.1% coriphosphine gave a moderately blue fluorescence. Similarly, acriflavine-SO_2 and auramine-O-SO_2 induced a greenish-yellow and an anomalous bright blue fluorescence respectively. Auramine O and auramine O-SO_2 normally produces an intense greenish-yellow fluorescence with basophils or with periodate-engendered dialdehydes.

The fluorescence in these experiments is not due to

the usual tissue basophilia or to "dialdehydes" from oxidised carbohydrate moieties. No fluorescence could be elicited from unoxidised, methylated sections stained in solutions of acriflavine, acridine yellow, auramine O or coriphosphine. Neither did methylated sections which had been oxidised in periodic acid give any visible colouration with Schiff's reagent or a fluorescent derivative with salicyloyl hydrazide. Moreover, the Schiff-stained sections exhibited/a blue fluorescence characteristic of colourless fuchsin adsorbed onto basophlic sites.

(b) The suspicion that tissue thiols were responsible for the positive fluorescent reactions in (a) was confirmed by incubating sections in sodium iodoacetate (sec.4.4) before carrying out the drastic methylation and oxidation in periodic acid: the induced basophilia was substantially reduced or abolished.

Confirmation for the existence of protein thiols was provided by the following experiments:

(c) The uptake of dinitrofluorobenzene (DNFB) at a pH below 5.5 (sec.6.3). Only thiols and phenols react with DNFB in acid media. As the sections were visibly stained yellow at pH 5.4, thiol groups must be reacting rather than phenolic groups (as in tyrosine) which have colourless derivatives. Very little visible colouration could be seen in sections stained at pH.3.6.

(d) The strong green fluorescence following the formation of the zinc complex of the salicyloyl hydrazone of sections previously incubated in N-ethyl maleimide (the NEM-SH/Z_n reaction; sec.4.2). Theoretically only amine and thiol groups can give a positive reaction but in practice only the latter ever gives a strong reaction.

The presence of tissue thiols was also inferred indirectly from the following experiments:

(e) Exposure of "drastically" methylated sections to sulphuryl chloride vapour (exp.sec.4.2) rendered them strongly basophilic; subsequent immersion in 0.01% solutions of acridine yellow or auramine O produced bright blue fluorescent colours.

Assuming that prolonged methylation completely methylates protein amine groups (sec.11.2) and extracts mucopolysaccharides, the only groups which are left for sulphation by sulphuryl chloride are the single hydroxyl or thiol groups of proteins. However, connective tissue, whose collagen contains relatively large amounts of hydroxyamino-acids (Fieser and Fieser, 1962), is not rendered basophilic by sulphuryl chloride vapour (Lewis and Grillo, 1959). In any event, hydroxyl groups sulphated in this way are not always basophlic (sec.13.5). By elimination, the only groups which could account for the fluorescence are thiols.

(f) The vic-glycols of neutral mucopolysaccharides can be prevented from being sulphated by previously oxidising them with periodic acid and condensing the engendered aldehydes with aniline. "Mildly" methylated sections (sec.11.2) with blocked vic-glycols were exposed to sulphuryl chloride vapour and stained in auramine O. As in (e), the anomalous blue fluorescence which is produced can only be attributed to thiol groups. Stained control sections, which had been methylated but not oxidised in periodic acid, exhibited the normal greenish-yellow fluorescence of auramine O after exposure to sulphuryl chloride vapour.

(8) The elimination of the interference due to oxidised thiols.

There is apparently only one way in fluorescence histochemistry of avoiding the interference due to thiols oxidised in the periodic acid treatment for vic-glycols, and that is to use salicyloyl hydrazide: it forms fluorescent complexes with engendered aldehydes (sec. 12.19), but does not react with oxidised thiol groups (sec.5.2/2ẅ).

If Schiff-type reagents (secs.12.6 and 12.7) are used for demonstrating the engendered aldehydes, the basophilic sulphinic acids formed by the oxidation of tissue thiols also react. The interfering thiols cannot be eliminated by methylation (Lillie et al., 1954) after the periodate oxidation, nor is there any suitable method of blocking them

beforehand without removing polysaccharide materials simultaneously.

Fortunately however, only a relatively small number of thiols are oxidised in methylated sections and they always form blue fluorescent conjugates with nearly all the fluorescent dyes tested so far. Therefore, if pseudo-Schiff reagents which form yellow-orange conjugates with engendered aldehydes (e.g. benzoflavine-SO_2 or auramine O-SO_2) are used, the oxidised thiol sites can be distinguished from those containing vic-glycols (Fig. 68).

(9) The recommended practice for using pseudo-Schiff reagents.

A recommended schedule for the use of pseudo-Schiff reagents in fluorescence histochemistry for visualising aldehydes revealed by the periodic acid reaction is given in exp. sec. 12.2.

To avoid confusion with the general tissue basophilic staining, sections are methylated before being oxidised in periodic acid: three to four hours treatment in methanolic thionyl chloride (sec.11.4) is usually sufficient.

The interference of thiols in the periodic acid reaction is not easily avoided, and tests (sec.12.7/7) should always be carried out to find out whether they co-exist in substantial quantities in periodic acid-reactive sites.

The oxidised, methylated sections are best stained

in 0.1% solutions of benzoflavine-SO_2 for at least 60 minutes. The pH of all these solutions should be adjusted to approximately 3. Fluorescence colours other than blue indicate sites containing vic-glycol groups.

Mucins of unusual activity can sometimes be demonstrated by oxidising an (unmethylated) section in periodic acid, followed by treatment in sulphurous acid and staining in 0.01% coriphosphine (Fig.64): an intense bright red fluorescence indicates reactive materials, which are not shown up by control sections in which the sulphurous acid stage has been ommitted. An explanation of this unusual fluorescence reaction is offered in the next section.

(10) The mechanism of the reaction between the engendered dialdehydes and pseudo-Schiff reagents.

There is no specific evidence in support of Kasten's hypothesis (1958a, 1960) that fluorescent amino-acridine-SO_2 solutions react with aldehydes by a mechanism analogous to the Schiff reaction.

Evidence reviewed earlier (sec.12.6/4) suggests that the Schiff reaction procedes essentially in two steps:

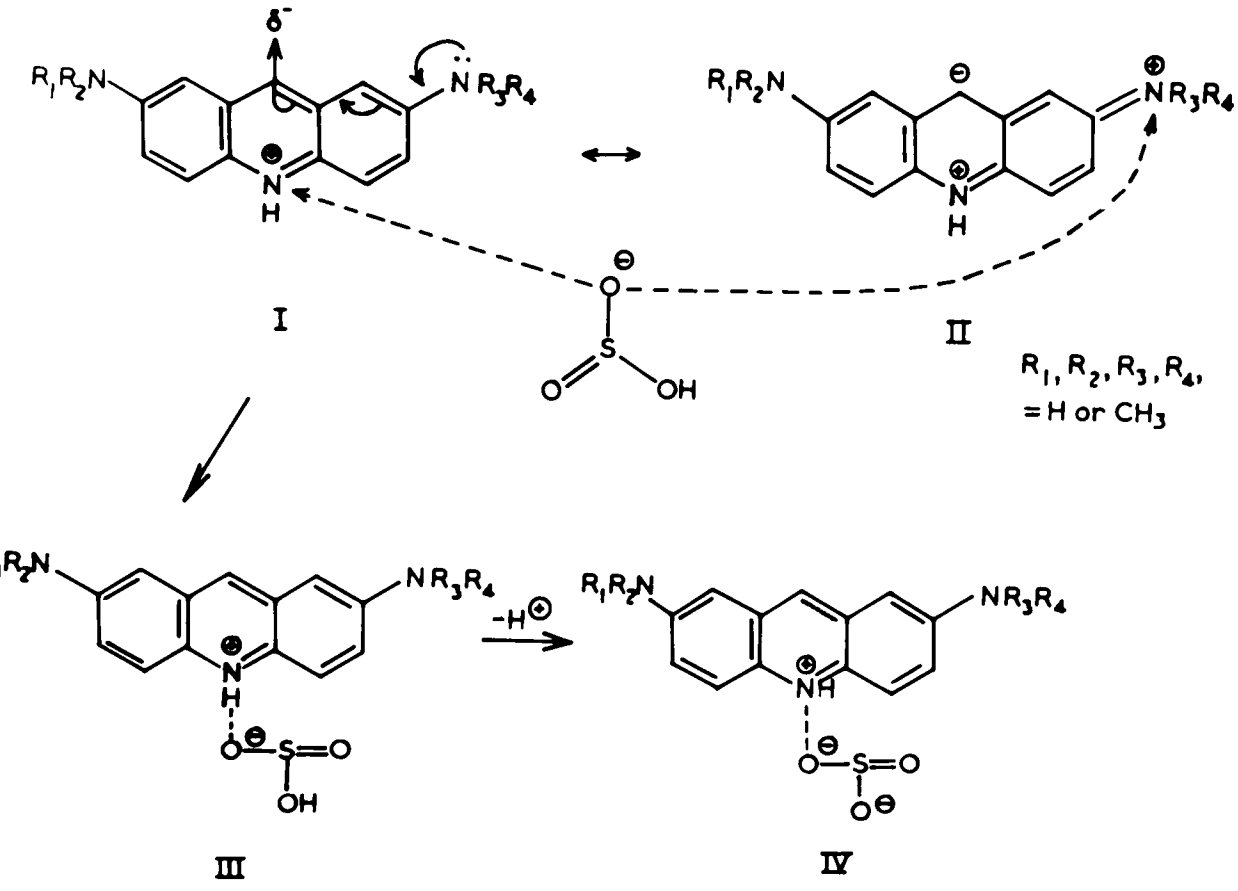
(i) The aldehyde reacts with sulphur dioxide or sulphurous acid to form a sulphonic acid.

(ii) The intermediate sulphonic acid condenses with the anilinium groups ($-C_6H_4.NH_3^+$) of the fuchsin dye to form a coloured complex.

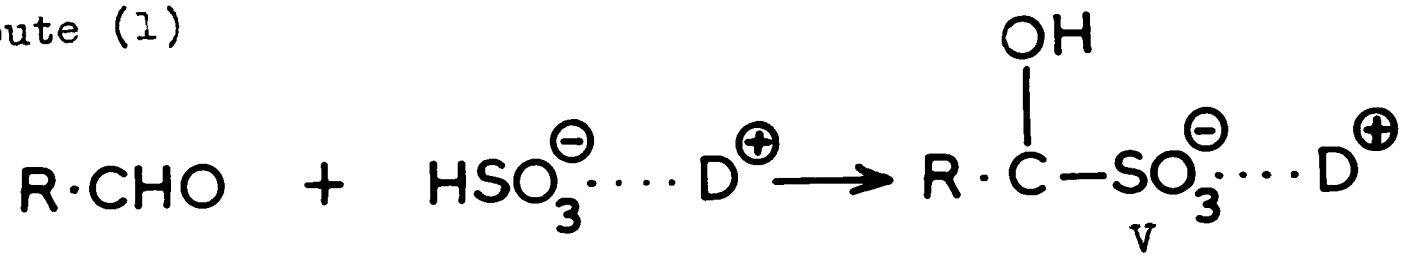
Stage (ii) is unlikely to take place with aminoacridine dyes; their absorption spectra have indicated that their amine groups do not take up protons (Craig and Short, 1945; Turnbull, 1945) except in highly acid media and are relatively inert in the chemical sense. Whenmonoaminoacridines form salts, the proton becomes attached to the ring nitrogen. As the resonance energy of the diaminoacridine dyestuffs is even higher than that of the monoaminoacridines (Albert and Goldacre, 1946), the amine groups of the former are even more inert and reluctant to take up hydrogen ions.

However, there is no reason why sulphur dioxide should not be attached to either the ring or the amine nitrogen atoms of acridine dyes by a salt linkage. Positive charges are carried on the ring and amine nitrogen atoms as shown on the two canonicals I and II of the resonance hybrid of the basic ion of 2:8-diaminoacridine dyestuffs. The negatively charged sulphite ion "neutralises" the electron-defficient centres on the dye to form the intermediate III, which presumably can ionise to IV.

It is unlikely that the oxygen atom of the sulphur dioxide moiety is attached to the nuclear nitrogen atom of the dye by a covalent linkage; the instability of this linkage is illustrated by the reduction of acridine-N-oxide to acridine with sodium metabisulphite (Lehmstedt and Klee, 1936).

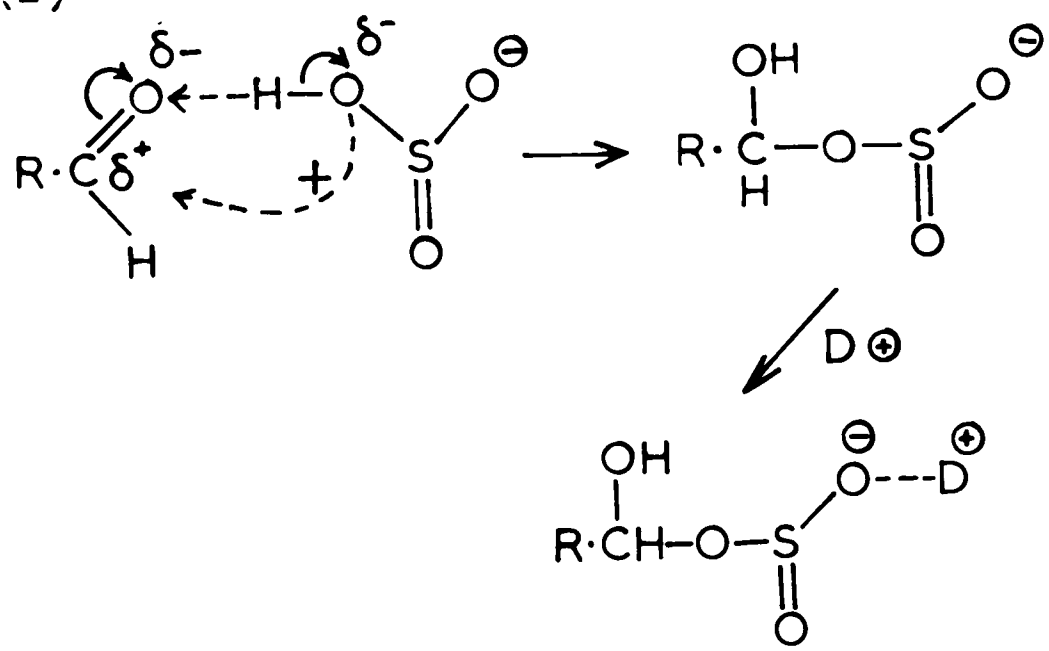


Route (1)



V

Route (2)



VI

The dye (D^+)-SO_2 complexes probably react with aldehydes either by route (1), or by route (2) which is similar to the step (i) in the Schiff reaction, or by both routes.

The final products V and VI are the same in both cases. Both are salt-like complexes similar to the precipitates which are formed between basic dyes and the phosphates or sulphates of nucleic acids and acid mucopolysaccharides respectively. The dye can always be extracted from tissue sections by alcohols in contrast to the fastness of true Schiff complexes.

The actual route followed by a given dye depends on the relative affinity for sulphur dioxide of the aldehyde and the dye; this can be illustrated by the histochemical evidence for route (2) using umbilical cord taken from a rabbit foetus (fixed in glycol ether). Sections of this tissue were oxidised in periodic acid and immersed in dilute (0.01%) solutions of (a) coriphosphine or acridine orange (pH adjusted to approx. 3), (b) coriphosphine-SO_2 or acridine orange-SO_2 (pH adjusted to approx.3), and (c) sulphurous acid followed by (a).

Neither (a) nor (b) produced any significant additional fluorescence, but (c) gave an intense, distinctive red fluorescence (Fig.64) which could not be quenched by ferric alum solution.

The reaction with acridine orange is especially

important for the hypothesis proposed here that pseudo-Schiff solutions react with aldehydes by salt linkages instead of through condensation of their amine groups. Acridine orange (Fig.60) has no free amine groups and therefore cannot react by a Schiff-type mechanism. Kasten (1958a) reported that two of his acridine orange batches gave a positive reaction. Betts (1961) has also reported that strong acridine orange-SO_2 solutions give good results for demonstrating periodate engendered aldehydes. On the other hand, Kasten (1962) claims that acridine orange which contains less than 1% primary amine impurity is non-reactive, but as his test for primary amine impurities is based on the negative staining of aldehydes in tissue sections (Kasten, 1958a), his proof is illogical.

On the other hand, the "dialdehydes" produced in tissues (other than the specimen of embryonic umbilical cord mentioned earlier) do not always form distinctive fluorescent complexes by method (c), but usually require sulphur dioxide to be present in the dye mixture (method b) before they stain appreciably. Similarly a comparison of the ease with which various reducing sugars combine invitro with sulphur dioxide shows that this interaction is increased when amino compounds are present (Ingram and Vas, 1950). This varying behaviour fits in with the concept of competing equilibria of sulphur dioxide for

aldehyde and amine groups where in a given instance, one may be chemically more dominant than the other.

Positive reactions for other dyes containing no primary amine groups have been observed for azure B and crystal violet (Kasten, 1958a). Moreover, the ability of some batches of pyronin B and pyronin Y to stain cytochemical aldehydes could not be correlated with dye impurities (Kasten et al., 1958, 1962). Only certain batches of methylene blue were reactive (Kasten, 1958a), although some solutions became reactive after a few days: the reactive dyes, azure A and thionin, are possibly produced as a result of the acidity of the solution and the auto-oxidation of methylene blue near the surface of the solution (Holmes and French, 1926).

All the experimental evidence cited here vitiates Kasten's hypothesis, that a primary amine is essential (in the 'chemical' sense) for a dye to react with aldehydes in the presence of sulphur dioxide. The role of the primary amine is more likely to act as an electrophilic centre for sulphur dioxide which is then adsorbed onto the aldehyde. An electrophilic centre will still be present when the amine group is alkylated (as in acridine orange) but there is less likelihood that aldehydes would be adsorbed at this centre because of steric hinderance: the relative reactivities of acridine yellow-SO_2 and benzoflavine-SO_2 for the engendered aldehydes of gastric mucins oxidised by periodic acid (sec.12. 7/5) support this hypothesis.

Further arguments against a Schiff mechanism for fluorescent dyes depend on the relatively low chemical activity of their amine groups as shown in both chemical and histochemical experiments. Amino-acridines require forcing conditions before they will condense with aldehydes to form anils. Attempts were made to simulate in tissue sections the few successful instances of anil formation *in vitro* recorded for 2 : 8-diaminoacridines (Glen, Sutherland and Wilson, 1936). The gastric mucin sites in sections of rabbit stomach, previously oxidised in periodic acid, did not appear to show any appreciable fluorescence after incubation in acridine yellow or

coriphosmine containing a basic catalyst (piperidine). It is just possible that an anil is formed at one stage during the incubation, but later dissolves out of the section; the facile hydrolysis of anils *in vitro* does not exclude this possibility.

Aromatic amines

Numerous aromatic heterocyclic amines form fluorescent products with aldehyde-sugars *in vitro*, but with the exceptions of 5-aminosalicylic acid and p-aminohippuric acid, none give fluorescent products with aldehydes *in situ*: some condense readily to form non-fluorescent anils, while others require sulphurous acid to promote a fluorescence reaction.

The following amines have been studied:

8. N-(1-naphthyl)-ethylene diamine.

Paper chromatograms of reducing sugars reach a maximal fluorescence after being sprayed with a slightly basic solution of this amine and heated to about 100° (Wadman *et al.*, 1954). However, only a moderate blue fluorescence could be obtained from liver sections oxidised in periodic acid and subsequently immersed in buffered solutions of the amin at room temperature. Neutral solutions produced a more intense fluorescence than acid solutions. The latter could be made to induce a considerably brighter blue fluorescence if the solution contained sulphur dioxide.

9. m-Phenylene diamine.

Chargaff et al.(1948) utilised alcoholic solutions of m-phenylene diamine hydrochloride for detecting reducing sugars on paper chromatograms: a yellow to orange fluorescence was produced in sugar spot areas when the chromatograms were heated to about 100°. Other wood fluorescence reactions have been reported for solutions of ~~the~~ aldehydes and diamine in vitro (Rosenthaler and Vegezzi, 1954; Tomita et al., 1956; Maruta and Suzuki, 1957), but only a diffuse bluish-green fluorescence could be obtained from liver sections previously oxidised in periodic acid, after immersion in a solution of the diamine at room temperature. Sometimes a brown, tarry substance was deposited on oxidised tissue sections: this is presumably a resin formed in a vigorous reaction of the type suggested by Meyer and Gross (1899).

The notorious susceptibility of phenylene diamines to auto-oxidation and complex intermolecular condensation makes the failure of m-phenylene diamine to react smoothly with aldehydes in situ not surprising. One characteristic feature of the reactions between carbonyl groups of reducing sugars and aromatic amines in vitro is the simultaneous production of brown, resinous products and/or fluorescent chromogens (the "browning" or Maillard reaction; Ellis, 1959). The last reaction is favoured

when sulphur dioxide is present. It is attended by a concomitant decrease in the browning reaction, and suggests that m-phenylene diamine might react with engendered aldehydes in tissue sections in the same way. This hypothesis has been investigated with glucose impregnated into filter paper (exp.sec.12.5). Aldehydes "in bulk" (i.e. those generated by periodic acid) reacted too vigorously with the purified diamine alone and produced brown, non-fluorescent resins. "Single" aldehydes (of unoxidised reducing sugars) react normally and give moderately fluorescent products of the Maillard type. In the presence of sulphur dioxide, however, even "dialdehydes" in vitro form intense yellow fluorescent complexes, although it is not known whether they are of the Maillard, pseudo-Schiff or diphenylmethane type. Unfortunately, the "dialdehydes" of neutral mucopolysaccharides in vitro (e.g. in umbilical cord or cock's comb) only form a weak bluish-green fluorescent conjugate which suggests that they react differently from those of simple sugars in vitro. Presumably only a little diamine is taken up by oxidised tissue sections and that by a pseudo-Schiff reaction.

10. p-N, N-dimethylphenylene diamine

The N, N- dimethylphenylene amines (or dimethylaminoanilines) have been used both as basic "dyes" for

sulphated acid mucopolysaccharides in mast cells (Bonomi, 1952) and as chromogenic agents for aldehydes in vitro (Utzinger and Regeness, 1954, Utermann and Ehemann, 1955). Spicer and Jarrels (1961) applied both properties for staining acid and neutral mucopolysaccharides in tissues following their oxidation in periodic acid. The former are slowly coloured black by the para-isomer, presumably by auto-oxidation of the adsorbed diaminine to Wurster's red and other insoluble pigments (Michaelis et al., 1939), whereas the engendered aldehydes of the latter form coloured anils comparatively early provided fresh solutions of the diamine are used. Some anils were also strongly fluorescent, such as those found in the thyroid colloid and the goblet cells of the duodenum of the mouse (but not those in the rat or rabbit). Surprisingly, other "dialdehydes", such as those originating from glycogen in the liver or muscle, did not form fluorescent compounds. Moreover, no fluorochemical reaction ever took place in the glycogen or neutral mucopolysaccharide areas in any of my test tissues. Spicer's observations must therefore be treated with some reserve especially as there are numerous theoretical arguments (sec.12.21/3) against fluorescent anil formation.

The dimethylphenylene diamines absorb strongly in the region around 366 mμ (Grammaticakis, 1951) so that it would not be unexpected for their anils to be

fluorescent when excited by ultraviolet light. However, as anils are not universally formed with all engendered aldehydes, the diamine cannot be used as a specific fluorochemical reagent. A positive reaction could be obtained if thionyl chloride was added to the diamine solution. Unfortunately the dull blue fluorescence induced in periodate-oxidised sections of liver, for example, could not be distinguished easily from tissue autofluorescence.

11. m- N, N- dimethylphenylene diamine

This diamine is less basic than the corresponding p- isomer judging by the relative values of their Hammett substitution constants (Sixma, 1953). The difference is reflected in its histochemical behaviour towards mixed mucins: only the dialdehydes of periodate-oxidised neutral mucopolysaccharides take up the diamine leaving acid mucopolysaccharides in the same sites unstained (Spicer, 1961). However, Spicer's results may be due in part to the considerable proportion of p- isomer in the sample which he used (Spicer, 1962).

No in vitro tests for aldehydes which involve the m-diamine appear to have been devised, but the fluorescence of the anils obtained with the p- isomer prompted an investigation into its use as a fluorochemical reagent. As the m- diamine reacts only with aldehydes and not with

basophilic, this reagent seemed especially promising for its specificity. Moreover, it is well-known that fluorescent dyes can be synthesised by heating aldehydes with solutions of this diamine in the presence of hydrochloric acid.

When dialdehydes engendered in tissues were heated in alcoholic solutions of the diamine to about 70 - 80°, no fluorescent compounds were formed in acid sites although there was a weak bluish-green fluorescence over the whole tissue. The fluorescence intensity was not increased by treating the sections with solutions of a ferric salt. Presumably the failure of the reaction is due either to the unreactivity of the engendered aldehydes (because of hemiacetal and hemialdal formation) or to the failure of the diaminophenylmethane intermediate (sec. 12.21/4) to cyclicise at the comparatively low temperatures used in this experiment.

Similarly, aqueous solutions of the m- diamine failed to form fluorescent compounds with engendered aldehydes at room temperature. When sulphur dioxide was present in the diamine solution, a moderate bluish-green fluorescence could be induced in tissues previously oxidised in periodic acid. The fluorescence seemed to be emitted from the whole tissue, which suggested that it might be due to the uptake of the diamine by oxidised protein thiol groups.

12. p- Aminohippuric acid

An orange fluorescence is formed in the areas containing reducing sugars on chromatograms sprayed with alcoholic solutions of p- aminohippuric acid and heated to about 140° (Salter and Zerbian, 1952). However, only a blue fluorescence can be obtained from liver sections, previously oxidised in periodic acid, that have been treated with aqueous solutions of p- aminohippuric acid. Asthe colour of the fluorescence is similar to that of tissue autofluorescence, the reagent is of little practical value.

13. 5 - aminosalicylic acid

The condensation products formed in vitro between aldehydes and p- ani osalicylic acid are fluorescent (Fujimori, 1953). When an aqueous solution of 5- aminosalicylic acid was tried as a histochemical reagent, only dialdehydes, such as those preformed in the periodate oxidation of umbilical cord, developed a bright yellowish-green fluorescence. The single aldehydes exposed by the acid hydrolysis of nuclei did not react at all. Like many other salicylic acid derivatives (e.g. the anils of salicylaldehyde, sec.3.5), the fluorescence of the glycoprotein "dialdehyde" conjugates faded after long exposure to air.

14. p- aminophenol

Although fluorescent products are formed by the condensation of aldehydes and p- aminophenol *in vitro* (Fujimore, 1953) the bluish-green fluorescence developed by periodate-oxidased glycoproteins in ovine umbilical cord was diffuse and weak. Attempts to increase the fluorescent output of the conjugate by coupling with a diazonium salt failed. The stable salt of tetrazised dianthornisidine was tried in view of the fact that it forms fluorescent complexes with thiol-HNI complexes (sec.4.1).

15. p- aminobenzoic acid.

No fluorescence has been observed in gastric mucin sites after they have been oxidised in periodic acid and incubated with an acidified solution of p- aminobenzoic acid. This is unlike the reactions of p- aminobenzoic acid *in vitro* (Fujimori, 1953). The conjugate formed *in situ* is weakly basophilic, however, and can take up basic fluorescent dyes such as coriphosphine.

16. Sulphanilic acid

Attempts to form fluorescent adducts between fluorescent basic dyes and the derivatives formed between engendered dialdehydes and sulphanilic acid were partly successful. A brown or blue fluorescence was produced with coriphosphine, but its intensity was insufficient to commend the method for general use.

17. p- phenylene diamine.

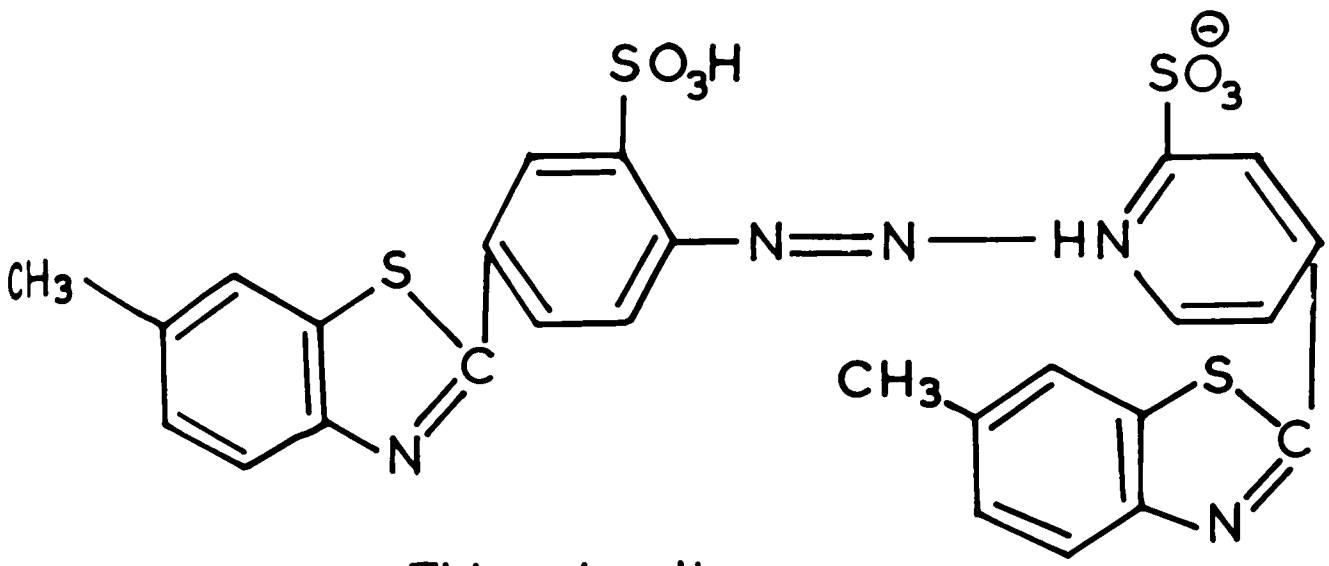
Engendered dialdehydes are completely blocked by p- phenylene diamine. The p- amino group of the anil can be diazotised, but unfortunately it would not couple with fluorescent amines (coriphosphine, acridine yellow, or the more sterically favourable phosphine) or fluorescent phenols (fluorescein or methyl β- umbelliferons, Fig. 69) although a visibly coloured azo-dye can be formed easily with H- acid.

Hydrazines

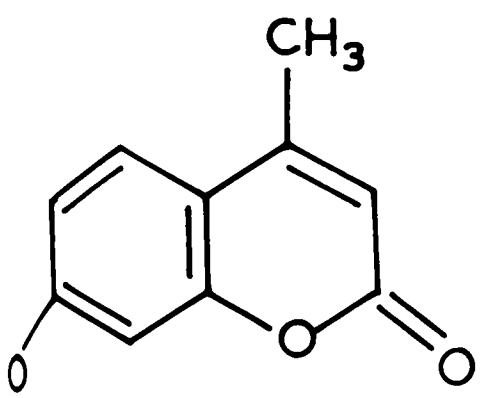
The amino groups of fluorescent aromatic amines are nearly always chemically unreactive, but this is not so in fluorescent hydrazines and hydrazides. This is particularly noticeable for:

18. Acridine hydrazines

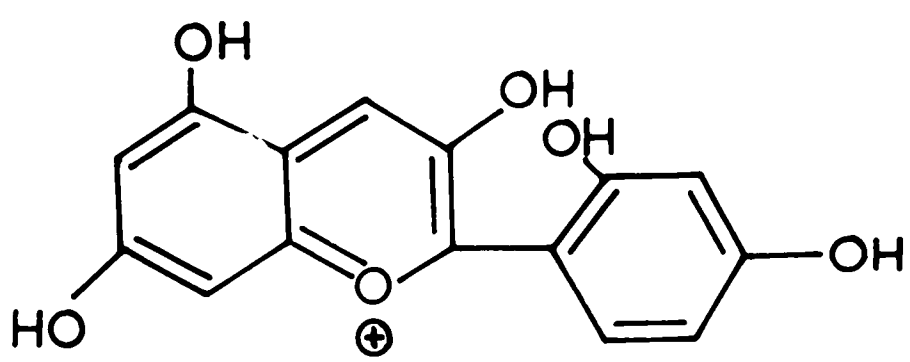
Hydrazines have been synthesised, but not isolated, from aminoacridine dyes and successfully condensed with the dialdehydes of rabbit gastric mucins previously oxidised by periodic acid. The hydrazones of acridine yellow and coriphosphine fluoresced a moderately intense greenish-yellow and dark-red colour respectively. The dark green fluorescence of the hydrazone derived from phosphine was much weaker. Unfortunately the excess acridine dye in the mixture stained the surrounding basophilic tissue, so that the contrast between the hydrazone of the mucin and the background is not particularly good.



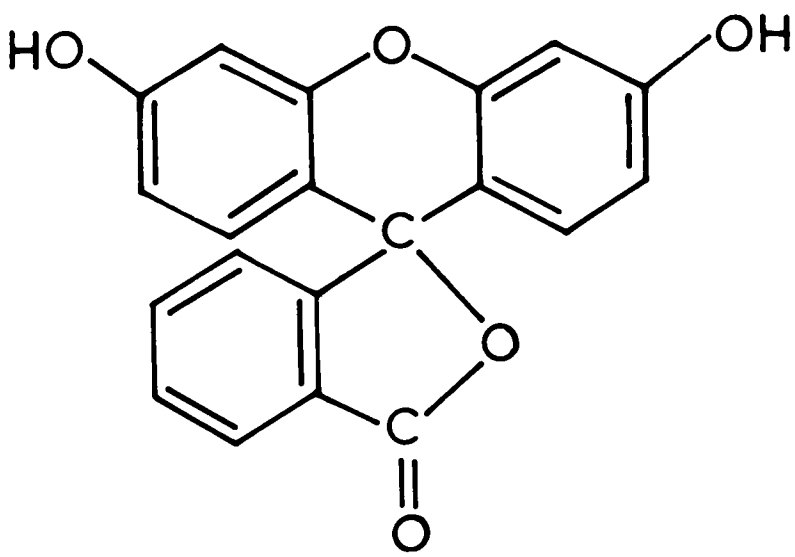
Thiazol yellow



β-Methyl Umbelliferone



Morin



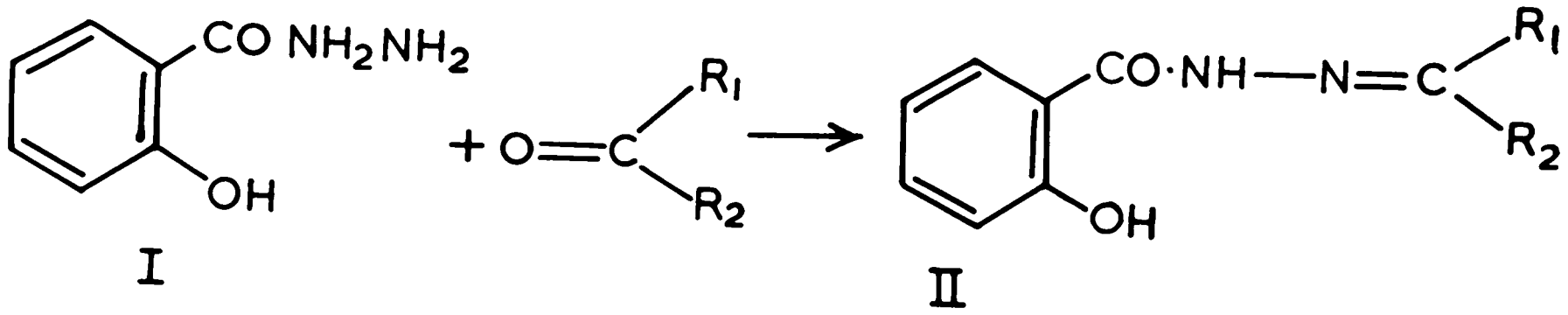
Fluorescein

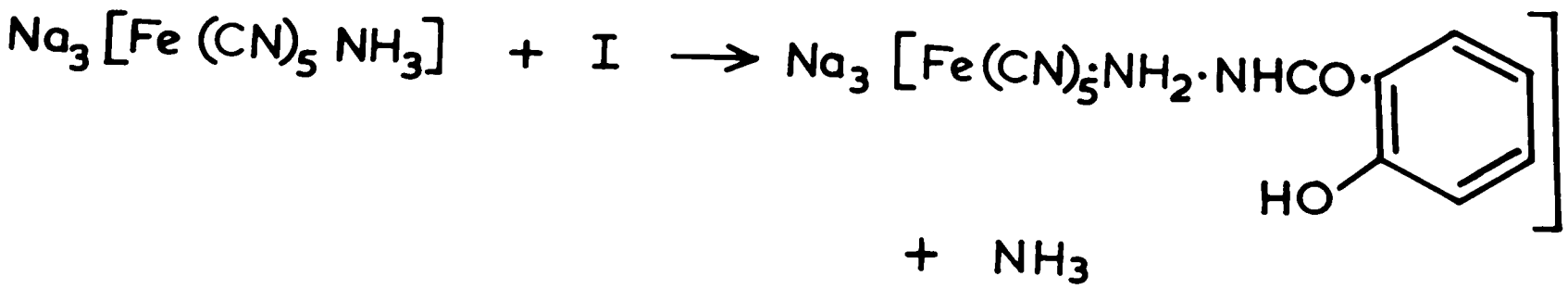
Fig.69. Fluorescent acidic and phenolic dyes.

19. Salicyloyl hydrazide

Camber (1954, 1957) introduced salicyloyl hydrazide (I) for the fluorochemical detection and estimation of aldehydes and ketones in solution, particularly for ketosteroids. Aldehydes and a few ketosteroids form intense blue fluorescent hydrazones (II), whereas simple ketone hydrazones exhibit no fluorescence until they are complexed with a zinc salt.

Acidified solutions of salicyloyl hydrazide have now been used to demonstrate the dialdehydes produced from neutral mucopolysaccharides in tissues oxidised by periodic acid. A very intense blue fluorescence can be seen in the neutral mucin sites. This fluorescence can be distinguished easily from tissue auto-fluorescence, which in many cases, is quenched by the histochemical procedure devised for this reagent. The most important advantage of this reagent is its ability as a hydrazide to react with all engendered aldehydes irrespective of whether they are in the hémi-acetal form or not, unlike pseudo-Schiff reagents which only react with dialdehydes in the "free" form. The extent and resolution of the fluorescence in neutral mucin areas is nearly always much greater and clearer than that obtained in pseudo-Schiff reactions. Moreover, salicyloyl hydrazide does not form fluorescent art facts with basophils or with sulphinic acids formed in the oxidation of tissue thiols


CO NH2NH2
OH
I
+ O=C
R1
R2
CO·NH—N=C
R1
R2
OH
II


Na3 [Fe (CN)5 NH3] + I → Na3 [Fe(CN)5:NH2·NHCO·
HO
+ NH3

by pera_cids (sec.5.2/21v). For these reasons, salicyloyl hydrazide is recommended as the standard reagent for demonstrating vic-glycols by the periodic acid reaction, and will be designated by the symbol PA-SH.

Figs. 51 and 58 show the neutral mucins in in guinea pig gut which has been oxidised in periodic acid. They should be compared with the gut sections stained with pseudo-Schiff solutions (Figs.62, 63 & 64), or with Schiff's reagent itself (Figs. 56 & 57).

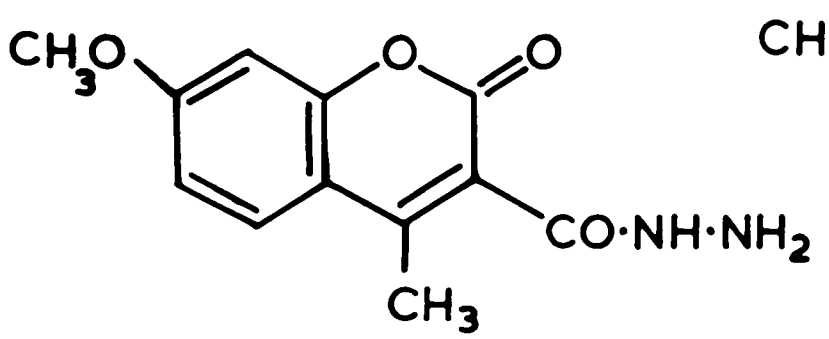
Chen (1959) found that aldehydo - salicyloyl-hydrazones had the same fluorescent characteristics as the unreacted hydrazide. Fortunately, excess salicyloyl hydrazide can be extracted from sections by rinsing them briefly in a freshly-prepared solution of tri-sodium penta cyano-ammine ferroate III (Camber, 1957) with which it forms a deep-purple, water-soluble compound (IV).

When tissues have been processed by the PA-SH technique, the tissue can be counterstained with a red acidic dye: the Solochrome Black-alum complex (sec. 3.10) is particularly suitable (Fig.58).

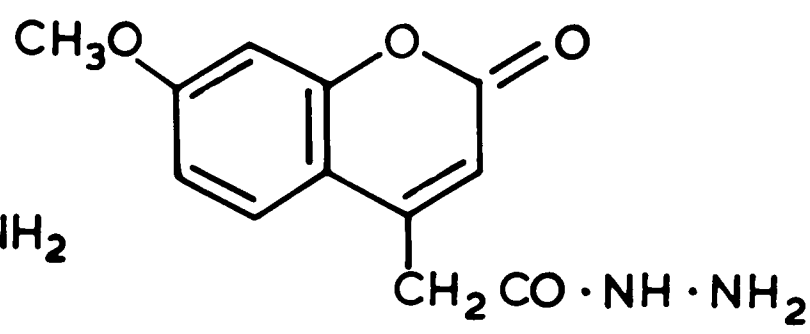
20. Acyl hydrazides

Wilson Baker, Haskar and McOmie (1950) synthesised a number of acyl hydrazides specifically as fluorescent reagents for carbonyl compounds. Unfortunately the most promising of these, 7-methoxy - 4-coumarin - 3 - carboxyhydrazide (I) and 7-methoxycoumarin - 4 - acetyl-

hydrazide (II), only formed bluish-purple fluorescent hydrazones with glycogen in liver sections, previously oxidised by periodic acid, whose intensities were much less than that of the corresponding salicyloyl hydrazones.



I



II

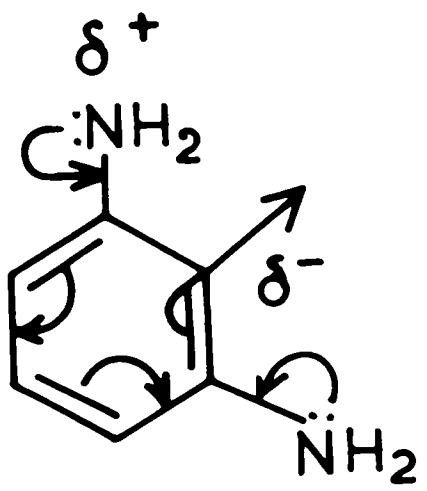
21. <u>The mechanism of the fluorochemical reactions between aldehydes and aromatic amines</u>

The failure of many amines to form fluorescen compounds with aldehydes <u>in situ</u> is both disappointing and inconclusive when compared with the success and diversity of the corresponding reactions <u>in vitro</u>. Many biologists would dismiss unsuccessful histochemical techniques on the grounds of some intrinsic inability of the chemical groups to react in tissues, but this approach is both unscientific and inhibiting to further progress. However, by enquiring as to whether there is any sound reason for the failure of reactions <u>in situ</u>, it can be decided whether any technique is worth re-examining.

When aldehydes react with amines, the following types of end-product can be formed:

(1) Brown tars and resins.

These originate in vigorous reactions between aldehydes and aromatic amines possessing nuclear centres of high electron density (Meyer and Cross, 1939):

e.g. 

+ R·CHO ⟶ tars

(2) Brown, non fluorescent pigments, such as those formed when reducing sugars are heated with amino compounds. Sometimes fluorescent products are formed as well. This reaction has been called the "browning" or the Maillard reactions indiscriminately, but it is now thought that they are distinct (Schroeder et al., 1955). Browning is due to the effect of pH on the sugar and can occur over a wide range, whereas the Maillard reaction takes place only in alkaline media.

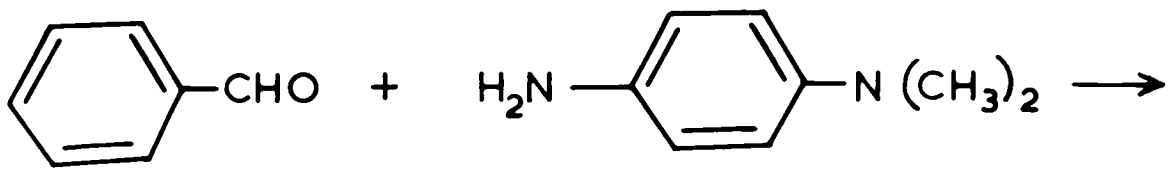
(3) Fluorescent anils.

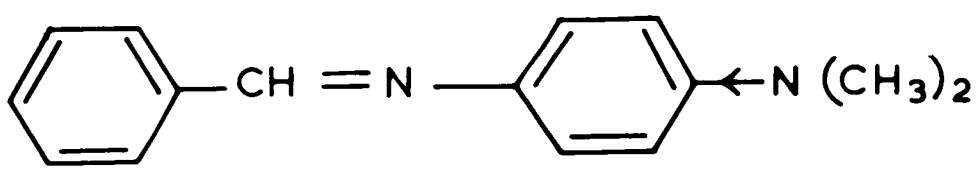
5- aminosalicylic acid and p-amino hippuric acid are the only aromatic amines which have been found to form fluorescent anils with non-aromatic aldehydes either in tissues or in vitro, unless forcing conditions

ere used. It is doubtful whether the fluorescent compounds found in mucin sites by Spicer and Jeanes (1961) are in fact anils in tissues which have been oxidised in periodic acid and subsequently treated with p- N, N- dimethylphenylene diamine: no fluorescent chemical reactions took place with "dialdehydes" produced from neutral sugars *in vitro* or in sites rich in neutral polysaccharide material (e.g. glycogen in the liver).

This failure of potentially fluorescent aromatic amines to condense with aldehydes to form anils with the generalised concept of the conditions necessary for a compound to fluoresce; *inter alia*, amino groups contribute to the resonance energy of an aromatic nucleus which, in turn, enhances the potential fluorescence of the whole molecule (Bowen, 1946), but this involves a concomitant loss of chemical reactivity of the amine groups themselves. In fluorescent aminoacridine dyes, for example, the amine groups are almost unreactive chemically, but if they are converted to a chemically active form, as in the acridine hydrazines, the fluorescence of the dye-aldehyde conjugate is considerably reduced. In other words, chemically active amine groups capable of condensation with aldehydes are inconsistent with the formation of highly fluorescent anils. However, this argument does not necessarily apply to reactions

with aromatic aldehydes *in vitro*. The π-electrons of the aldehyde will be conjugated with those of the amine or an anil (e.g. II) is formed, but as aromatic aldehydes are unlikely to be present in tissues, this reaction is of no histochemical value.

eg. 



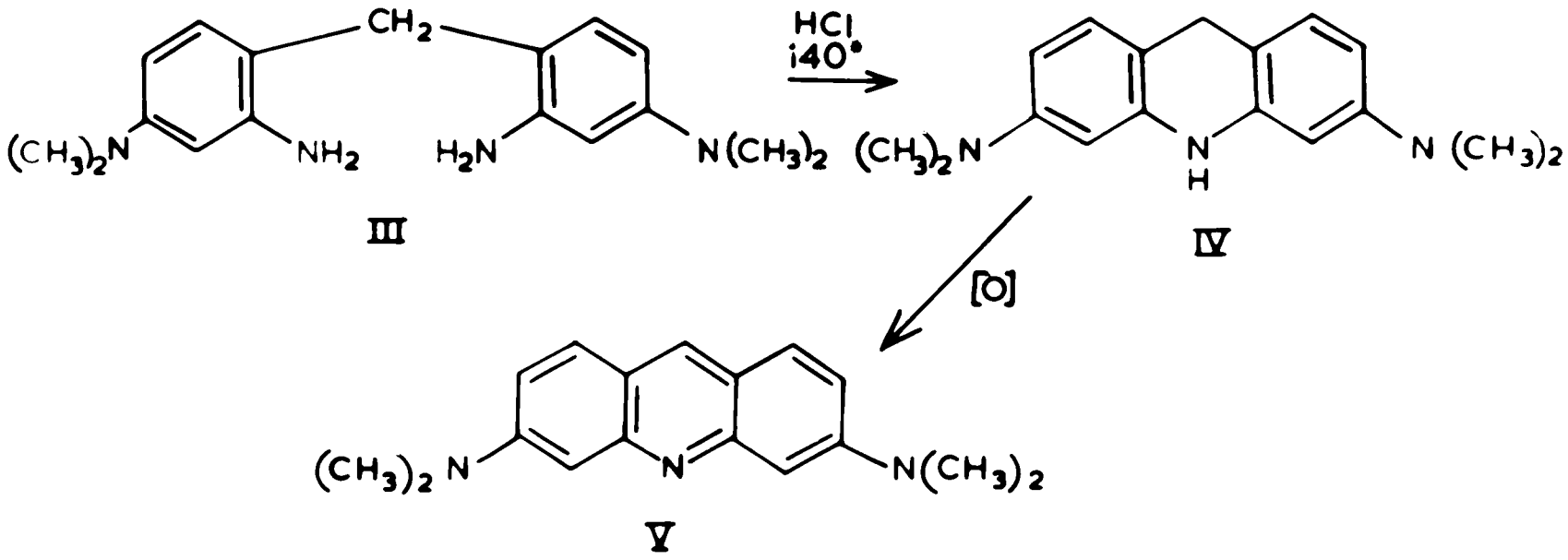
II

On the other hand, the amine groups in 5-amino-salicylic acid and p-aminohippuric acid are essentially normal in chemical activity and play little part in promoting the ability of their parent molecules to fluoresce. Consequently, both compounds can condense with aldehydes to form fluorescent anils.

(4) Aminoacridines.

Many aromatic amines form fluorescent complexes with aldehydo-compounds when heated to comparatively high temperatures (100 - 140°) in the presence of air. The detection on chromatograms of reducing sugars with p-phenylene diamine is a typical example. Although it is tacitly assumed that fluorescent anils are formed, it seems more likley that they are aminoacridine derivatives.

When simple aldehydes (e.g. formaldehyde) are heated with solutions of substituted phenylene diamines acidified with hydrochloric acid, intermediates of the diamino-phenylmethane type (III) are formed. The aldehyde condenses at the centres of high electron density ortho to the amine group instead of condensing with the amine (Cp. secs. 1 and 3). Hydrochloric acid can cyclicise III to the acridan (IV) at ~~the~~ comparatively high temperatures. The latter is susceptible to aerial oxidation and forms a fluorescent dye, e.g. acridine orange, V (Biehringer, 1896).



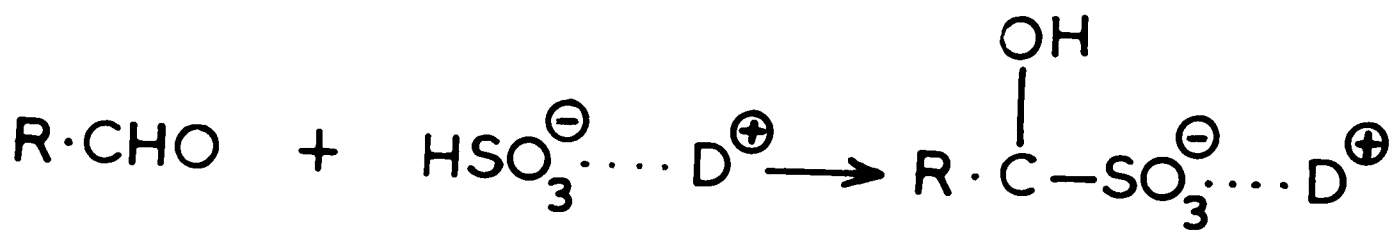
III IV V

If the amine solution contains sulphur dioxide, two other reactions can take place:

(5) a pseudo-Schiff reaction.

A mechanism for this reaction has been proposed earlier (sec. 12.7/10). It depends essentially on an electrostatic linkage between the basic amine (D^+) and

an aldehyde - sulphur dioxide conjugate.



This reaction works with aminoacridine dyes because they possess centres of high electron deficiency (e g. at their ring nitrogen atoms). However, the simple homocyclic amines (e.g. the phenylene diamines) only carry relatively small positive charges (c. .I) and so are less likely to take up sulphurous acid or be adsorbed onto the aldehyde-sulphur dioxide complex. Moreover the synchronising effect of the electron shifts by the amine groups in I, for example, makes them less likely to take up protons than the p- isomer. Consequently the probability is reduced for such amines to form positively charged anilinium ions which might form part of a fluorescent conjugate.

(6) A reduction in the browning reaction with a simultaneous increase in fluorescence.

The mechanism by which sulphur dioxide does this in the reaction between reducing sugars and amine compounds is incompletely understood (Ellis, 1959). The compounds responsible for the fluorescence are unknown except that they are precursors of the brown pigments (Patton and Chism, 1951). It is generally supposed that N-glycosylamines are formed at some stage (Iacobellis, 195[illegible])

At one time it was thought that one of the isolated reaction products, furaldehyde, might be significant because of its ready combination with sulphur dioxide (Ingram and Vas, 1950). However, it gave no fluorescent products with amino acids (Swain and Pardee, 1956).

The fluorescence output of the reaction reaches a maximum and then decreases, (Overby and Frost, 1952) and has been correlated in one instance with the production of 5- (hydroxymethyl)- 2- furfuraldehyde (Nomura, 1955) which is now known not be be the main factor in the browning reaction itself (Yoshiro and Nomura, 1960). If this compound is the principle agent in the fluorescent Maillard reaction, it is not surprising that the quite different dialdehydes exposed in the periodate oxidation of mucins do not form fluorescent products in the same way.

Conclusion.

Although a large number of fluorescent reactions can take place between aldehydes and amines in vitro, very few can ever be applied to the histochemical detection of aldehydes engendered in tissues. The only amines suitable for fluorescence histochemistry are the chemically active ones whose aromatic moiety is intrinsically fluorescent. The hydrazines derived from salicylic acid are the best examples of this type available at the present time.

22. Confirmatory tests for aldehyde groups

The presence of aldehydes engendered in tissues has been confirmed by showing that they no longer react

with potentially chromogenic or fluorogenic reagents after they have been reduced to primary alcohols, or alternatively, after they have been blocked with specifically reactive compounds.

The reduction of aldehyde groups is discussed in sec. 3.14/4.

Blocking reagents can be used to block pre-existing aldehydes before exposing others, as in the plasmal reaction (Cain, 1949; Belt and Hayes, 1954), or to block aldehydes selectively in the presence of the chemically similar ketones. Sulphanilic acid has been used for the latter in the histochemical demonstration of ketosteroids in the presence of lipid aldehydes (sec.15.4).

10% solutions of aniline dissolved in 10% hydrochloric acid (Lillie, 1954) or glacial acetic acid (Lillie and Glenner, 1957) have been used as the routine reagent for reversibly blocking aldehydes. Sections were treated with this reagent for 2 - 6 hours at room temperature. These conditions are unlikely to lead to the extraction of the aldehyde components from the tissue sections.

Other blocking reagents which have been used in histochemistry are sodium bisulphite (Feulgen and Voit, 1924), cyanides (Gomori, 1953), semicarbazides (Bennett, 1940), thiosemicarbazides (Pearse, 1953) and hydroxylamine (Danielli, 1949). However, the conditions required

by some of these reagents may also lead to the simultaneous extraction of the aldehydes (vide sec 12.7/3). Alternatively the block may be easily reversed by reagents used for testing the efficacy of the blocking procedure. For these reasons, none of the reagents named in this paragraph are reliable.

Phenylhydrazines also condense with tissue aldehydes to form phenylhydrazones which are resistant to further oxidation (Bennett, 1940; Danielli, 1949). The only important difference between phenylhydrazine and aniline is that the former condenses equally well with aldehydes and ketones, whereas the condensation of the latter with some ketones may be sluggish.

Dimedone (5:5- dimethylcyclo-hexane-1:3-dione) forms stable conjugates with some tissue aldehydes. Cain (1949) presumed that both ketones and aldehydes would react, although Wild (1947)had stated earlier that no condensation would take place with the former.

Pearse (1953, 1960) found that dimedone rapidly abolished the Schiff reaction of the periodate-engendered aldehydes in the gastric mucosa, connective tissues and muscle (except for glycogen and mast cell granules), but not the mucinogeous granules of the mucosa. The conditions of high aqueous acidity and temperature under which he used this reagent are likely to extract the aldehyde substrate from the tissue and would give the same end result of negative Schiff staining.

Staple (1957) therefore recommended using solutions of dimedone in 80% alcohol to avoid this possibility. Bulmer (1959) has suggested that dimedone blocks the dialdehydes engendered from the oxidation of all neutral mucopolysaccharides with periodic acid except those from glycogen and therefore the reagent could be used in a specific test for that carbohydrate. In view of the discrepancy of the results cited earlier, dimedone cannot be used as a universal blocking reagent for aldehydes or even for demonstrating glycogen specifically.

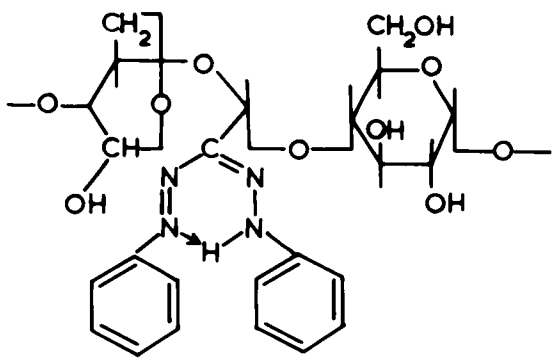
23. The Formazan Reaction

One of the principal points put forward in sec. 12.4/1 is the explanation for the failure of some periodate-engendered "dialdehydes" to react with pseudo-Schiff's reagents because of their transformation into unreactive hemiacetal and hemialdal forms. This explanation could be confirmed by showing that those sites which react with hydrazides but not with Schiff-type reagents also do not form formazans. Only the phenylhydrazones of aldehydes containing active methine groups can couple with diazonium salts and ultimately form formazans (Mester, 1955). The phenylhydrazones derived from hemiacetals (Mester and Major, 1955a; Guthrie and Honeyman, 1959) or from ketones (Hünig and Boes, 1953) cannot couple. The structures of the formazan (I) derived from oxidised starch and diazotised aniline

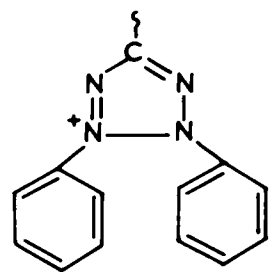
(Mester, 1955; Mester and Major, 1955 a, b, 1956) is shown in Fig. 70.

Formazans were first prepared by Pechmann (1892) and Bamberger and Wheelright (1892), but their importance in carbohydrate research has only been recognised comparatively recently (Mester, 1958). The formazan reaction was introduced into histochemistry by Seligman et al., (1949) as part of the synthesis of an iodinated tetrazolium salt. Seligman and Ashbel (1951) were unable to obtain positive results with the lipid aldehydes in formalin-fixed sections of adrenal using tetrazotised dianisidine as the coupling agent. Similarly Pearse (1960) was unable to obtain a formazan with formalin-fixed adrenals or with the aldehydes exposed by the Feulgen hydrolysis of DNA, but he was able to demonstrate the "dialdehydes" as formazans in the mucin granules of the gastric mucosa previously oxidised by periodic acid.

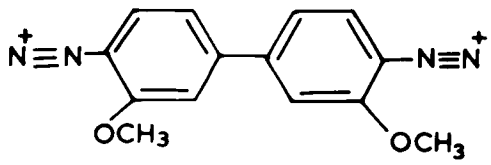
I have never been able to synthesise formazans *in situ* by coupling the "dialdehydes" of neutral muco-polysaccharides oxidised by periodic acid with the stable diazonium salts (e.g. II or III) which are available commercially. This failure was thought at first to be due to the ortho- or meta- substituted groups of the diazonium salt which sterically hinder the approach to the methine group of the hydrazone zone, but this



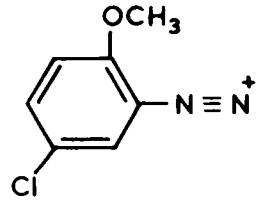
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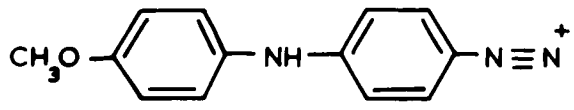
V



II



III



IV

Fig. 70.

explanation was vitiated by finding that neither the diazonium salt of 4- amino- 4[1]- methoxydiphenylamine (IV) nor freshly prepared diazotised p- nitroaniline would couple. An alternative explanation for this failure is that the mucin phenylhydrazones have mutarotated to a form unsuitable for coupling with diazonium salts (Mester, 1958). On the other hand, it is not known whether phenylhydrazones can couple with such salts in vitro: coupling with compounds other than diazotised aniline has only been reported for diazotised o-, m-, and p- aminobenzoic acids (Mester and Moczar, 1958) and diazotised sulphanilic acid (Rosenthaler and Vegezzi, 1954).

Positive reactions were, however, always obtained in situ by coupling "dialdehydes" with diazotised aniline in cold aqueous pyridine, provided the following experimental conditions were observed;

(a) the aldehydo-phenylhydrazone was prepared by immersing sections, previously oxidised in periodic acid, in fresh solutions of phenylhydrazine, of half the strength used by Pearse (1960), for 2 - 6 hours at room temperature. Very little formazan was ever produced when oxidised sections were treated with the hot solutions recommended by Pearse (1960). Barry and Mitchell (1954) found similar difficulties in vitro which they attributed to the decomposition of the

phenylhydrazones by the excess acid at the comparatively high temperatures used.

(b) the phenylhydrazones are coupled with an aqueous solution of diazotised aniline which has been added to cold pyridine not more than 30 minutes previously. Presumably in older solutions the alkaline conditions convert the diazonium salt to a variety of rearrangement products. This point has not been mentioned in Mester's papers.

The colour of the aniline - formazans derived in situ from neutral mucopolysaccharideswhich had been oxidised by periodic acid were always yellow or yellowish-orange, unlike the deep red colours of the formazans synthesised in vitro (Mester, 1955; Mester and Major, 1956). Unfortunately the contrast of the formazan was poor in sections examined in visible transmitted light. Furthermore these formazans did not fluoresce which is surprising in view of their apparently favourable structure and their high absorption in the ultra-violet and visible regions. The failure to couple with stable diazonium salts was therefore especially disappointing as it was hoped that their formazans would be fluorescent by analogy with the thiol- hydroxynaphthylisomaleimide-diazonium salt complexes which can be formed in tissues (Pearse, 1960). However, it is known that ultraviolet light can reversibly alter the visible colours of the

formazans; this has been attributed to changes in the equilibria of their cis-trans configurations (Hausser et al., 1949, 1951; Foffani and Stupiarch, 1953), from which it can be inferred that formazans do not possess the rigid extended planar structure essential for a compound to fluoresce. The structurally rigid tetrazolium salts (V) formed by the oxidation of formazans by N- bromosuccinimide (Mester and Moczar, 1956) also did not fluoresce in situ.

The only way of improving the visible contrast or inducing fluorescence from the formazans in situ is to use an aniline-type coupler containing a functional group para to the diazonium group which can then be demonstrated by another histochemical method.

This approach has been tried without success using solutions of freshly-prepared diazotised p- nitroaniline and p- aminoacetophenone on the phenylhydrazones of periodate-oxidised glycogen in liver sections. The former did not condense at all (exp.sec. 12. 16). The latter, however, did form a visible pale yellow conjugate which did not seem to be confined to the glycogen sites. The whole section appeared to be coloured. When such sections were treated successively with solutions of salicyloyl hydrazide and zinc acetate (exp.sec. 7.1 stages 6 - 10), no fluorescent complex was formed, as might have been expected with the p-methyl ketone group

of the formazan complex. This failure might be attributed to the inability of the glycogen phenylhydrazone to form a formazan with diazotised p- aminoacetophenone in the first stage of this experiment.

Experimental Section 12. vic-Glycols

1. Periodic-Acid-Schiff Method (PAS, f and v).

Preparation of Schiff's reagent (after de Tomasi, 1936).

1 g of basic fuchsin was dissolved in 200 ml boiling distilled water. The mixture was shaken for 5 mins, cooled to 50° and filtered. 10ml 2 N hydrochloric acid and 1 g sodium metabisulphite were added to the filtrate at room temperature. This solution was stored overnight. The excess fuchsin was removed by shaking with sufficient activated powdered charcoal (about 2 g). The colourless filtrate from this mixture was stored at 0 - 4° C, but was allowed to reach room temperature before use.

Method. (after McManus, 1946, and Hotchkiss, 1948).

(1) Bring sections to water.

(2) Oxidise with 1.% periodic acid for 8 mins.

(3) Rinse in SO_2-water (0.1% thionyl chloride) for 1 min. to destroy excess periodic acid.

(4) Rinse in Schiff's reagent for 20 - 40 mins.

(5) Rinse twice in SO_2-water, 1 min. each.

(6) Rinse in water, take through alcohols, clear in xylene and mount in DPX.

Result

PA+ sites are stained in various shades of purplish red when observed by visible light. When the same

sections are examined for fluorescence, the PA+ areas fluoresce a characteristic purple colour which does not always contrast well with the blue fluorescent background due to colourless fuchsin adsorbed onto tissue basophils.

The PAS method for visible light microscopy serves as a control for the fluorescent techniques.

2. Pseudo-Schiff methods (f)

Preparation of pseudo-Schiff solutions

0.01% and 0.1% aqueous or 30% alcoholic solutions of various aminoacridine dyes (e.g. acridine yellow, acriflavine or coriphosphine) were saturated with sulphur dioxide by adding 2-3 drops of thionyl chloride to 100 ml. of the dye solution. The pH was adjusted to 2.5 - 3.5 with dilute sodium hydroxide. The solutions were filtered before use, but were not decolourised with charcoal as recommended for Schiff's reagent.

Method

(1) Dewax and rehydrate sections until their basophilia is abolished. Treatment with 2% thionyl chloride in ethyl alcohol for 4 hours is usually sufficient.

(2) Take to water

(3) Oxidise with 1.2% periodic acid for 8 mins.

(4) Wash with SO_2-water (0.1% thionyl chloride) OR water for 3 rins.

(5) Rinse briefly in water

(6) Immerse in either (a) pseudo-Schiff reagent,

or (b) dye solutions containing no SO_2, for 20-90 mins.

(7) Wash twice in water

(8) Mount in water and examine for fluorescence.

Results

The PA+ areas usually fluoresce yellow or blue depending on the concentration of the pseudo-Schiff solutions or the particular mucin under examination. The reactive areas are not always as extensive as indicated by the salicyloyl hydrazide technique (exp. sec.12.14). Acriflavine - SO_2 appears to be the most sensitive reagent: it gives a greenish-yellow fluorescence with reactive sites.

Controls with unmethylated sections show that it is difficult to distinguish the PA+ sites from tissue basophils, particularly nucleic acids (Cp.Kasten, 1958a).

At least one periodate-oxidised mucin gives a distinctive red fluorescence with coriphosphine or acridine orange alone (6b), which is not exhibited if the preliminary SO_2 treatment is omitted.

3. Experiments with aminoacridines (f)

Fluorescence staining was hardly ever observed in the mucin sites of sections which were oxidised with periodic acid and subsequently immersed in 0.01% and 0.1% solutions of aminoacridine dyes or the diphenylmethane dye, auramine O

Unsuccessful attempts were made to form a fluorescent product under conditions known to give anils with 2:8-di-

aminoacridines *in vitro*; sections oxidised by periodic acid were incubated in 0.1% solutionsof acridine yellow or coriphosphine dissolved in 70% alcohol containing a few drops of piperidine for 6 hours at 60°C.

4. N-(1-Naphthyl)-ethylene diamine (f)

0.1% solutions of the diamine in M/10 phosphate buffers gave a blue fluorescence after 18 hours treatment with glycogen in liver sections previously oxidised by periodic acid. The solution at pH 6.1 produced a stronger fluorescence than the solution of pH 3. When sodium metabisulphite and dilute hydrochloric acid were added to a 0.1% solution of the diamine, the resulting mixture induced an intense, bright blue fluorescence in oxidised liver sections.

5. m-Phenylene diamine (f)

0.5% solutions of commercial samples of m-phenylene diamine hydrochloride (m-PDA) which were acidified with hydrochloric acid to pH 3 produced only a weak, diffuse blue-green fluorescence after 1 hour in sections of liver oxidised with periodic acid.

In view of the successful applications of this reagent on paper chromatograms reported by Chargaff *et al.* (1948), *in vitro* experiments were carried out with purified samples of diamine. The brown, commercial sample was warmed with an aqueous suspension of activated

charcoal, acidified with dilute HCl and filtered. Small yields of colourless rhombic crystals were obtained from the filtrate cooled to 5° for 24 hours.

Spots of glucose solution on filter paper were subjected to the following tests:

(a) Oxidised with one drop of PA for 5 mins., followed by a 1.5% solution of m-PDA in 70% alcohol, and heated to 60° for 15 mins.

(b) as (a), omitting PA oxidation.

(c) Oxidised with PA, followed by 1.5% solution of m-PDA in M/20 phosphate buffer (pH 6.8) containing 1.5% w/v sodium metabisulphite, and heated to 60° for 15 mins.

(d) as (c), omitting the PA oxidation.

The spot (a) produced a brown, non-fluorescent stain (i.e. a "browning reaction"), while the control (b) gave some yellow fluorescence as also did reaction (d).

The pseudo-Maillard reaction (c) gave a yellow fluorescence that was much more intense than either (b) or (d).

6. <u>m- or p-N, N-dimethylphenylene diamine</u> (f)

(After Spicer and Jarrells, 1961; Spicer, 1961)

A 0.1% solution of either isomer in M/10 phosphate buffers at pH 3 or 6.1 produced, at most, a weak, diffuse blue fluorescence even after 18 hours condensation

with glycogen in liver sections oxidised by periodic acid. The blue fluorescence was enhanced when thionyl chloride was added to the solution of the m-isomer.

7. p-Aminohippuric acid (f)

A 1% solution gave a strong blue fluorescence after condensation for 2 hours with liver sections containing glycogen which has been previously oxidesed by periodic acid.

8. 5,-aminosa_licylic acid (f)

Method

(1) Bring sections to water.

(2) Oxidise with 1.2% periodic acid for 8 mins.

(3) Rinse in wa_ter

(4) Immerse in 0.5% solution of 5-aminosalicylic acid, acidified with dilute HCl (pH 3), for 1 hour.

(5) Wash with water, take to alcohol, dry and examine for fluorescence.

Result

PA+ sites exhibit a bright yellow-green fluorescence, tha_t fades after long exposure to air.

9. p-Aminophenol (f)

A filtered, acidified 0.5% solution (pH 3) was used for 1 hour on liver sections oxidised with PA. A bluish fluorescence with a slight greenish tinge was produced.

10. p-Aminobenzoic acid and basic dye sequence (f)

Sections oxidised by PA were condensed with 5% p-aminobenzoic acid, dissolved in 0.5N HCl, for 2 hours. This was followed by staining in 0.1% coriphosphine for 15 mins. The sections were washed twice in water, once in 30% alcohol, dried and examined for fluorescence.

Some brown fluorescence was observed in PA+ sites, although it was sometimes indistinguishable from the fluorescence of the nuclei.

11. Sulphanilic acid and basic dye sequence (f)

Engendered aldehydes were condensed with a saturated solution of sulphanilic acid in N HCl for 2 hours. The sulphonic acid group of the sulphanilic acid conjugate was demonstrated by a technique suitable for acid mucopolysaccharides. When the sections were subsequently stained with o.1% coriphosphine and washed with 30% alcohol, a brown fluorescence appeared in some PA+ sites.

12. p-Phenylene diamine and Danielli sequence (v)

Method

(1) Bring sections to water

(2) Oxidise with 1.2% periodic acid for 8 mins.

(3) Rinse in water for 2 mins.

(4) Immerse in 5% p-phenylene diamine in 0.5 N HCl for 3 hours.

(5) Rinse in water

(6) Diazotise the diarine conjugate with fresh nitrous acid at 0-5 °C for 30 mins. The nitrous acid is made by slowly adding a cold solution of fresh 5% sodium nitrite to three times its volume of cold N HCl, keeping the temperature below 5°C.

(7) Wash briefly in cold water

(8) The diazonium conjugate is coupled with a fresh saturated solution of H-acid (1-amino-8-naphthol-3:6-disulphonic acid) in 2% sodium barbitone (pH 9) at 0-4°C for 15 - 30 mins.

(9) Wash in water, take through the alcohols, clear in xylene and mount in DPX.

Result

An intense reddish-purple colour is formed in PA+ after when the sections are examined in visible light. The remainder of the section is faintly stained.

No fluorescent colours were developed when the phenolic fluorescent dyes, fluorescein or methyl β-umbelliferone (0.1% in barbitone buffer, pH 9) were substituted for H-acid. Similarly amine dyes, such as coriphosphine or phosphine (0.1% in dilute acetic acid), failed to couple.

(13) Acridine hydrazines (f)

0.1% solutions (pH 1.5 - 2.8) of hydrazines derived from acridine yellow, phosphine or coriphosphine always formed fluorescent derivatives after reacting with enzyme red aldehydes for 18 hours at 37°. The relatively weak fluorescence and poor contrast of the fluorescent derivative, however, did not commend the general use of these hydrazines.

Preparation of acridine hydrazines.

0.2g of an aminoacridine dye was dissolved in 1 ml concentrated HCl and 3 ml cold water, and diazotised with 0.25g sodium nitrite dissolved in 2 ml cold water. 2 g sodium sulphite and 0.25g sodium carbonate, dissolved in 6 ml water, were then added and the mixture stirred continuously for 10 mins. at 30°, followed by the dropwise addition of thionyl chloride until the mixture was saturated with SO_2. The mixture was stirred for a further 5 mins., warmed slowly to about 80° on a water bath and filtered. The filtrate was acidified with 3 ml concentrated HCl and cooled to room temperature.

The following sequence of reactions took place:

$$R\cdot NH_2 \xrightarrow{HNO_2} R\,N_2Cl \xrightarrow{Na_2SO_3} R\cdot N_2SO_3Na$$

$$R\cdot N_2SO_3Na \xrightarrow{SO_2\,(Redn.)} R\cdot NH\cdot NH\cdot SO_3Na \longrightarrow Cl)$$

(14) Periodic Acid - Salicyloyl hydrazide method (PA - SH, f).

Method

(1) Bring sections to water

(2) Oxidise in 1.2% periodic acid for 8 mins.

(3) Wash in water, 2 mins.

(4) Immerse in 1% salicyloyl hydrazide (SH) at pH 3 (in 5% acetic acid) for 20 - 40 mins. A brown, resinous precipitate is slowly deposited in the SH solution after a few weeks, but the effectiveness of the reagent remains unaltered.

(5) Rinse once in water.

(6) Rinse in a fresh dilute solution of trisodium penta-cyano-ammine ferroate (PCAF) until any purple colouration disappears.

(7) Wash twice in distilled water

(8) Counterstain the acidophilic background with Solochrome Black-Alum complex (exp.sec. 3.8) for 10 mins.

(9) Rinse once in water.

(10) Take through the alcohols, clear in xylene, and mount in Fluormount.

Result

PA+ sites exhibit an intense blue fluorescence which contrasts against the red fluorescence of the protein background.

(15) Acyl hydrazides (f)

1% solutions of 7-methoxycoumarin-4-acetyl-hydrazide or 7-methoxy-4-methylcoumarin-3-carboxyhydrazide acidified with acetic acid sometimes yielded blue or purple fluorescent products with periodate-engendered aldehydes after 30 mins. condensation.

(16) Formazan Formation

Sections, taken to water, were oxidised with 1.2% periodic acid for 8 mins. and washed with water. The engendered "dialdehydes" were converted to their phenylhydrazones by incubating them in a fresh 5% solution of phenylhydrazine in 10% acetic acid for 2 - 6 hrs.

The hot 10% solutions of phenylhydrazine in 20% acetic acid which have been recommended By Pearse (1960) tended to detach sections from the slide as well as causing some distortion in the tissue structure; in addition they sometimes gave negative formazan reactions with test liver sections as might be expected if the excess acetic acid broke down the phenylhydrazones which were initially formed.

The phenylhydrazones were coupled with freshly-prepared diazotised aniline in pyridine at 0° for 10-30 mins. The diazotisation mixture was made up as follows: to 3ml aniline dissolved in 9 ml water and 9 ml concentrated hydrochloric acid cooled to 0°, 2.5g sodium nitrite dissolved

in 12ml cold water were slowly added, keeping the temperature below 5°. Half of this mixture was added to 20 ml cold pyridine, and used within 30 mins.

A deep yellow formazan was formed from liver glycogen. It remained fast towards the alcohols prior to mounting the sections in DPX. The periodate-reactive neutral mucopolysaccharides of rabbit umbilical cord, cock's comb and guinea-pig gut gave formazans with an orange hue. The weak bluish-green fluorescence of the formazans was not very distinctive and could not be used as a reliable test. The alcohol fastness of the formazans was in contrast with the ready extraction of the simple phenylhydrazones.

The intensity of the formazan colour could be increased by immersing the sections containing the phenylhydrazones in 1% alkaline ascorbic acid (pH 8) for 10 mins. before the coupling stage.

The formazans derived <u>in situ</u> from diazotised aniline in pyridine were oxidised to non-fluorescent pale-yellow or colourless tetrazolium salts by immersion in 2% N- bromosuccinimide in methanol for 2 hours.

Fresh, cold 0.3% solutions of stable diazotates in a 1 : 3 water-pyridine mixture used for 5 - 30 mins. (Pearse, 1960) did not couple with the phenylhydrazones derived from liver glycogens oxidised by periodic acid as seen by the appearance of sections examined in both

the visible light and fluorescence microscopes. The following stable diazotates were tested (purchased from L.Light & Co.):tetrasized O-dianisidine (Fast Blue B Salt), diazotised 4-chloro-o-anisidine (Fast Red RC salt) and the diazonium salt of 4-amino-4^{1}-methoxy-diphenylamine (Fast blue VB salt).

17. The detection of N-acetylamino hydroxyl groups

Method

(1) Take sections to water.

(2) Rinse in 30% ortho-phosphoric acid for 1 min.

(3) Rinse in 60% phosphoric acid for 90 mins. (to complete the hydrolysis of N-acetyl groups)

(4) Immerse in 5% periodic acid dissolved in 60% phosphoric acid (= Warren's reagent, 1959) for 20 mins.

(5) Wash in 60% phosphoric acid for 1 min.

(6) Wash in 30% phosphoric acid for 1 min.

(7) Wash in water for 1 min.

(8) Wash in aqueous solution (10%) of ethylene glycol (to destroy excess periodic acid).

(9) Wash in water for 1 min.

(10) Incubate in 0.6% thiobarbituric acid at the temperature of a boiling water bath for 15 mins.

(11) Wash in water for 1 min.

(12) Examine for fluorescence as a water mount.

Result

Using test tissue sections of mouse salivary gland which were known to contain sialic acid, a diffuse

and non-discrete fluorescence was observed in what roughly corresponded to the chromidial sites.

The intensity of the final fluorescence was very much less if stage (3) was omitted. This stage is omitted in Warren's (1959) procedure for sialic acids _in vitro_.

Section 13

Hydroxyl Groups

1. Introduction

The only way to demonstrate hydroxyl groups in tissues, irrespective of whether they are primary(1^{o}), secondary (2^{o}) or tertiary, is to replace their acidic hydrogen atoms with an anionic group, such as sulphate or phosphate, which can be subsequently detected with a basic dye. The acidic hydrogen atoms of other groups, such as phenols, thiols and amines, may also be substituted and it is usually necessary to consider the possibility of these reactions interfering with the specificity of the reaction for hydroxyl groups.

Most reagents for sulphating hydroxyl groups are so acidic that they sometimes hydrolyse O- sulphates off again as soon as they are formed (Lewis and Grillo, 1959). Glycogen, for example, is especially difficult to sulphate *in situ* with acidic reagents. This secondary complication can be avoided by carrying out the sulphation in a non-acidic environment, such as by exposing dry sections to sulphuryl chloride vapour (Lewis and Grillo, 1959; Grillo and Lewis, 1959). Glycogen is satisfactorily sulphated by this technique but the sulphation of other neutral mucopolysaccharides *in situ* is more complex.

Alternatively sulphation can be carried out in basic media. Strong bases cannot be used because desulphation

would occur simultaneously (Jorpes et al., 1950). The complexes formed between sulphur trioxide and certain Lewis bases sulphate the hydroxyl groups of polysaccharides very efficiently, including those of glycogen, when dissolved in weak bases such as pyridine or dimethylformamide (DMF). Unfortunately proteins are sulphated at the same time.

All the methods that have been tried for avoiding the sulphation of amine groups in tissues, by removing or blocking them beforehand, have had unsurmountable drawbacks. The sulphation of amine and hydroxyl groups can be distinguished, however, by preferentially removing the N-sulphate groups with methanolic hydrochloric acid (sec. 13.8) after sulphation.

The induced sulphates can be stained metachromatically with azure A or toluidine blue for visible light histochemistry. The sulphation-metachromasia technique seems to be more sensitive than the PAS method for demonstrating neutral mucopolysaccharides in tissues (Sulkin, 1960). The improvement may be real because of the relative insensitivity of Schiff's reagent or because some periodate-engendered "dialdehydes" are converted to unreactive hemiacetals and hemialdals (sec. 12.4/1). On the other hand, some of the additional basophilia after sulphation may be artefacts introduced by the sulphation of protein groups.

At the present time, sulphation basophilia techniques are not very useful in fluorescence histochemistry for two reasons:

(1) It is difficult to distinguish the fluorescence of the induced basophilia towards coriphosphine or acridine orange from that of the normal tissue. Attempts to avoid this complication by methylating the pre-existing basophils beforehand only prevented the attack of the sulphating agent on the hydroxyl groups. Presumably the protein amine groups are methylated to bulky polymethylamines which sterically protect the hydroxyl groups of the mucin.

(2) The fluorescence of coriphosphine adsorbed onto the induced sulphate groups is heavily quenched: a dull-brown fluorescence is always produced. The fluorescence of other dyes is quenched completely. The only way to obtain a satisfactory fluorescent appearance from sulphated sections is to use a dye whose fluorescent part is independent of its cationic centre. Unfortunately such dyes are not yet available (sec. 9.3).

The sulphation of hydroxyl groups.

The following methods for sulphating hydroxyl groups have been investigated:

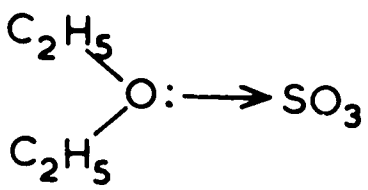
2. Concentrated sulphuric acid

Although the hydroxyl groups of simple alcohols (Sutur, 1944), phenols and proteins (Reitz et al., 1946) can

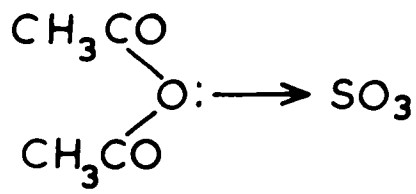
sulphated with concentrated sulphuric acid *in vitro*, there is no evidence that carbohydrates can be similarly sulphated. On the contrary, concentrated sulphuric acid degrades carbohydrates to hydroxy-furfuraldehyde and other aldehydes (Rice and Fischbein, 1956). It is therefore difficult to understand reports which claim that polysaccharides can be sulphated in tissues (Bignardi, 1940a; McManus and Mowry, 1952; Kramer and Windrum, 1953, 1954). The observed sulphation is more likely to occur in the protein constituents. The tyrosine residues, for example, may be both sulphated (to R.O. SO_3.H) and sulphonated (to HO.R.SO_3.H) the second process being the predominant one (Haworth and Lapworth, 1924). However Pearse (1960) argues that the negative metachromasia of collagen which has been sulphated in this way *in situ* disposes of the idea that protein hydroxyl groups play much part in sulphation-metachromasia, because this protein contains relatively large amounts of hydroxy-proline (Bowes and Kenten, 1948). Another possible explanation for the induced basophilia may involve the sulphation of protein thiol groups to thiosulphonates (Reitz *et al.*, 1946). As most proteins contain less than 1% thiol groups (Hess and Sullivan, 1943), this interference in the sulphation of tissues should be small, but it may be significant in certain tissues (Cp. sec. 12.7/7). The amine (Reitz *et al.*, 1946) and disulphide (Harris *et al.*, 1934) of proteins do not react with concentrated sulphuric aci

Kramer and Windrum (1953, 1954), Gomori (1954), and Mowry (1958) found that tissues were sulphated better if ether or acetic anhydride was added to the acid. The improvement is undoubtedly due to the formation of a Lewis addition complex between the sulphur trioxide present in the acid and the oxygen atoms of ether (I) or acetic anhydride (II). Aschan and Schwalbe (1932), called the ether complex "diethyl oxonium sulphate", and found that it was a good sulphating agent for monohydric alcohols. It only reacted with one hydroxyl group in polyhydric alcohols, including dextrose.

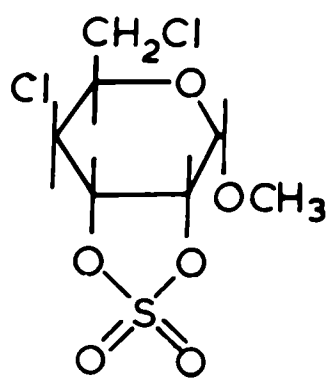
The structure of sections that have been sulphated with sulphuric acid-ether mixtures become seriously distorted. Moore and Schoenberg (1957) therefore recommended that sulphation should be carried out at low temperatures. However, much of the tissue distortion takes place, not during the immersion in the sulphating medium, but during the washing stages afterwards due to the large amounts of heat which are liberated locally in the tissue when the water mixes with the acid remaining in the section. The heat of dilution can be substantially reduced by washing the sulphated sections in appropriately graded mixtures of sulphuric acid and a suitable diluent, such as acetic acid (Lewis and Grillo, 1959). By combining the advantages of Moore and Schoenberg's technique with those of Lewis and



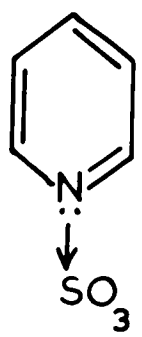
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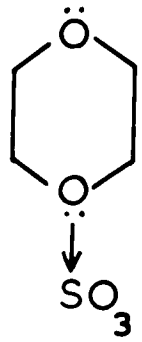
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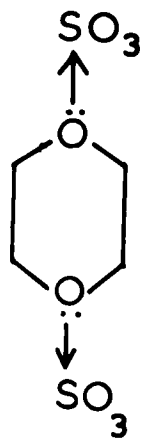
III



IV



V



VI

Grillo's, sections can be satisfactorily sulphated without distorting the cytological structure. The details are given in exp. sec. 13.1.

Nevertheless, sulphating reagents which contain excess concentrated sulphuric acid will always possess certain disadvantages for the exclusive sulphation of hydroxyl groups of carbohydrates in tissues:

(i) Nucleic acids are extracted simultaneously. The difference between the basophilia of sulphated sections and that of control sections cannot be wholly attributed to induced sulphates.

(ii) Carbohydrates are degraded to various aldehydes, including hydroxy-furfureldehyde (Rice and Fischbein, 1956), which may subsequently be amenable to sulphation.

(iii) Some polysaccharides, such as glycogen, may be sulphated initially by the ether complex, but the excess acid would hydrolyse the O-sulphates soon after they had been formed.

(iv) The glycosidic linkages of polysaccharides may be hydrolysed. The low molecular weight oligosaccharides which are formed will diffuse out of the section so that they are unavailable for sulphation.

(v) If sulphuric acid can behave like other oxidising mineral acids, such as nitric acid, the 1^{o} hydroxyl groups of polysaccharides may be oxidised to basophilic uronic acids.

Bignardi (1940, c, d and e) has expressed a substantially similar opinion.

(vi) The hydroxyl groups of serine, threonine, hydroxyproline and hydroxylysine residues of proteins may be sulphated. Reitz et al., (1946) found that these sulphates were unstable if kept in the acid mixture for too long so that their interference in the sulphation of tissues may not be very important. The thiol groups of cysteine may be sulphated.

3. Pyridine-sulphur trioxide complex

The feasability of using the pyridine-sulphur trioxide complex IV on tissues was first investigated by Kramer and Windrom (1953, 1954) in two guises. They called their first preparation "chlorosulphonic acid in pyridine" and the other "pyridine-sulphur trioxide complex". Both preparations contain essentially pyridine-sulphur trioxide complex mixed with variable amounts of contaminants, such as pyridine sulphate, pyridine chloride, and hydrochloric acid which can seriously affect their performance as sulphating agents. Sutur (1944) states that the complex formed between pyridine and chlorosulphonic acid is not the same as that formed between pyridine and sulphur trioxide directly, on the grounds that the former, a chloresulphonate, requires higher temperatures than the trioxide complex before it acts as a sulphating agent. It is now known, however, that the chloride ions in the mixture can

themselves alter the course and extent of the sulphation reaction (Guiseley and Ruoff, 1961).

Chlorosulphonic acid in excess dry pyridine will sulphate *in vitro*, not only the hydroxyl groups of carbohydrates, but also the functional groups of protein residues, including primary amide and thiol groups, with the exceptions of the imidazole, carboxyl and peptide groupings (Reitz *et al.*, 1946a). If water is present in the sulphating mixture, hydroxyl groups are not sulphated. (Baumgarten *et al.*, 1932; Reitz *et al.*, 1946a).

Kramer and Windrum (1953, 1954) aclaimed the chlorosulphonic acid reagent for producing very little distortion of tissue sections and for giving an excellent metachromatic reaction with the ground substance of connective tissue and with glycogen, but in my hands, the reagent did not give very good results, particularly if it was more than 24 hours old. The mixture takes up moisture very quickly, and no doubt the free acids which are subsequently formed promote the desulphation and distortion of the tissue. It is difficult to understand how Kramer and Windrum obtained good results, particularly when they obtained less satisfactory results with their second preparation containing less hydrochloric acid.

The preparation designated by Kramer and Windrum as pyridine-sulphur trioxide was first described by Baumgarten

(1926) and used by Duff (1949) for the sulphation of monosaccharides *in vitro*. The complex was synthesised and used in almost the same way as the preceding preparation, except that the solid was filtered from the mixture, washed with cold chloroform to remove pyridinium chloride (Baumgarten and Marggraff, 1931), and resuspended in dry, excess pyridine. In my hands, such preparations usually gave better results than the chlorosulphonic acid mixture but nevertheless, at least one preparation failed to sulphate glycogen in liver sections. This failure may be due in part to the inclusion of water and hydrochloric acid in the complex. These impurities are best avoided by distilling dry sulphur trioxide directly into dry pyridine. If the complex is prepared in this way, it can be stored without deterioration at 5° in air-tight containers and has been successfully used for the selective sulphation of primary hydroxyl groups (sec. 14).

When carbohydrates are treated with chlorosulphonylating reagents in excess pyridine, three important side-reactions occur in addition to the main one. Some of the hydroxy-sulphonyl esters initially formed can be replaced with chlorine or pyridinium ions or they can be eliminated during intramolecular ether formation (Ferns and Lapworth, 1912; Reynolds and Kenyon, 1951). As there is a close parallel between the chemical behaviour of the sulphates

and sulphonic acid esters of carbohydrates (Percival, 1949), the induced sulphates in tissues may undergo similar fates during the sulphation process. This may also explain why even under optimal conditions *in vitro*, only about 30% of disulphate can be obtained from glucose with the pyridine complex (Dodgson and Spencer, 1954).

The variability and lack of specificity of pyridine-sulphur trioxide complexes militates against their use as routine reagents for the detection of hydroxyl groups of polysaccharides in tissues.

4. Chlorosulphonic acid

Kramer and Windrum (1954) claimed that chlorosulphonic acid mixed with a suitable diluent, such as acetic anhydride and chloroform, was a satisfactory sulphating reagent. Its main disadvantage is the rapidity with which the reagent loses its potency.

In my experience, chlorosulphonic acid does not sulphate glycogen very satisfactorily. This failure was not unexpected in view of the acidity of the medium. Furthermore it is well established that chlorosulphonyl compounds or even chloride ions can displace 2^{o} hydroxyl groups and substitute chlorine in their place (Roberts, 1957; Guiseley and Ruoff, 1961). Chlorosulphonic acid itself preferentially sulphates primary hydroxyl groups *in vitro* (Saito *et al.*, 1961) particularly at low temperatures. For these reasons,

chlorosulphonic acid cannot be recommended as a reliable sulphating agent for hydroxyl groups.

5. Sulphuryl chloride.

When dry sections are exposed to sulphuryl chloride vapour, glycogen is sulphated rapidly and specifically (Grillo and Lewis, 1959; Lewis and Grillo, 1959). Isolated neutral and acid mucopolysaccharides in the form of dried smears can also be sulphated rapidly in this way. They subsequently exhibited different fluorescence spectra in the solid state after they had been stained in 0.01% solutions of acridine yellow dissolved in 30% alcohol. Heparin exceptionally did not take up acridine yellow either before or after sulphation. The spectra were unaffected by changes in the pH of the dye solution. No noticeable difference could be detected in the fluorescence spectra of sulphated mucin smears stained in 0.01% coriphosphine. The differences in the fluorescence spectra of solutions of these dyes containing dissolved acid mucopolysaccharides were the reverse of those observed in the solid state. Kuyper (1957) found that the fluorescence spectra of coriphosphine mixed with acid mucins varied uniquely with pH, but solutions of acridine yellow had the same spectra irrespective of the pH or mucin content (Kuyper, 1962).

On the other hand, neutral mucopolysaccharides in situ are not rendered basophilic by sulphuryl chloride vapour,

with the notable exception of glycogen. Mucins "sulphated" in this way took up neither azure A nor coriphosphine as shown by the absence of visible staining and fluorescence respectively, nor could they be oxidised by periodic acid. This behaviour is consistent with the chlorination of some carbohydrate (1^{o}) hydroxyl groups and the formation of non-basophlic cyclic sulphates with others (2^{o}) in a way analogous to what happens *in vitro* when simple carbohydrates are treated with solutions of sulphuryl chloride (Bragg *et al.*, 1959).

Glucopyranoside, for example, gives the derivative III (Jones *et al.*, 1960). The cyclic sulphate group can be opened up with a base *in vitro* (Helferich *et al.*, 1923), but a similar hydrolysis could not be established *in situ*: there was no fluorescence in the neutral gastric mucin sites in sections of rabbit stomach, for example, that had been exposed to sulphuryl chloride vapour, hydrolysed in 0.5% alcoholic potash for 10 minutes, taken down to 30% alcohol and stained in 0.01% coriphosphine. It is feasible that the O- sulphate groups, if formed at all, are hydrolysed off by the alkali as soon as the cyclic sulphate is opened up.

The differing behaviour towards sulphuryl chloride of mucopolysaccharides *in situ* from those *in vitro* remains an enigma. A preferential attack on the proteins associated with mucopolysaccharides *in situ* may be partly responsible.

Even glycogen *in situ* cannot always be sulphated, particularly if sections are exposed to sulphuryl chloride that has been left in a loose-capped Coplin jar for more than a week. Sulphuryl chloride rapidly takes up moisture, so that sections are in effect exposed to a mixture of sulphuryl chloride and moist hydrochloric acid. The latter would hydrolyse glycogen sulphates initially formed.

Sulphuryl chloride will also attack the thiol and disulphide groups of proteins (Speakman *et.al.*, 1938). Its solution in white spirit has been used commercially for imparting an unshrinkable finish to wool by breaking down the cystine disulphide bonds to S,S-disulphoxides, chlorides (R.Cl) and sulphonyl chlorides ($R.SO_2.Cl$). The thiol groups (of cysteine) are either oxidised to disulphides (Courant and Richter, 1885) or are chlorosulphonylated in exactly the same way as hydroxyl groups (Simon and Kunrath, 1959, 1961a and b). The thiosulphonyl chlorides which are formed readily react with water or alcohols to give the corresponding thiosulphonates.

$$R{\cdot}SH + Cl{\cdot}SO_2{\cdot}Cl \longrightarrow R{\cdot}S{\cdot}SO_2Cl + HCl$$

$$R{\cdot}S{\cdot}SO_2Cl + R'OH \longrightarrow R{\cdot}S{\cdot}SO_2{\cdot}OR' + HCl$$

An important application of its use for detecting low concentrations of thiol groups in tissues is described in sec. 12.7/7).

The complexity of the reactions of sulphuryl chloride with carbohydrates, coupled with the fact that it also reacts with thiols, militates against its use as a specific reagent for hydroxyl groups.

6. Dioxan-sulphur trioxide complex.

The dioxan-sulphur trioxide complex has a greater sulphating activity than the pyridine-sulphur trioxide complex, although it is less stable in solution than the latter (Sutur et al., 1938). The β-chloroethyl ether-sulphur trioxide has an even greater sulphating ability (Sutur and Evans, 1938).

The dioxan complex was prepared directly by distilling sulphur trioxide into a cooled mixture of chloroform and dioxan. The white solid complex consisted of both the mono- and di-adducts (V) and (VI).

A suspension of the adduct in chloroform induced some γ-metachromatic azurophilia in sections of liver after 3-5 minutes at room temperature. Better metachromatic staining could be induced by using solutions of the complex dissolved in dimethylformamide; the intensity of γ-metachromasia was moderate after 1 or 2 minutes treatment, but then the staining became orthochromatic for sulphation times in the range of

4-9 minutes. 10-15 minutes reproduced the δ-metachromatic staining in full intensity.

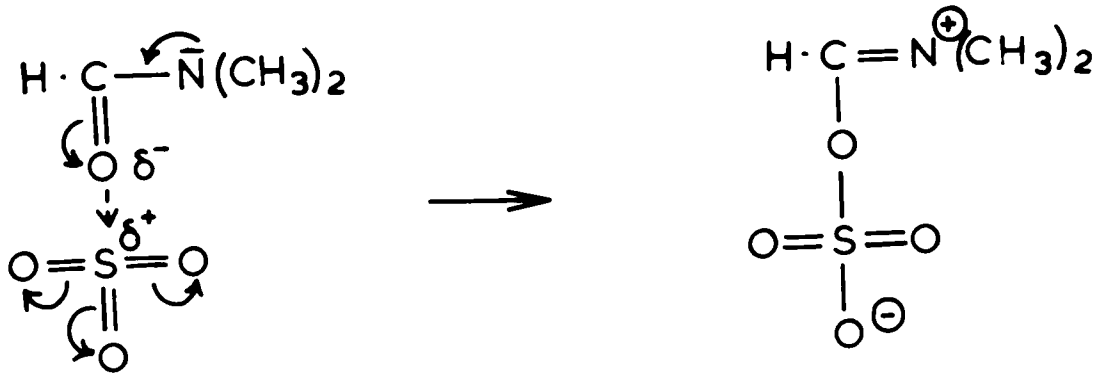
7. Dimethylformamide-sulphur trioxide complex

The advantage of the complex formed between sulphur trioxide and dimethylformamide (DMF) is that it is soluble in excess DMF and can be used at room temperature. It was first used for the sulphation of leuco vat dyes (Coffey et al., 1948, 1950) and in carbohydrate research, for the complete sulphation of chitosan (Wolfrom and Shen-Man, 1959).

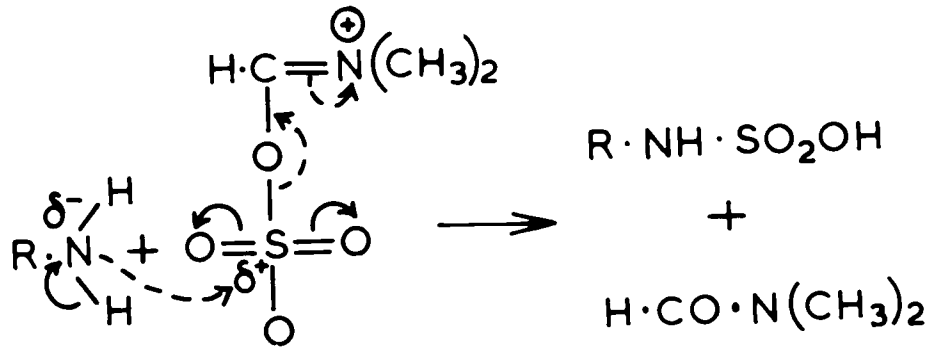
Two methods for preparing the complex were investigated. The conventional method of adding chlorosulphonic acid to dry DMF produced a clear, colourless solution which lost its sulphating power towards glycogen 24 hours after preparation. The loss of potency is probably due to the presence of hydrochloric and sulphuric acids. This prompted the preparation of a complex free from acids by distilling sulphur trioxide directly into dry DMF. A stable, yellow solution was obtained, which subsequently induced very strong δ-metachromatic azurophilia in sections of liver and of other tissues after 3-5 minutes at room temperature. The structure of the tissues was not altered, nor were the nucleic acids affected. The solutions retained their full sulphating abilities for 1-2 months, particularly if they were stored at 5^{o}.

The complex not only sulphates all the available mucin hydroxyl groups ih tissues, but also sulphates the amine and

hydroxyl groups of proteins. The same reaction takes place with amino-acids <u>in vitro</u> (Kenner and Steedman, 1952) according to the following mechanisms:



VII



Carboxylic acids can also form a 1:1 adduct with the DMF complex.

The dipolar complex (VII) therefore seems to be the best one for sulphating hydroxyl groups in tissues, provided the simultaneous sulphation of amine groups can be avoided or selectively reversed.

8. <u>The elimination of the interference due to amine groups in sulphation experiments</u>.

The obvious way to avoid the sulphation of amine groups is to remove them beforehand. This could be done by first diazotising the amine groups and then deazotising them with warm ethanol (sec. 3.13). Most tissues require 2-6 days before all their amine groups are ~~converted~~ removed. Unfortunately by this time, most neutral mucopolysaccharides will have been leached out of the section.

The chemical reactivity of amine groups can be "blocked" by specific fluorochemical reactions, such as the König-Sassi or alkaline salicylaldehyde reactions, (secs. 3.3 and 3.5), or by methylation (11.4). Although all the amine groups can be effectively blocked in this way, the hydroxyl groups of the tissue carbohydrates become sterically protected and are insensitive to the DMF complex.

Amine groups can be destroyed by oxidative deamination. Efficient oxidants such as hypochlorites or tungstate-hydrogen perioxide mixtures (secs.3.14 and 3.15) replace the amine groups with strong basophilic groups whose conjugates with coriphosphine have exactly the same intensity and colour as would be obtained ultimately with sulphated sections. Non-basophilic aldehydes can be obtained with amines with milder oxidants, such as ninhydrin, but unfortunately not all the amine groups are converted (as can

be shown by a positive König-Sassi reaction); a sufficient number of amine groups remain after oxidation to interfere in the induced sulphation of hydroxyl groups.

Because all methods for removing or blocking amine groups in tissues also remove or sterically block the chemical reactivity of polysaccharides before sulphation, the only way of eliminating the interference of the former is to remove them after sulphation, by invoking their substantially greater lability towards acids. The N-sulphates of natural sulphated mucopolysaccharides (e.g. heparin) can be hydrolysed in vitro even by weak acids, such as nitrous or acetic acids (Charles and Scott, 1936; Wolfrom et al., 1943a; Doherty et al., 1943). The comparative lability of N- and O-sulphates in synthetic sulphated polysaccharides was measured by Wolfrom, Shen and Summers (1953): N-sulphates were lost within 30 minutes after heating in 0.004 N hydrochloric acid at 95°, but O-sulphates required more than 12 hours.

0.1N aqueous hydrochloric acid at room temperature removed not only induced N-sulphates from sections after 16 hours treatment, but also extracted O-sulphates and DNA (Cp.sec.9.7/1). 1% (N/10) hydrochloric acid in methanol at room temperature removed only the N-sulphates and is therefore the reagent of choice. The γ-metachromatic azurophilia was completely abolished in liver sections that had been

incubated in diastase to remove glycogen, sulphated and left in methanolic hydrochloric acid for 4-16 hours. Fig. 71 shows the γ-metachromasia in the glycogen sites of sulphated sections hydrolysed for 4 hours in methanolic hydrochloric acid.

If sections were incubated in methanolic hydrochloric acid at temperatures higher than that of the room, the O-sulphates would be methylated and/or removed (sec. 9.15). Even prolonged treatment at room temperature may also lead to the desulphation of O-sulphates. Wolfrom and McNeely (1945) claimed that heparin, for example, could be completely desulphated *in vitro* after 24 hours treatment at 5° with 0.006 M hydrochloric acid in methanol. Danishefsky *et al.*, (1960), however, state that not all the O-sulphates of chondroitin sulphate can be removed by this process.

Fig. 71. Guinea pig liver. Fixed glycol ether. Dimethylformamide-sulphur trioxide complex; methanolic hydrochloric acid, 4 hours.; azure A. Transmitted white light. Water mount, X 180.

The γ-metachromatically stained sites containing glycogen can be distinguished from the nuclei which are stained blue.

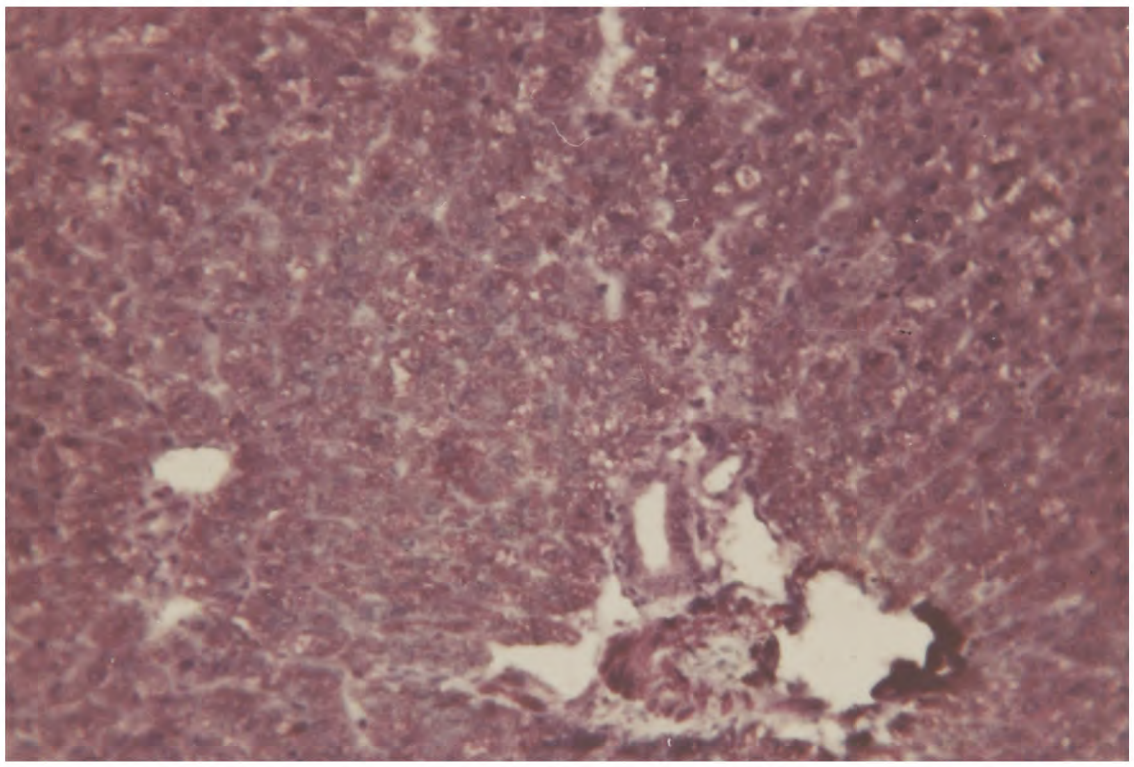


Fig. 71

Experimental Section 13. Hydroxyl Groups

The efficacy of the following sulphation techniques on sections containing neutral mucopolysaccharides was tested by observing the induced β-metachromasia towards azure A. Sulphated sections were stained in 0.02% solutions of the dye in 30% alcohol for 20 - 30 minutes.

In method (13.6), the sulphated amine groups were hydrolysed before staining, by leaving the sulphated sections in 1% hydrochloric acid in methanol for 4 hours.

Sulphation techniques

1. Sulphuric acid - ether complex (after Mowry, 1958).

The complex was prepared by adding 40ml fuming sulphuric acid carefully to 10 ml diethyl ether.

Method

Three variations were tried:

(a) Dry, dewaxed sections were sulphated for 2 - 4 mins. (after Kramer and Windrum, 1954). The excess acid was drained off and the sections washed in running tap water for 5 - 10 mins.

(b) Sections were taken to water, and then through various strengths of acetic acid to glacial acetic acid itself before sulphation in a 50 : 50 mixture of the complex and glacial acetic acid for 5 mins. at 0° (after Moore and Schoenberg, 1957). They were then washed in running tap water for 10 mins. and finally in distilled water.

(c) Sections were sulphated as in (a) or (b), but then washed with the following graded solutions of acetic acid: concentrated sulphuric acid: water by volume:- 6 : 3 : 1; 6 : 2 : 2; 5 : 1 : 4; 4 : 0 : 6 (after Lewis and Grillo, 1959). The sections were finally washed in running tap water and distilled water.

Results

Sulphated sections showed an increase in visible γ-metachromasia and a reddish-brown fluorescence in sites containing neutral mucopolysaccharides other than glycogen.

Method (b) distorted the tissue structure much less than method (a). With method (c), little distortion of the sections took place.

2. Pyridine - sulphur trioxide complex (after Kramer and Windrum, 1954).

Preparation (after Ricketts, 1952)

5.5ml chlorosulphonic acid were added dropwise to 50 ml ice-cold pyridine (previously dried over barium oxide) with vigorous stirring. Two liquid layers and a white solid were formed in the mixture.

Method

Two techniques were tried:

(a) Dry, dewaxed sections were immersed in the Rickett's mixture at 65 - 70^{o} for 2-5 mins., rinsed twice in acetone, hydrated to 30% alcohol and stained.

(b) The white solid was filtered from Rickett's mixture, washed several times with cold chloroform, and dissolved in dry pyridine as a 5% solution which was used immediately.

Results

A little (2+)% metachromatic azurophilia was induced after 2 mins. by method (a) on test sections of liver containing glycogen. No induced basophilia could be observed if sections were sulphated for more than 2 mins. The tissue structure was distorted to some extent.

Less distortion of the tissue structure occurred in method (b). Sections sulphated for 5 mins. were subsequently stained metachromatically (2+).

3. Chlorosulphonic acid (after Kramer and Windrum, 1954).

Sections were immersed for 5 mins. in a freshly-prepared mixture of :- 5 ml. chlorosulphonic acid, 12 ml chloroform and 10 ml glacial acetic.

Very little additional basophilic staining could be observed subsequently in sections of guinea pig liver.

4. Sulphuryl chloride (after Grillo and Lewis, 1959).

Method

Dry, dewaxed sections were exposed to sulphuryl chloride vapour for 30 minutes and then stained without any intermediate washing.

A little liquid sulphuryl chloride was kept in a dry Coplin jar placed inside a large beaker which stood

on a hot plate (40°). The sulphuryl chloride was replaced occasionally as the vapour reacted with atmospheric moisture and became saturated with hydrogen chloride. Concentrated acids collected on the loose-fitting lid of the Coplin jar.

Results

Glycogen in liver sections was sulphated very efficiently, but other neutral and acid mucopolysaccharides reacted indifferently or not at all.

Dried smears of neutral and acid mucopolysaccharides exhibited characteristic fluorescence spectra after they had been exposed to sulphuryl chloride vapour and stained in 0.01% solutions of acridine yellow in 30% alcohol.

5. Dioxan: sulphur trioxide complex.

Preparation

Sulphur trioxide was distilled from fuming oleum over phosphorus pentoxide into a cold, 25% mixture of dioxan (previously dried over barium oxide) and carbon tetrachloride until the precipitation of the white solid was completed and excess sulphur trioxide fumes appeared.

The adduct was filtered and washed four times with cold carbon tetrachloride.

Results

A suspension of the complex in carbon tetrachloride led to only a little metachromatic staining towards toluidine blue when used on liver sections for

3 minutes or longer.

A 5% solution of the complex in DMF gave variable results. One or two minutes sulphation gave fairly good metachromasia (about 50% of that given by the DMF:SO_3 complex), but longer treatment of up to 8 mins. resulted in very little staining. Longer sulphation (10 - 15 mins.) restored the metachromatic reaction.

6. Dimethylformamide-sulphur trioxide complex (DMF : SO_3).

Preparation

(a) A procedure equivalent to Rickett's preparation of the pyridine complex, in which 50 ml DMF (dried over barium oxide) was substituted for pyridine, resulted in a clear, colourless solution. This reaction was less vigorous than the reaction with pyridine.

(b) The DMF:SO_3 complex dissolved in excess DMF was prepared free from hydrochloric acid by distilling sulphur trioxide from 65% oleum over phosphorus pentoxide into cold, dry DMF until a permanent precipitate just persisted. The saturated solution had a pale yellow-brown colour.

Method

(1) Rinse dewaxed sections in alcohol.

(2) Rinse once in DMF

(3) Immerse in DMF:SO_3 in excess DMF (preparation b) for 3 mins.

(4) Wash once each in DMF (1 min.) and acetone (30 secs.)

(5) Immerse in 1% hydrochloric acid in methanol for 10 hours.

(6) Hydrate to 30% alcohol or water, and stain.

Results

Fresh, acidic solutions of the complex (preparation a) show some sulphation properties, but they lose their potency after 24 hours storage at 5°.

Preparation (a) retains its full reactivity after standing for 1 month at room temperature. If stored at 5°, it remains potent for 2 - 3 months. This complex is the preferred sulphating reagent as it introduces very little distortion in the morphology of tissue sections.

7. Desulphation experiments

Removal of N- sulphate groups.

(a) Methanolic hydrochloric acid.

Sections containing neutral mucopolysaccharides that had been sulphated in the DMF-sulphur trioxide reagent were immersed in N/10 hydrochloric acid in methanol for 4 - 10 hours.

Only the sites corresponding to neutral mucopolysaccharides showed any γ-metachromasia after the sections were stained subsequently in toluidine blue. The nuclei were stained orthochromatically and were clearly discernable. The sulphate groups on the protein amine groups were therefore presumably all removed.

(b) Aqueous hydrochloric acid

A procedure analagous to method (a) was tried using N/10 aqueous hydrochloric acid.

Hydrolysed sections which were subsequently stained in toluidine blue exhibited a weak orthochromasia over the whole section. The nuclei could not be clearly seen. Presumably both the N- and O- sulphate groups were hydrolysed, and this stage was followed by a dissolution of the neutral mucopolysaccharides and some nucleoproteins from the section.

Section 14. Primary hydroxyl groups

1. Introduction

No specific histochemical test for primary (1^{o}) hydroxyl groups has been reported in the published literature. Their presence in carbohydrates *in vitro* is usually inferred by indirect tests which involve the identification of the fragments produced in the drastic oxidation of the sugar. However, if a specific test could be devised, it would be very useful for investigating *inter alia* the mechanism of histochemical reactions, such as the reduction of uronic acids (sec. 10.2/2).

2. Selective sulphation.

Methods for selectively sulphating the primary hydroxyl groups of tissue neutral mucopolysaccharides in the presence of secondary (2^{o}) hydroxyl groups have been investigated. The new O- sulphate groups were detected by their basophilia towards coriphosphine (f) or azure A (v).

Weak acid sulphating agents, such as sulphamic acid ($\overset{+}{N}H_3\text{-}\overset{-}{S}O_3$) will sulphate primary alcohol groups preferentially *in vitro* (Cupery, 1938). Unfortunately liver sections containing glycogen showed no additional azurophilia after they had been treated with 10% aqueous solutions of sulphamic acid for periods varying from 10 minutes to 1 hour.

Relatively weak sulphating agents might be expected to sulphate primary hydroxyl groups specifically

and effectively in basic media. For example, although the pyridine-sulphur trioxide complex dissolved in excess dry pyridine attacks nearly all hydroxyl groups *in vitro*, particularly if the solution contains chloride ions (Reitz *et al.*, 1946b), only the primary hydroxyl groups of polysaccharides are sulphated if the complex is dissolved in dimethylformamide (Guiseley and Ruoff, 1961). The specificity of this reagent was therefore investigated on tissue sections. The glycogen in sections of guinea pig liver was used as test material.

A fresh 5% solution of the complex (exp.sec.13.2b) in dry dimethylformamide induced less δ-metachromasia towards azure A than the DMF-sulphur trioxide complex (sec.13.7). However the induced metachromatic reaction was not altered either by previously oxidising the tissue sections in periodic acid (to destroy the 2^{o} hydroxyl groups) or by post-treating the sulphated sections in methanolic hydrochloric acid for 4 hours. If the dialdehydes engendered in sections of liver by periodic acid were condensed with aniline (sec.12.22) before carrying out the sulphation, no induced metachromasia was observed. Presumably the large number of phenyl groups in the region of the 1^{o} hydroxyl groups protected them from attack. This is comparable to the steric interference which takes place if the protein groups are "protected" during the sulphation of both 1^{o} and 2^{o} hydroxyl groups by the DMF complex (Sec.13.8).

Section 15. Lipids

1. Introduction

Only a perfunctory investigation into the fluorescence histochemistry of lipids has been carried out. Lipids are not likely to be encountered in many routine histological studies because they will be extracted during the dehydration and clearing stages in alcohol or chloroform before tissues are embedded in paraffin wax

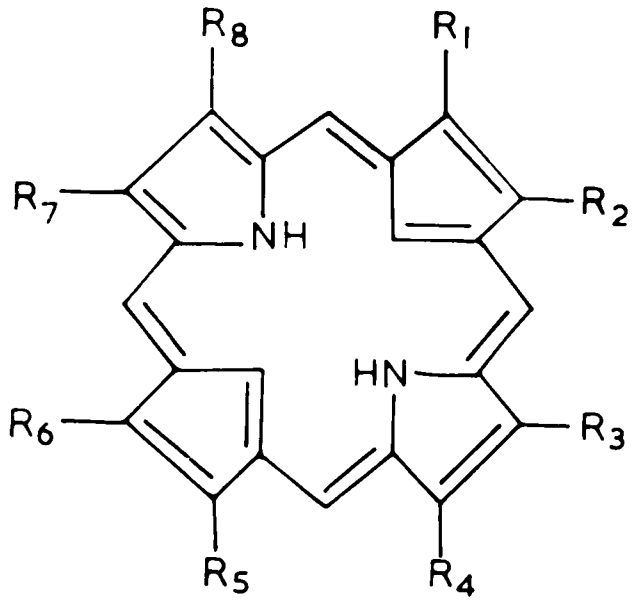
However three techniques for the identification of lipids were devised for fluorescence histochemistry in order that lipids could be shown not to be interfering in histochemical reactions for other chemical groups. For example, sphingomyelin (sec.12.3/5) interferes with the specificity of the periodic acid reaction for 1,2-glycols. Therefore in critical studies of mucopolysaccharides, it is essential to show that sphingomyelin is absent (by the protoporphyrin test; sec.15.2) before carrying out the periodic acid test.

For the purposes of this investigation, test materials for lipids (rat adrenals, ovaries and testis) were fixed in neutral 10% formalin containing 0.01% hydroquinone, washed in running water for 5 mins. to remove formalin bound in excess, and sectioned on the freezing microtome. The hydroquinone was added to prevent the oxidation of unsaturated lipids to aldehydes during the fixation process (Karnovsky and Deane, 1955).

The auto-oxidation of the tissues was checked daily by examining them for fluorescence after condensing them in salicyloyl hydrazide (exp.sec.12.14, stages 3 - 7). Control sections which had been fixed in formalin without the hydroquinone additive showed that the antioxidant was necessary and was effective at the concentration stated earlier.

2. Tests with lipophilic dyes

Lipids have a marked affinity for many fluorescent dyestuffs (sec.9.1/4) and hydrocarbons, particularly for those having an extensive planar aromatic structure such as benzpyrene (Berg, 1951). The most convenient, sensitive and selective lipophils are the protoporphyrins. Kosaki et al. (1954, 1957) claimed that copropoporphyrin or propoporphyrin III (as 0.03% solutions in water or 0.05N hydrochloric acid) are rapidly taken up by sphingomyelin only and have no affinity for other lipoidal substances found in the tissues of normal higher animals. Copropoporphyrin I and its methyl esters have no affinity for sphingomyelin. The structurally similar protoporphyrin IX is the only porphyrin commercially available in this country (from L.Light & Co., Colnbrook). It has been used for the detection and estimation of serum lipoproteins on paper electrophorograms (Sulya and Smith, 1960; Searcy and Bergquist, 1960). The sample used in this investigation would not dissolve in



	Proto-porphyrin IX	Copropo-porphyrin III	Copropo-porphyrin I
R_1	M	M	M
R_2	V	P	P
R_3	M	M	M
R_4	P	P	P
R_5	P	P	M
R_6	M	M	P
R_7	M	M	M
R_8	V	P	P

M = $\cdot CH_3$; V = $\cdot CH{=}CH_2$; P = $\cdot CH_2CH_2CO_2H$

The structures of some natural porphyrins (after Lamberg and Legge, 1949)

Fig. 72. Rat ovary. Fixed in formalin.

Protoporphorin IX. Water mount, X 90.

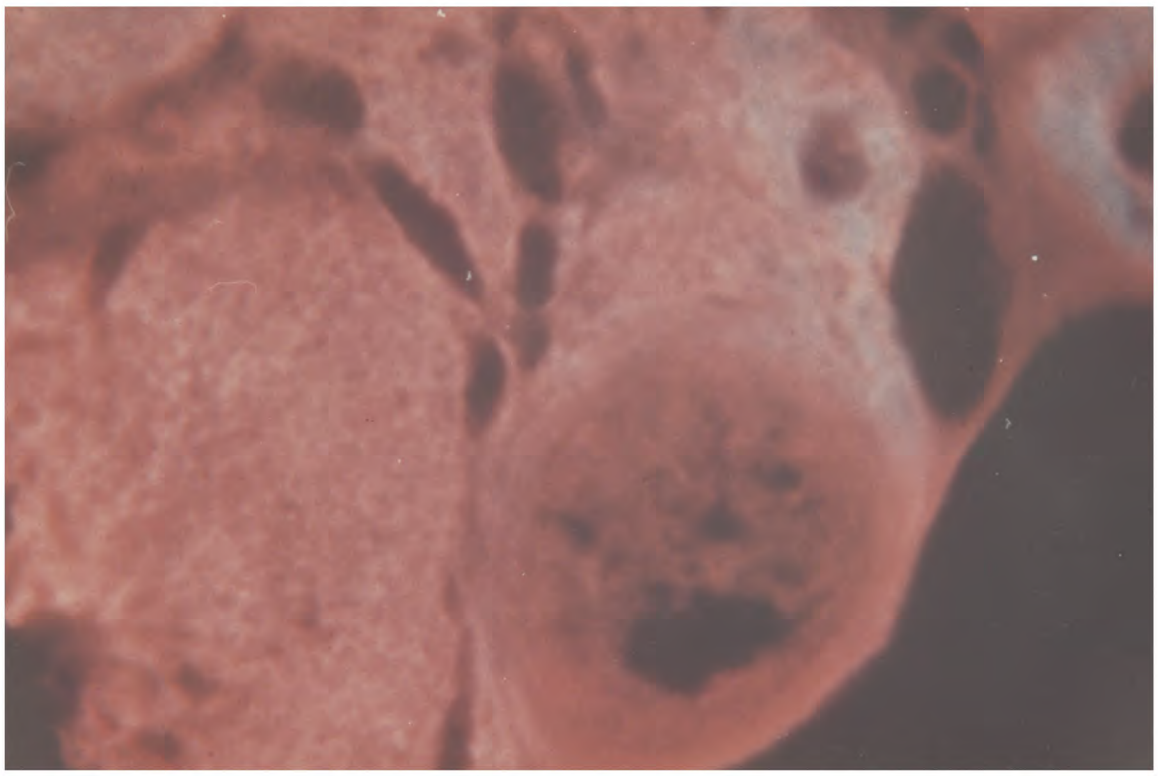


Fig. 72

dilute acids to the concentrations stated by the workers cited earlier. It required more acid to effect its solution. A filtered (nominal 0.05%) solution of the porphyrin in 0.5N hydrochloric acid stained the lipids in tissue sections maximally after about 30 mins. Such sections exhibited a strong purplish-red fluorescence initially which faded to a pink colour after about 5 mins. exposure to ultraviolet light or after leaving them exposed to the air overnight. Some purplish-blue areas were also apparent in the 'exposed' sections. Proto-porphyrin IX is also unstable in solution when exposed to light (Lemberg & Legge, 1949).

3. Oxidation of olefinic groups

The olefinic bonds in unsaturated lipids were oxidised by peracetic acid to aldehydes which were subsequently demonstrated by the strong blue fluorescencee of their salicyloyl hydrazones. The theory of this method is discussed in sec. 5.2/211.

4. Ketosteroids

In the preliminary tests for the auto-oxidation of unsaturated lipids described in sec. 15.1, ketosteroids simultaneously gave fluorescent complexes with salicyloyl hydrazide unlike their aliphatic counterparts (sec. 7.2). which required post-treatment with zinc salts before they would fluoresce. It is, therefore, difficult to distinguish between the fluorescence originating from auto-oxidised

lipids and that of ketosteroids. Boscott and Mandl (1949) tried various specific techniques for the latter and failed. This problem is still unresolved. However two modifications of the fluorescence reaction with salicyloyl hydrazide have now been carried out and have given promising results. Freshly-cut tissue sections were first left in a saturated solution of sulphanilic acid overnight to block the aldehydes engendered by the auto-oxidation of unsaturated lipids. The less-reactive ketone groups in ketosteroids should not condense with this reagent (Boscott and Mandl, 1949), and therefore only they should yield blue fluorescent salicylhydrazones.

Most of the lipoidal tissue, however, fluoresced a moderately strong green colour which implied that the blocking of the lipid aldehydes was imperfect. Droplets of highly fluorescent material could be observed in the follicles of ovary treated in this way. Presumably these droplets contain ketosteroids.

Another difference between the salicyloylhydrazones of simple (aliphatic) aldehydes and ketosteroids is the stability of their fluorescence towards alkali. That of the former is destroyed immediately <u>in vitro</u>, whereas that of ~~the~~ certain ketosteroids is not and may even be enhanced (Camber, 1957) by this treatment. The pre-treatment of the ketosteroid salicyloylhydrazones with zinc salts before

the alkali treatment can be used to distinguish certain steroids from others. When sections were treated according to the technique outlined in the previous paragraph, and then immersed in a 0.5% solution of potash in 80% alcohol for 20 minutes, most of the blue and green fluorescence (due to the lipid aldehydes?) was destroyed. The fluorescence which remained was tentatively attributed to ketosteroids.

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