SOME STRUCTURAL AND BIOCHEMICAL

STUDIES OF NUCLEIC ACIDS

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submitted for the degree of

DOCTOR of PHILOSOPHY

by

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SOME STRUCTURAL AND BIOCHEMICAL STUDIES OF NUCLEIC ACIDS

INTRODUCTION

Although it is nearly a century ago since Miescher published the first account of the preparation of a nucleic acid, the chemical structures and biological properties of these compounds are still matters for some speculation. Early concepts of nucleic acid structure were based on the results of work on extensively degraded samples of the compounds. Extremes of pH and temperature used in extraction procedures helped to produce samples having very different properties from the high molecular weight samples of later years. Another limitation to progress was the adherence to the tetranucleotide theory which influenced thought on the structure of nucleic acids for nearly forty years, until about 1948. Pioneers of the early 20th century, such as Levene, Kossel and Steudel, may now be considered as having contributed sound evidence for the fine structure of the basic component of nucleic acids, the nucleotide, but their views on the structure of the final polymer have been greatly altered as the result of the last ten years' work.

The general concept of nucleic acid structure is shown in fig.1.

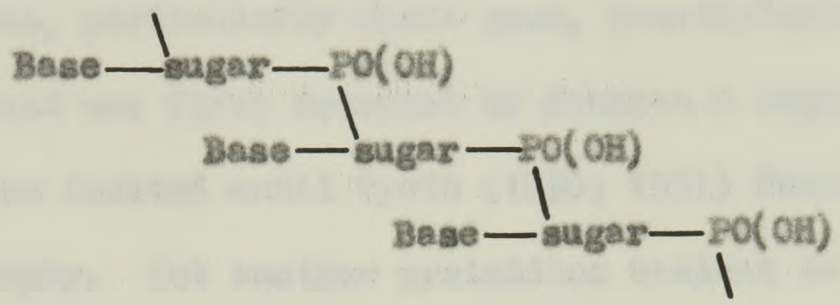


Fig.1

The most marked difference in nucleic acids of different biological origin is in the sugar moiety. Only ribose and 2-deoxyribose have been found and no other sugars have yet been identified in nucleic acids. The ribonucleic acids (RNA) appear to be biologically as well as chemically distinct from the deoxyribonucleic acids (DNA). It is emphasized that the existence of other sugar components is not precluded but a range of nucleic acids of plant, animal and bacterial origin have been examined without finding a trace of other carbohydrate constituents (Chargaff, Vischer, Doniger, Green & Misani, 1949; Chargaff & Lipshitz, 1953).

The bases present in nucleic acids are confined in their variety to purines and pyrimidines. In both RNA and DNA the purines guanine (2-amino-6-hydroxypurine) and adenine (6-aminopurine) have alone been found but the pyrimidine bases show greater variation in

number and quantity. In RNA cytosine (2-hydroxy-6-aminopyrimidine) and uracil (2-6-dihydroxypyrimidine) can only be detected but in DNA the uracil is replaced by thymine (5-methyluracil). In DNA from some sources, particularly wheat germ, 5-methylcytosine is present. This compound was first detected by Johnson & Coghill (1925) but its presence was doubted until Wyatt (1950; 1951) demonstrated it by paper chromatography. Yet another pyrimidine variant is found in the DNA's of T-even bacteriophages of Escherichia coli. First it was evident that cytosine was absent (Marshak, 1951) and a further investigation by Wyatt & Cohen (1952) revealed the presence of 5-hydroxymethylcytosine in place of cytosine in these phages.

The mode of linkage between base and sugar and the configuration of the sugar in nucleic acids is well defined. A glycosidic type link to position 3 of the pyrimidine bases (Levene & Tipson, 1934) and position 9 of the purine bases (Gulland & Story, 1938) appears universal, whilst the sugars are β-D-ribofuranose in RNA and β-D-2-deoxyribofuranose in DNA (Levene & Tipson, 1932; 1933: Bredereck, 1932; 1933; 1934). The sugar-phosphate polymeric linkages are more complex.

The general formula of fig.1 involving phosphodiester links between successive sugar molecules was first derived by Levene & Simms (1925, 1926) using the technique of electrometric titration on both DNA and RNA, on what must have been very degraded samples. Some twenty years later these measurements were repeated on higher molecular weight samples (Fletcher, Gulland & Jordan, 1944; Gulland & Jordan, 1948;

Gulland, 1947) when it was found that the number of dissociating secondary phosphoryl groups were too great for a linear polydiester of the type in fig.1. Gulland and his colleages therefore proposed the structure shown in fig.2 in which a branched chain polymer introduces a number of phosphomonoester as well as phosphotriester linkages.

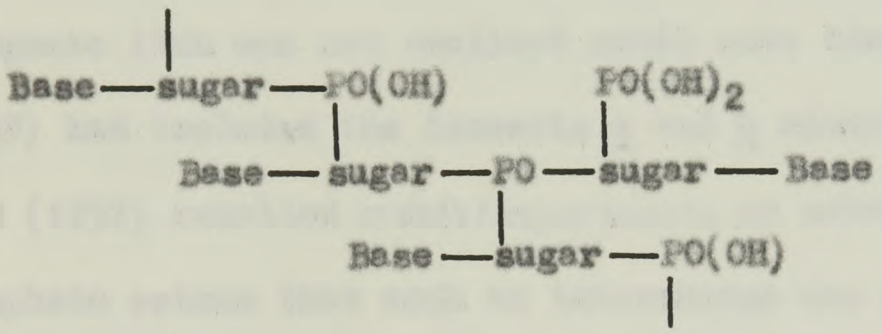


Fig.2.

Among the latest views on chainbranching and the type of sugar-phosphate links are those inferred from the results of chemical and enzymic degradation of nucleic acids. Much more work has been done on RNA than DNA despite the fact that it is easier to get DNA in a less degraded form. This is due to the ready lability of RNA to alkali and the greater understanding of the mode of action of ribonuclease.

Examination of the D-ribofuranose molecule reveals that only carbon atoms 2, 3 and 5 are available for phosphoester formation and until recently hydrolysis of RNA had only yielded the 3' phosphates of the four nucleosides present. It was not considered that internucleotide links could involve the 3'5' positions since muscle adenylic

acid (adenosine 5' phosphate) appeared as resistant to alkaline degradation as yeast adenylic acid. The 3'3' position was also impossible, so in the absence of information of the chemical lability of nucleoside 2' phosphates, Levene & Tipson (1935) proposed a 2'3' linked structure.

The ready interchange between 2' and 3' position for the phosphate link was not realised until some time after Carter & Cohn (1949) had isolated the isomeric a and b adenylic acids. Brown & Todd (1952) recalled in model experiments on substituted α- and β-glycerophosphate esters that such an interchange was inevitable under the alkaline conditions used in RNA hydrolysis experiments. The system functions as shown in fig.3.

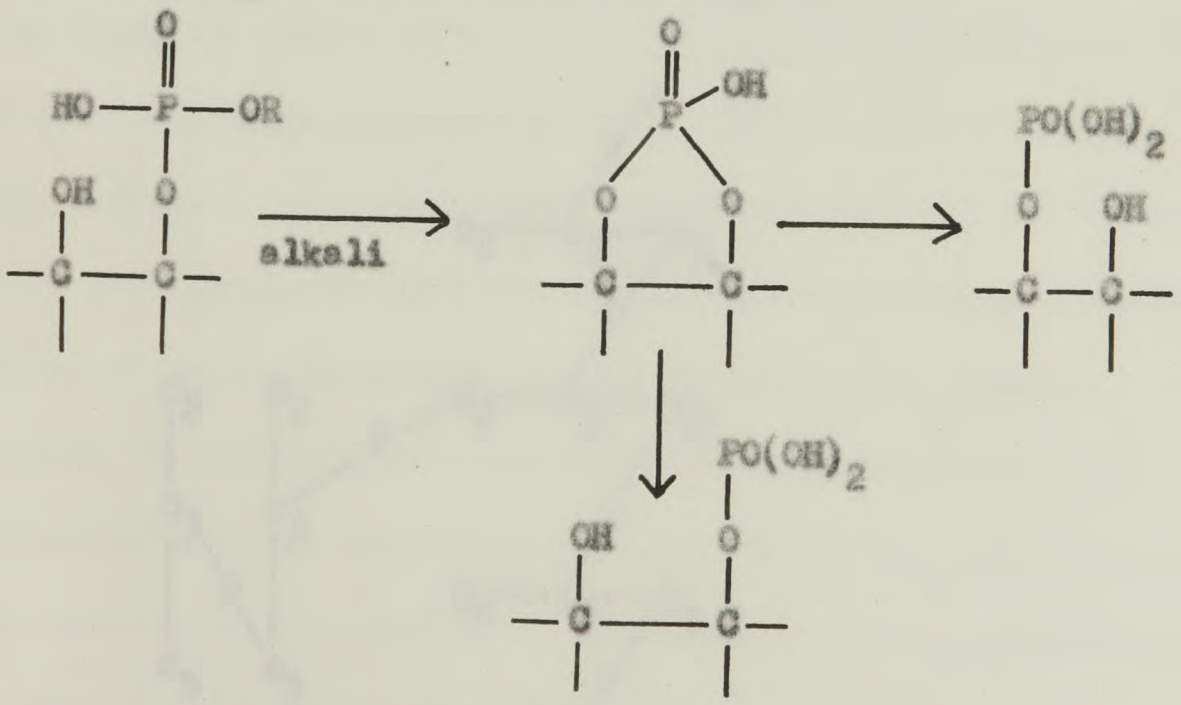


Fig.3.

The essential features required are a <u>cis</u> hydroxyl adjacent to a phosphodiester, just the conditions found in RNA, and not only has this function been used to account for the products of chemical hydrolysis of RNA, but it has also been used to infer its structure with some success. The absence of nucleoside 5' phosphates from hydrolysis products is consistent with the cleavage of the 5' bond in the formation of 2'3' cyclic nucleoside phosphates which in turn are sufficiently labile to hydrolyse further to 2' and 3' nucleotides. It is now possible to impose limitations based on chemical evidence for the type of internucleotide link and the extent and kind of side chain formation possible. The only permissible internucleotide links are, for a linear polymer, phosphodiester links between 2'5' or 3'5' positions of adjacent ribose molecules, or, in the event of measurable side chain formation, the structure of fig. 4.

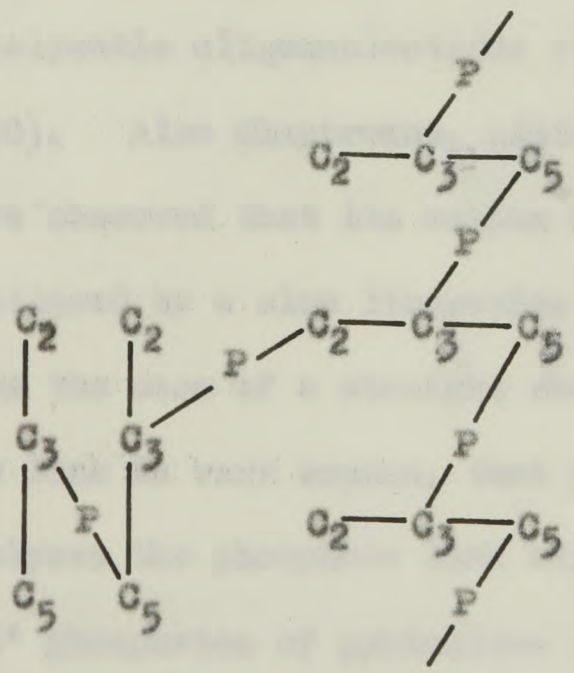


Fig. 4.

The notation C_2-C_3-C_5 represents the functional ribose carbon atoms (Brown & Todd, 1952).

Since the nucleoside 2' and 3' phosphates are present in almost equivalent quantities in alkaline hydrolysates of RNA, it is surprising that the 2' phosphate was not discovered by the very elegant method of Levene & Harris (1932). In this the ribose phosphates from nucleotides were reduced to ribitol phosphates of which the ribitol 2 phosphate would be optically active and ribitol 3 phosphate optically inactive. Levene & Harris were unable to measure any optical rotation and concluded that RNA hydrolysates contained only 3' nucleoside phosphates.

Structural results obtained from the study of pancreatic ribonuclease action are in close agreement with chemical degradation studies. Model experiments performed on cytidine and uridine 2' and 3' benzyl phosphates by Brown & Todd (1953) showed that this ribonuclease only attacked the 3' esters, yielding pyrimidine 3' phosphates. With RNA, it is found to give a mixture of dialysable pyrimidine mononucleotides and non-dialysable oligonucleotides rich in purine content (Bacher & Allen, 1950). Also Chantrenne, Linderstrøm-Lang and Vandendriessche (1947) have observed that its action has two stages, a quick depolymerisation followed by a slow liberation of acidic groups. These results indicate in the case of a straight chain polymer that the 3'5' internucleotide link is very common, that pancreatic ribonuclease specifically hydrolyses the phosphate link adjacent to a pyrimidine nucleotide, and that 3' phosphates of pyrimidine nucleosides are formed by slow breakdown of the corresponding 2'3' cyclic phosphates. These cyclic phosphates have been isolated (Markham & Smith, 1951).

Postulations on side chain formation have been made from degradative enzyme studies, notably by Cohn & Volkin (1953). The RNA's from three different sources were degraded with crude snake venom, which contains mainly diesterase and 5' nucleotidase activities. The production of pyrimidine nucleoside 2'5' and 3'5' diphosphates in significant quantity and much free phosphate occurred, but when the venom was freed of 5' nucleotidase, the free phosphate greatly diminished with a corresponding increase in all four nucleoside 5' phosphates. Cohn & Volkin have interpreted this work on the basis of Brown & Todd's structure of fig.4.

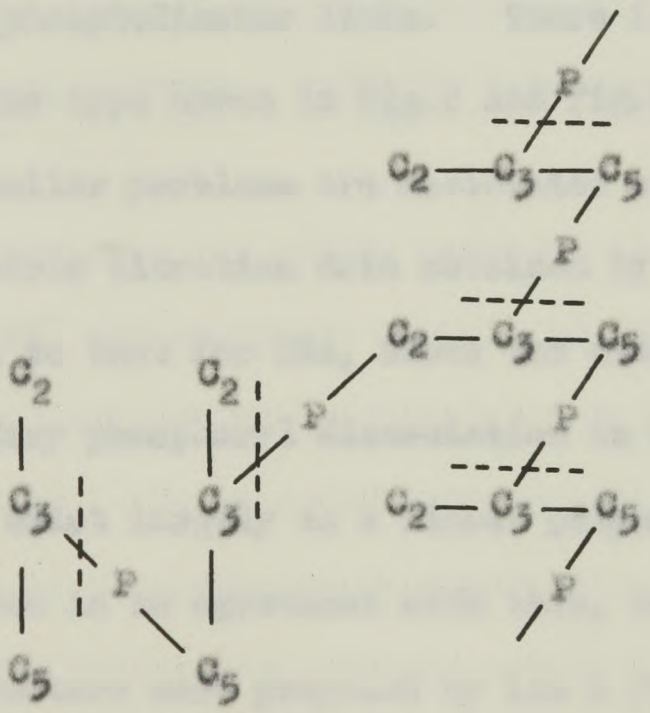


Fig.5

If fission by the diesterase were to occur at the C_3-O-P link as in fig.5, large quantities of nucleoside 5' phosphates would be produced whilst a pyrimidine diphosphate would be formed from the branch

point nucleotide, which, as found in pancreatic ribonuclease studies, only occurs at a pyrimidine nucleotide. In this paper, however, no explanation is afforded for the production of 3'5' diphosphates. In view of the structure of fig.5, it is difficult to account for these especially as it appears from the ready production of both 2'5' and 3'5' diphosphates from DNA that the cyclic 2'3' phosphate formation does not take place with venom diesterase.

Thus numerous features of the general polymeric structure of RNA still await elucidation. Recent results suggest a long chain structure linked between 3' and 5' positions of adjacent ribose molecules by phosphodiester links. There is evidence for chain branching both of the type shown in fig.2 and fig.4.

Similar problems are associated with the structure of DNA. Electrometric titration data obtained by Gulland, Jordan & Taylor (1947) analogous to that for RNA, shows the existence of much less tendency to secondary phosphoryl dissociation in DNA. It was concluded that DNA must exist largely as a linear polymer as in fig.1. A great deal of evidence is in agreement with this, but results favouring a branched chain structure were proposed by Lee & Peacocke (1951) and by Reichmann, Varin & Doty (1952). Also a group of workers in Birmingham produced evidence based on the Feulgen reaction for a C_1' phosphoryl link to the deoxyribose molecule (Li, Overend & Stacey, 1949; Overend, Stacey & Webb, 1951; Overend Peacocke & Stacey, 1952; Lee & Peacocke, 1952). Although this is in contradiction with the general structure of fig.1.,

no confirmatory evidence appears to be forthcoming for an alternative structure. The resistance of DNA to alkaline hydrolysis is expected from a 3'5' linked polydiester since there is no free hydroxyl group on position 2 of the deoxyribose molecule.

The original claim by Levene & Jacobs (1912) to have isolated pyrimidine 3'5' diphosphates from the acid hydrolysis of DNA has been confirmed and extended by Dekker, Michelson & Todd (1953), who have synthesized and isolated these compounds as the barium salts. The action of enzymes on DNA although much less clearly understood than with RNA, decisively confirms the 5' phosphate link in the molecule (Heppel & Hilmoe, 1951; Sinsheimer & Koerner, 1952; Sinsheimer, 1954) and at least it would seem that the 3'5' internucleotide link is a common one in DNA.

There is as yet little evidence on the sequence of nucleotides in RNA or DNA, and available enzymic results appear complex and without satisfactory explanation (Zemenhof & Chargaff, 1949, 1950; Smith & Markham, 1952). An important property of DNA which may facilitate the elucidation of the sequence of nucleotides is the preferential lability of the purine nucleotides towards acidic hydrolysis. Products of acid hydrolysis of DNA were first isolated by Kossel & Neumann (1893), who termed them "thymic acid" because they could discover thymine as the only nitrogenous base. These early compounds were too degraded for quantitative work and the use of acid hydrolysis as a technique was not deemed important until Tamm, Hodes & Chargaff (1952) re-examined it.

Using calf thymus DNA, these workers were able to cleave off with hydrochloric acid all the purines that were chromatographically detectable without altering the pyrimidine ratios. The highly polymerised structure remaining after dialysis was termed "apurinic acid". The presence of a free aldehydic group in position 1 of the 2-deoxyribose not only makes such residues highly reactive but allows these sugar molecules to decyclise. Carbon atom no.4 now exhibits a free hydroxyl group rendering the C-O-P bond at position 3 labile to alkali. The mechanism of cyclic phosphate formation is now possible with consequent splitting of the polymer in a controlled manner as with RNA. (Tamm, Shapiro, Lipshitz & Chargaff, 1953). The presence of the free reactive position 1 carbon atom of the 2-deoxyribose, however, leads to uncertainty in the degradation mechanism, and part of the work recorded in this thesis is devoted to the examination of this type of procedure for determining nucleotide sequence but with the aldehydo-sugar residues blocked with ethyl mercaptan.

The extraction of nucleic acids from tissues confronts the structural and biological investigator with a difficult problem. There is no means of measuring how closely extracted samples resemble nucleic acids in living cells. The very act of removing nucleic acids from the proteins to which they appear to be bound may modify them considerably. There are a number of properties to which a sample must conform before it can be considered worth studying, but these only fix a lower limit to its integrity. DNA is rather easier to

extract than RNA since it was discovered that its corresponding nucleoproteins show a marked solubility in solutions of strong electrolytes. This property forms the basis of a number of well-used methods for the separation of this material from other cellular components (Daly, Allfrey & Mirsky, 1950; Signer & Schwander, 1949; Gulland, Jordan & Threlfall, 1947). The other recognised method depends on the use of anionic detergents to extract and dissociate the nucleoprotein (Kay, Simmons & Dounce, 1952). Some interesting work on the relative merits of these methods leads Frick (1954) to the conclusion that there are important faults in all of them, and the choice is between a slightly more degraded sample (detergent method) or an incompletely deproteinised sample (strong electrolyte methods). Methods of RNA extraction appear even less exacting. When animal tissue is the source, the sole method using mild reagents is that of Volkin & Carter (1951), or the modification by Grinnan & Mosher (1951). In less complex sources such as viruses, heat treatment is often used to separate the protein from the RNA, whilst in one of the richest sources of RNA, namely yeast, it is doubtful whether any of the present methods successfully extract it, because of the difficulty in breaking the cell wall.

Having chosen the method of isolation, there are certain criteria which must be satisfied before a sample is worthy of further investigation. In the case of DNA, freedom from RNA is examined by means of the available colour reactions and the analytical values for

nitrogen and phosphorus are determined. Published figures from mammalian sources seem to vary about a mean 14.5% for nitrogen and 8.5% for phosphorus. Chargaff (1955) states that the phosphorus content should approximate to 9.2% yet in his paper (Chargaff, Vischer, Doniger, Green & Misani, 1949) he gives figures as low as 7.6% on a dry weight basis. More important is the atomic nitrogen to phosphorus ratios, which vary between 3.5 to 4.0.

The absorption in the ultraviolet region should show a smooth symmetrical peak with a maximum at 259 to 261 millimicrons at pH 7.0, and an atomic exinction coefficient with respect to phosphorus (ϵ_p) around 6,600 (Chargaff & Zamenhof, 1948). A high value for the extinction coefficient is considered an indication of degradation since it is universal that when a sample of DNA is degraded by deliberate hydrolysis the final product has a considerably higher extinction coefficient. Other criteria of nativity include electrophoretic homogeneity, a high viscosity in solution and the presence of streaming birefringence.

In recent years, since DNA has been presumed an important component of the genetic material of a cell, the possibility has been considered that preparations of it may consist of a great number of closely related molecular species (Chargaff et al, 1948) giving a constant composition as determined by analysis. It has been observed that preparations from the same site and from different sites within the same species show remarkable constancy of composition, but

considerable variations exist between different species (Chargaff, 1951; Wyatt, 1952). Further evidence in favour of the species specificity of DNA is found in the ratios of the nitrogenous bases. In a large number of preparations the quantities of adenine have closely equalled those of thymine and guanine those of cytosine plus 5-methylcytosine. In addition to this the ratio of adenine plus thymine to guanine plus the cytosines varies greatly from species to species but not within the same species. This ratio ranges from 0.4 for M.tuberculosis (avian strain) to 1.9 for sea-urchin sperm. At first methods of fractionation were largely unsuccessful (Wyatt, 1950; Zamenhoff & Chargaff, 1951; Bendick & Russell, 1953) until almost simultaneously Chargaff, Crampton & Lipshitz (1953) in America and Brown and Watson (1953) in London published successful fractionation procedures for calf thymus and other types of DNA. Both methods depend on the selective affinity of the DNA molecules for a histone type protein, and both showed an increase in the proportions of adenine and thymine with respect to guanine and cytosine on increasing the salt concentration in the solution used to break down the DNA/histone complex. At the present time therefore it is necessary to regard any sample of nucleic acid as a gross mixture of polynucleotides probably accompanied by degradation products giving statistically constant analytical figures.

As regards the orientation of DNA molecules in space, several models of a somewhat speculatory nature have been devised. The first by Ronwin (1951) was a revolutionary structure having as its core a

polymer of alternate phosphorus and oxygen atoms. In 1953 Pauling & Corey suggested a helical structure and shortly afterwards Watson & Crick (1953) proposed the most attractive scheme whereby two molecules of DNA are intertwined in two coaxial helices with respect to their sugar-phosphate chains. The gap between the two helices is bridged by inward directed base molecules, such that they are bound by hydrogen bonds. Only certain combinations of bases will fit in the structure: thus adenine will be hydrogen bonded to thymine and guanine hydrogen bonded to cytosine. This structure is particularly attractive because not only does it agree well with present X-ray analytical data, but it provides an explanation for the existence of adenine-thymine predominant (AT) and guanine-cytosine predominant (GC) types. Furthermore, a template scheme may be devised from it whereby the essential function of exact self duplication required by a genetic material like DNA can be carried out.

So far it is universally recognised that the site of DNA is within cell nuclei. Whenever DNA has been isolated, chemists have by choice used tissues such as thymus gland, pus cells or sperm, which are known to be rich in nuclei. The term "nucleic acid", first used in 1889 by Altmann, would probably not be a modern choice now that cytoplasmic nucleic acids have been discovered. The discovery of the Feulgen stain specific for DNA and the characteristic ultraviolet absorption of DNA have been valuable tools not only for discovering the site of it, but also estimating its quantity. In addition, the DNA content of nuclei after they have been isolated may be measured

using the methods of Schmidt & Thannhauser (1945) by estimating the phosphorus, and of Schneider (1945) by estimating the deoxyribose colour with diphenylamine. Using these methods, Boivin, Vendrely and Vendrely (1948) and Vendrely & Vendrely (1948) were able to measure the nuclear DNA content of a number of beef tissues. They found great similarity in the DNA content of all the tissues except the sperm which had approximately half the normal content. This conclusion developed into the far-reaching one that the nuclear DNA in diploid somatic cells is a constant for a particular species, and that haploid sperm cells have half the corresponding diploid DNA content. Certain factors tend to complicate this hypothesis rather, not the least of which is polyploidy. Cells which exhibit polyploidy have a greater DNA content than diploid cells, and when mixed with the latter give a DNA value higher than expected. The liver cells of rodents are a good example. The surprising values obtained by Schrader & Leuchtenberger (1949) for the differing nuclear DNA content of various tissues of that fashionable drawing room plant Tradescantia have been explained as the existence of polyteny in these tissues. Although the number of chromosomes is constant, the thread-like chromonemata are said to reduplicate. What is more difficult to explain is the unaccountably large store of DNA in the eggs of a number of different species, particularly sea urchins (Schmidt, Hecht & Thannhauser, 1948; Vendrely & Vendrely, 1949). Instead of the normal haploid content as in the sperm, over a hundred times the amount has

often been found. Since such large amounts of DNA could not be contained within the nucleus the existence of cytoplasmic DNA in eggs is inevitable, but its presence is regarded by many as being a start for future mitoses. That the DNA content of the nucleus is doubled during mitosis is indicated by the slightly higher DNA content in rapidly proliferating tissues such as intestine and liver after partial hepatectomy (Thomson, Heagy, Hutchison & Davidson, 1953; Price & Laird, 1950). A great deal of circumstantial evidence also exists to help show that the nuclear DNA content is constant. When animals are fasted or put on a protein-free diet, even in extreme cases the DNA content as measured in their liver nuclei remains markedly unchanged. Campbell and Kosterlitz (1952) and others have in fact noted a very slight increase in DNA content. The action of hormones and vitamin deficiencies though greatly affecting protein synthesis has no effect on DNA. Even cancerous and pathologically degenerate tissues all show this unchanging property.

In some careful work by Mirsky, Pollister and Ris (Pollister, 1952), chromosomes were isolated in sufficient quantity to enable analysis. Their material has been subjected to sufficient scrutiny to make it very likely that it is truly chromosomal in character. Most of the mass (90%) was soluble in molar sodium chloride solution and proved to be nucleoprotein containing about 45% DNA. The residue, insoluble in sodium chloride solution, was mainly protein

but contained a little RNA and DNA. Although these results must be regarded with a good deal of caution, at least they give direct proof that DNA is a major component of chromosomes. This conclusion is one of many resulting from recent work that support Miescher's original hypothesis that his nucleic acid was genetic material. The apparent homogeneity of DNA for a long time disfavoured this hypothesis, but upon the fractionation of DNA in 1953 and the conception of the Watson Crick structure in the same year it was possible to visualise sufficient structural variation between molecules for them to play this role.

Along with analytical data much evidence from biological observation supports the theory that genes are DNA. Genetic mutations in fungi have been produced by irradiation with ultraviolet light, and the effectiveness of the process increases to a maximum at 260 millimicrons, the absorption maximum for DNA (Hollaender & Emmons, 1941). The toxicity of the sulphur and nitrogen mustards and X-rays is related to their ability to cause chromosome breakage, and their effect on isolated DNA is shown by Butler & Smith (1950) to cause depolymerisation and loss of viscosity in solution. The most impressive work in support of this theory, however, is the discovery of bacterial transformation by Griffith in 1928, subsequently investigated by Avery, MacLeod and McCarty in 1944. The important features of these transformations are the permanent conversion of a rough pneumococcal strain to a smooth variety upon exposure to the

smooth type DNA, the production of the type specific polysaccharide of the smooth variety by the transformed rough strain, and the indefinite propagation of the smooth variety without further exposure to DNA. Development of this work has revealed so far that mutations involving over thirty different properties have been induced in bacteria by the use of the characteristic DNA. Within the bacterial cell therefore can be found the closest parallel between the action attributed to the hypothetical "genes" and by material that has all the properties of DNA. The action of bacteriophage particles is also of significance in the biological role of DNA. These small particles appear under the microscope as tail-bearing spheres and consist chemically of two-thirds protein and one-third DNA. Hershey & Chase (1952), using isotopic methods, were able to demonstrate the role played by these two major components. ^{35}S-labelled protein from T_2 phage was shown not to enter Escherichia coli cells, whereas the corresponding ^{32}P-labelled DNA did enter. The DNA appeared to be contained within a protein membrane that made it insusceptible to deoxyribonuclease action, but on releasing the DNA from the membrane it was rapidly depolymerised by the enzyme. The overall picture of phage action is one in which the DNA from these particles infects the host cell and reorganises its genetic mechanism with the resultant formation of phage particles at the expense of the host cell's components.

There is considerable evidence of the important relation between DNA and hereditary characteristics, but just how DNA is able to propagate the similarity between an organism and its progeny is a question to be answered in the future. The answer well might not be forthcoming until the study of DNA on a monomolecular basis becomes possible. In this event the structural difference between isolated molecules may then be directly related to their biological specificity.

Turning now to the problem of the biological properties of RNA an equally complex picture is obtained. There has long been a correlation between RNA and growth or protein synthesis. This is obvious from examination of the best sources of RNA. Micro-organisms such as yeast which are capable of rapid multiplication, proliferating tissues such as the root tips of plants, and embryonic tissues, are all rich in RNA and are sites of marked protein synthesis. Perhaps the most striking example of this correlation is found in the silk gland of the silkworm. This organ, adapted only to production of the protein, silk, has one of the largest known contents of RNA (Denucé, 1952). That the function of this type of nucleic acid is essentially different from DNA is shown by the contrasting effects that abnormal conditions have upon them. Whereas it was seen that the DNA content of liver cells was unchanged in the event of a protein-free diet (Campbell & Kosterlitz, 1952), a considerable drop in RNA occurred under the same conditions (Davidson, 1947). Also Jeener & Jeener (1952)

when studying the growth of Bacterium acidophilus showed that removal of DNA from the culture medium did not prevent growth though nuclei were underdeveloped. On the other hand, removal of uracil from the medium had a marked effect on the growth of the whole organism.

The biological site of RNA is much less well defined than that of DNA. The isolation of nucleoli, which contain RNA (Vincent, 1952) has led to the as yet unsubstantiated belief that this is the only site of nuclear RNA. However, as Dounce (1955) has pointed out, only a small percentage of these particles represents this nucleic acid, as shown by published figures, and even if they were solid RNA they would not account for the total nuclear RNA. In spite of this, nuclear RNA is low (approx. 1.5%) and the largest quantities are to be found in the cytoplasm. Of the cytoplasmic particles, the microsomes are particularly rich in RNA, and although both the cell sap and mitochondria are not devoid of it, the latter is a particularly poor source. Recent experiments show marked differences between nuclear and cytoplasmic RNA. Experiments on the incorporation of ^{32}P, first carried out by Marshak (1948) indicate that the nuclear RNA has a much higher specific activity. This would infer that it might be the precursor of cytoplasmic RNA. If this is so, then the formation of cytoplasmic RNA cannot be merely a diffusion through the nuclear membrane of nuclear RNA, since the composition of the two nucleic acids is different (Elson &

Chargaff, 1952). Of the mechanisms whereby RNA is linked to protein synthesis, the "template" hypothesis appears to receive the greatest support at the present time. Although the hypothesis has no proof from experimental data, certain very significant experiments fit in well with it. Gale & Folkes (1955) were able to give the first direct demonstration that nucleic acid is essential for protein synthesis, and extending this work on cells of disrupted Staphylococcus aureus Gale (1956) was able to devise a mechanism for this process. Using a ribonuclease digest of the RNA of this organism, he separated nucleotidic fractions capable of promoting the incorporation of specific amino acids into the protein of the disrupted cells. Gale suggests that these nucleotidic fractions are loci for the combinations of specific amino acids. In the intact nucleic acid whole chains of amino acids are joined to their loci in this specific manner and by means of exchange reactions with the loci, peptide bonds may be formed to link the amino acids into a protein. Further results indicating that proteins in the form of enzymes are built up at the expense of free amino acids come from the work of Halvorson & Spiegelman (1953). A preliminary report by Binkley (1952) may well have a bearing on the role of RNA in protein synthesis. The dipeptidase hydrolysing cysteinylglycine was thought to be pentose polynucleotide and without detectable protein. From these results Binkley postulates a method of protein synthesis in which RNA synthesises dipeptides which are linked together with the agency of glutamic

acid. This highly speculatory proposal has met with no substantiation except the observation by Linderstrøm Lang (1940) that dipeptidases are found in large amounts in high protein synthesising cells. The precise role of RNA in cellular metabolism is still not clear. Its duplication and its biochemical relation to DNA represent frontiers of investigation in biology.

SECTION I.

SEQUENCE OF NITROGENOUS BASES IN DNA.

Present Knowledge of RNA and DNA Structure. Evidence for the molecular structure of both RNA and DNA is still fragmentary. At the present time more work has been done on RNA, and this is due partially to the greater knowledge of the mode of action of pancreatic ribonuclease compared with deoxyribonuclease, and the greater lability of RNA to alkaline hydrolysis. On the other hand, the extraction of DNA is easier and samples used for structural analysis are more likely to resemble their in vivo state. Although present evidence for the structure of the nucleic acids has been surveyed above, it is convenient to summarise the most important deductions with respect to DNA at this stage. It has been generally accepted that the "tetranucleotide" hypothesis is untenable. The majority of evidence from physical studies, such as electrometric titration, X-ray diffraction, etc., indicate that DNA is an essentially linear polymer of high molecular weight. On the other hand, some evidence based on electrometric titration, light scattering, and the Feulgen reaction favours some degree of chain branching. The internucleotide links are effected by phosphate esters joining adjacent 2-deoxyribofuranose molecules in their 3' and 5' positions. This hypothesis is supported

by chemical evidence based on strong acid hydrolysis first used by Levene & Jacobs (1912) and confirmed by Dekker, Michelson & Todd (1953) and also by enzymic hydrolysis. The sequence of DNA nucleotides is the least studied. Examination of the oligonucleotides from deoxyribonuclease digests suggest an irregular sequence with no apparent order. Both Zamenhoff & Chargaff (1949) and Smith & Markham (1952) are unable to indicate any simple regularity when using enzymic methods. This may be a reflection on the lack of knowledge of the mode of action of deoxyribonuclease or an indication of its possible reversible action. Recently Heppel, Whitfeld and Markham (1954) have shown that both pancreatic and spleen ribonuclease react reversibly. A chemical method of approach to the problem of nucleotide sequence is therefore desirable because of its less ambiguous nature.

Chemical Degradation of DNA. DNA is particularly resistant to the action of alkali. This has been attributed to the unreactive properties of the CH_2 group in position 2 of the deoxyribose molecule. The lack of an adjacent hydroxyl function to the phosphodiester groups prevents the type of reaction suggested by Brown & Todd (1952) which results in extensive depolymerisation, as with RNA. The action of acid on DNA, on the other hand, has a great effect. Hot dilute mineral acid, used by Kossel & Neumann (1893) in the preparation of "thymic acid" leads to ill-defined products of varying composition, but a re-investigation of the action of acid on DNA by Tamm, Hodes & Chargaff (1952) led to the important discovery of "apurinic acids". Under

carefully controlled conditions, the optimum being pH 1.6 at 37°C, the purine bases could be quantitatively removed from thymus DNA by scission of the deoxyribosyl purine links, but the more stable deoxyribosyl pyrimidine links remained intact. The resultant apurinic acid, obtained after dialysing away the free purines, contained aldehydo sugar groups at the sites of former purine attachments.

Mercaptalated DNA. Although apurinic acid provides a valuable approach for the structural study of DNA, disadvantages arise by reason of its highly reducing nature. A large number of derivatives of monosaccharides may be prepared in which the aldose properties are removed, but many of these are unstable to alkali while others may involve closure of the sugar ring structure. An acyclic sugar residue with non-reducing properties is a desirable feature, as will be seen later. It is well known that thioalcohols react with monosaccharides yielding nonglycosidic thioacetals (dimercaptals) which are acyclic, e.g.

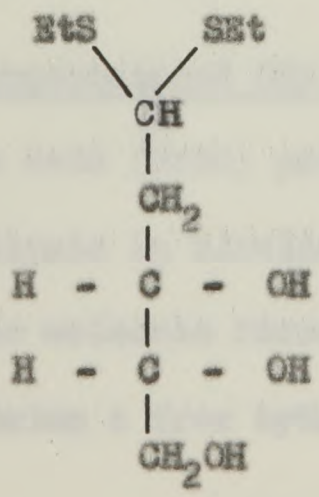


Fig.6. 2-deoxyribosediethylmercaptal.

Compounds of this type from calf thymus nucleic acid were first prepared by Kent (1950). The formation of both sugar mercaptals and apurinic acids is facilitated by acidic conditions. If therefore DNA is treated with hydrochloric acid in the presence of, say, ethyl mercaptan, then as the purines are removed, the free deoxyribose molecules formed will react with the ethyl mercaptan to form acyclic diethylmercaptal derivatives.

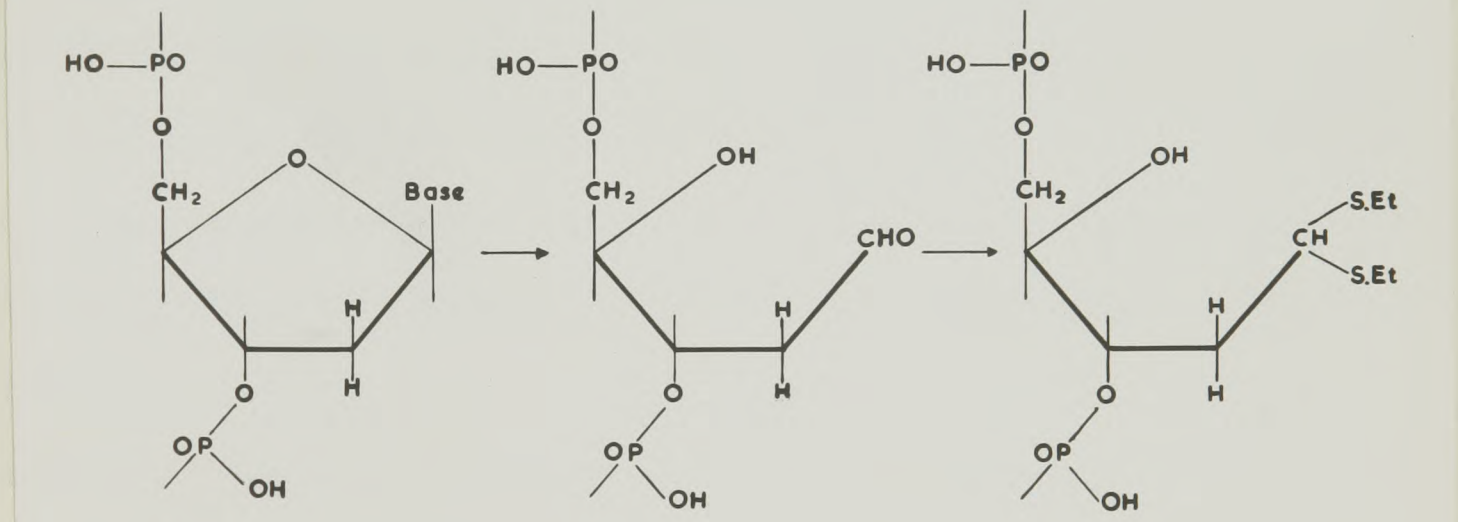
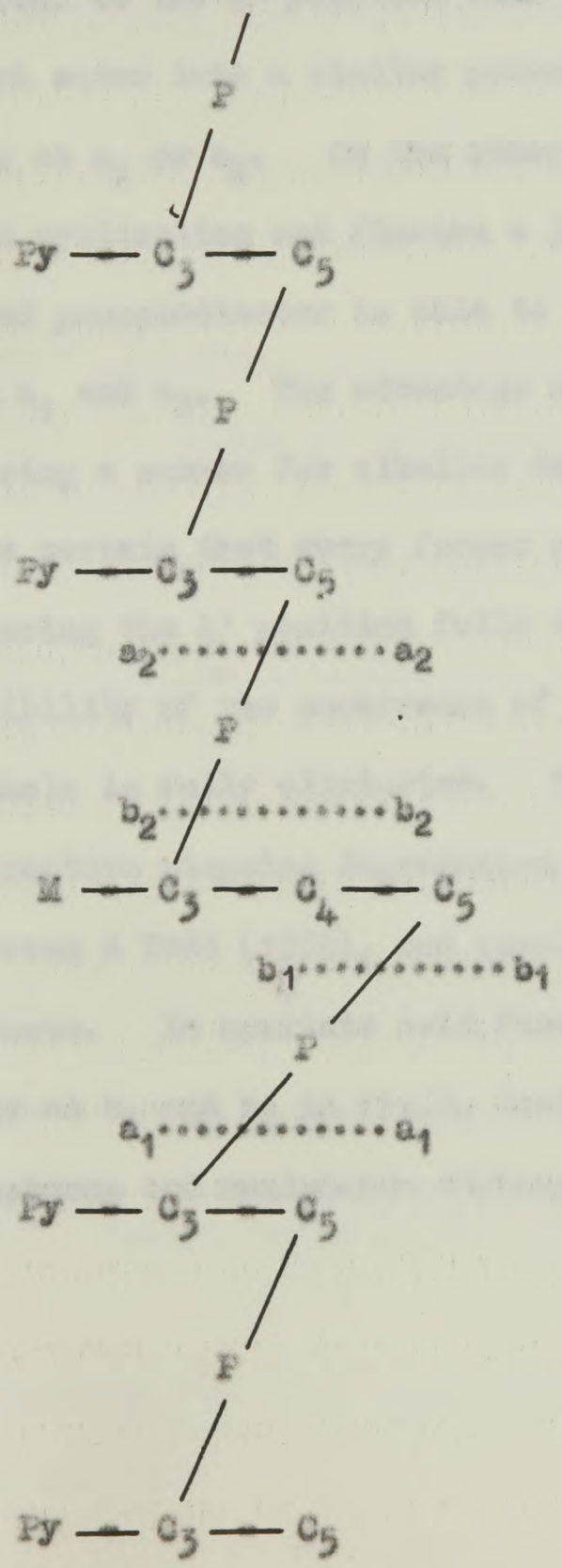


Fig. 7.

Hydrolysis of Mercaptalated DNA. The polymeric mercaptalated deoxyribonucleic acid (MDNA) prepared in this way is theoretically capable of hydrolysis in alkaline conditions in a controlled manner. Every deoxyribose molecule formerly linked to a purine base is now acyclic and contains a free hydroxyl function in position 4 which

was previously involved in the furanose ring structure. Upon the addition of alkali, cyclic phosphate formation is likely at every phosphoester link adjacent to an acyclic sugar residue. The ultimate result will be fission at either or both of the bonds marked with the dotted lines a_1 and a_2 in fig.8.



Py = pyrimidine

M = diethylmercaptal

C_3, C_4, C_5 = functional sugar carbon atoms.

Fig. 8.

It is seen that there is an equal chance for cyclic ester formation by either adjacent phosphoryl groups whether attached to the 3' or 5' carbon atoms, but they cannot both react simultaneously. If the first unstable cyclic phosphate cleaves leaving the phosphoryl group attached to the 4' position then the second adjacent phosphodiester cannot enter into a similar process. Fission will therefore only occur at a_1 or a_2. On the other hand, if upon completion of the first cyclisation and fission a 3' or 5' phosphate is formed then the second phosphodiester is able to react, with the ultimate cleavage at both a_1 and a_2. The advantage of MDNA over apurinic acid when devising a scheme for alkaline degradation products is now more obvious. It is certain that every former purine bound sugar molecule is acyclic rendering the 4' position fully available for reaction, and the possibility of the occurrence of labile phosphates of β-aldehydo alcohols is fully eliminated. This latter factor is the basis of an alternative stepwise degradation mechanism for apurinic acid proposed by Brown & Todd (1955), and complicates the possible degradation products. In apurinic acid, fission of sugar phosphate bonds could occur at b_1 and b_2 in fig.8, leading to large amounts of inorganic phosphorus and nucleoside diphosphates.

Degradation products of MDNA. Assuming the general structure of MDNA as in fig.8, it is possible to predict the products theoretically arising from alkaline hydrolysis (causing cleavages at a_1 and a_2). Whatever the distribution of purines and pyrimidines in the parent DNA, only eight different types of products are expected to be formed:

P.1 pyrimidine oligonucleotides with terminal pyrimidine nucleosides

P.2 pyrimidine oligonucleotides with terminal mercaptalated sugar 4' phosphates

P.3 pyrimidine nucleosides

P.4 pyrimidine nucleosides attached by phosphodiester link to a mercaptalated sugar 4' phosphate

P.5 2-deoxyribose diethyl mercaptal 3' phosphate

P.6 2-deoxyribose diethyl mercaptal 4' phosphate

P.7 2-deoxyribose diethyl mercaptal 5' phosphate

P.8 2-deoxyribose diethyl mercaptal 3'5' diphosphate

In addition, five more types of molecules may definitely be excluded. These are:

P.9 pyrimidine oligonucleotides with terminal phospho-monoesters

P.10 2-deoxyribose diethylmercaptal 3'4' diphosphate

P.11 2-deoxyribose diethyl mercaptal 4'5' diphosphate

P.12 pyrimidine nucleoside monophosphates

P.13 pyrimidine nucleoside diphosphates

If the distribution of purines and pyrimidines in the parent DNA is not random then it should be possible to observe the predominance of the favoured sequences from the relative amounts of the eight possible degradation products. Single purine nucleotides flanked by pyrimidine polynucleotides will give large amounts of products P1, P2 and P8, and none of P3, P4, P5, P6 and P7, whilst single pyrimidine nucleotides surrounds by purine polynucleotides will give types P3, P4, P5, P6 and P8 and not types P1, P2 and P8. Partial separation of the products may be effected by dialysis against distilled water or buffer solutions. Most molecules of P1 and P2 will be non-dialysable whilst the other six will be freely dialysable with the possible exception of P8. This molecule may be somewhat slower in dialysing as it is highly ionic. It is seen that the alkaline degradation of MDNA can theoretically give

evidence that will lead directly to the predominant pyrimidine linkages in the parent DNA and will allow the frequency of purine polynucleotides to be inferred.

Preparation of DNA. The source originally chosen for the DNA to be used in the degradation experiments was herring roe, but this quickly proved unsatisfactory. The roes were difficult to get in a fresh condition and those commercially available contained little or no DNA. The method of extraction of nucleoprotein used was essentially that of Pollister & Mirsky (1946). Out of six preparations only one yielded any nucleoprotein and although this was deproteinised by the method of Sevag, Lackman & Smollens (1938), dialysed against distilled water and freeze dried, it was used only for preliminary work. The most readily available source appeared to be ox spleen, and this proved a very successful one. The extraction procedure was modelled on that of Daly, Allfrey & Mirsky (1950) (see p. 69). It was found that the removal of some of the cellular material by straining through gauze after washing with physiological saline was unnecessary and gave rise to lower yields of DNA. Centrifugation of the DNA-containing cellular fraction and washing with physiological saline three times was necessary to remove pentose-containing material. The Spinco preparative ultracentrifuge was found invaluable for the effective sedimentation of débris after strong sodium chloride solution had been used to extract the nucleoprotein. Centrifugation of 1 litre

of extract at 65,000 g. for 30 minutes gave a perfectly clear supernatant solution of nucleoprotein, and the use of this centrifuge obviated the straining procedure used in the early stages of the extraction by Daly et al. Although the protein may be more readily removed from the nucleoprotein by raising the pH of the solution to 9 before shaking with chloroform and amyl alcohol, it was found that at pH 7.0 all the protein that could be detected by the Sakaguchi test could equally well be removed but at a slower rate. Finally the nucleic acid was not precipitated by alcohol, but dialysed free from small molecules (mainly sodium chloride) against distilled water, then freeze dried to a white solid.

The method of Kay, Simmons & Dounce (1952) using sodium dodecyl sulphate was also investigated for the extraction of DNA from beef spleen, but the two samples selected for mercaptalation and degradation were both prepared by the method similar to that of Daly et al. The analytical composition of these samples is given in Table 1. It is convenient to record the base ratios in mols per 4 atoms of phosphorus, as this brings the figures close to unity.

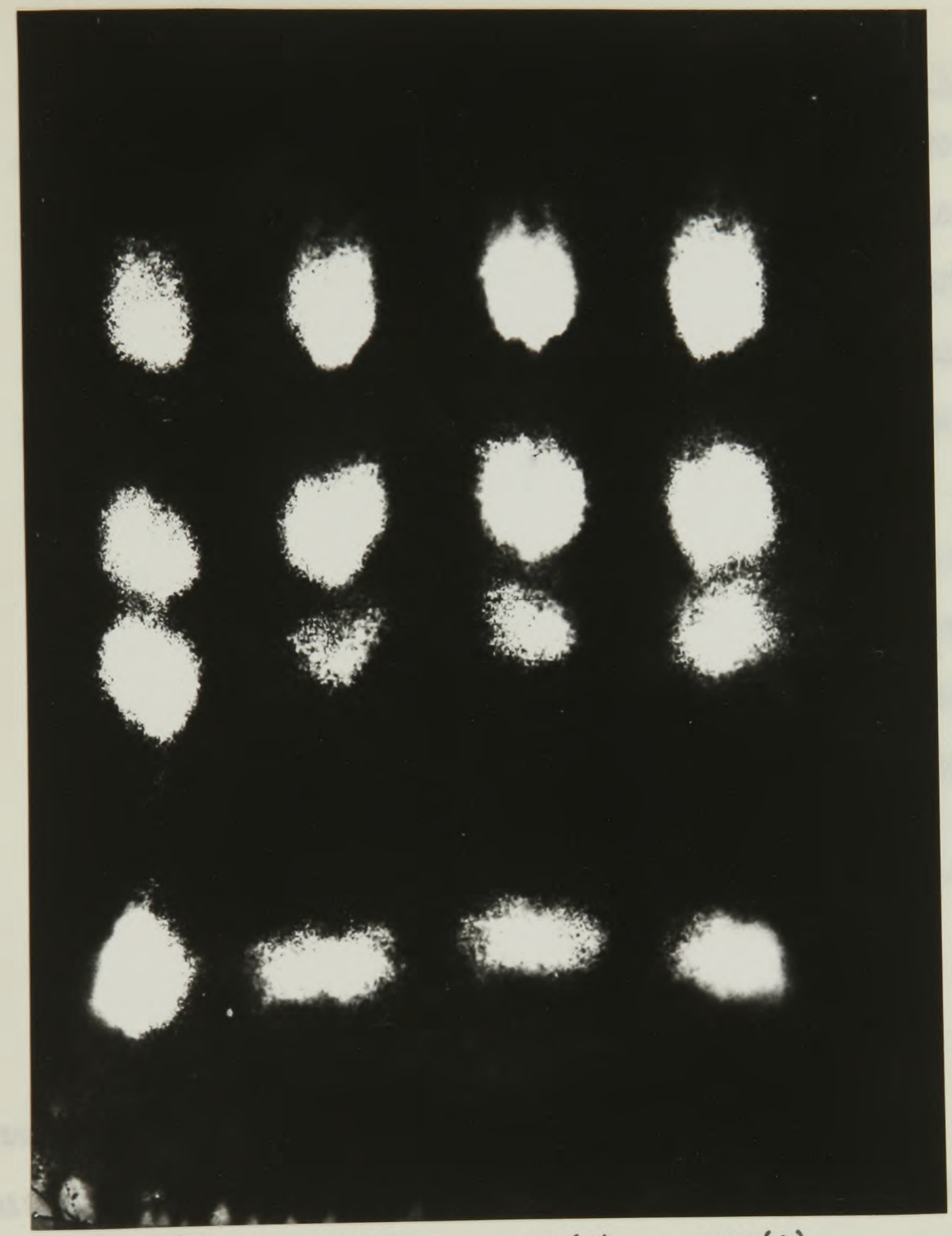
	DNA (1)	DNA (2)
nitrogen (% dry weight)	14.55	14.20
phosphorus (% dry weight)	8.88	8.55
nitrogen (atoms/4 atoms P)	14.52	14.71
guanine (mols/4 atoms P)	0.88	0.88
adenine (mols/4 atoms P)	1.02	1.05
cytosine (mols/4 atoms P)	0.89	0.81
thymine (mols/4 atoms P)	1.05	1.03
Total bases (mols/4 atoms P	3.84	3.77
thymine/cytosine (mol.prop.)	1.18	1.27
Dische extinction coeff. (per atom P)	1460	1510
ϵ_p (Extinction coeff. (per atom P)λ_{max} 259 mμ)	5830	5640

Table 1.

If the theory of Bowin, Vendrely & Vendrely (1948) that the composition of DNA from a single species is constant is correct, then the analytical data given above should closely correspond with those given by other authors for ox tissues. The nitrogen and phosphorus content is somewhat lower than that given for ox spleen DNA by Wyatt (1951) and for calf thymus DNA given by Gulland, Jordan & Threlfall (1947), Tamm, Hodes & Chargaff (1952) and Kay, Simmons & Dounce (1952), but the atomic nitrogen/phosphorus ratio agrees closely with all these authors. The predominance of adenine and thymine over guanine and

cytosine is also very apparent. The purine and pyrimidine analyses were performed by the hydrolytic and paper chromatographic methods described by Wyatt (1951). Fig.9 shows a chromatogram of these bases found in DNA (1).

Mercaptalation of DNA. In the first mercaptalation experiment, 1 g. of DNA (1) was suspended in 40 ml. of ethyl mercaptan, after which 1 ml. of hydrochloric acid (sp.gr. 1.18) was added. The mixture was shaken vigorously by a mechanical shaker for 48 hours at room temperature, then 100 ml. water were added. The aqueous layer was removed and neutralised with sodium bicarbonate. The unchanged ethyl mercaptan was extracted with ether and the aqueous solution was dialysed twice against 5 litres of distilled water at 2°C. Freeze drying of the dialysed solution gave 48.6 mg. of white solid (MDNA(1)). It was obvious from the low yield that the reaction had not proceeded along the theoretical lines of fig.8, and uncontrolled degradation had occurred. The analytical data of the freeze-dried solid is given in Table 2. Figures for the parent DNA are also given for convenience.



markers DNA(1) DNA(1) DNA(1)

Solvent: iso-propanol(138 ml.) HCl(sp.gr.1.18, 31.7 ml.) water(30.5 ml.).

Downward solvent development.

G = guanine

A = adenine

C = cytosine

T = thymine

Fig.9. Ultra-violet Chromatography of Hydrolysed Ox Spleen DNA(1).

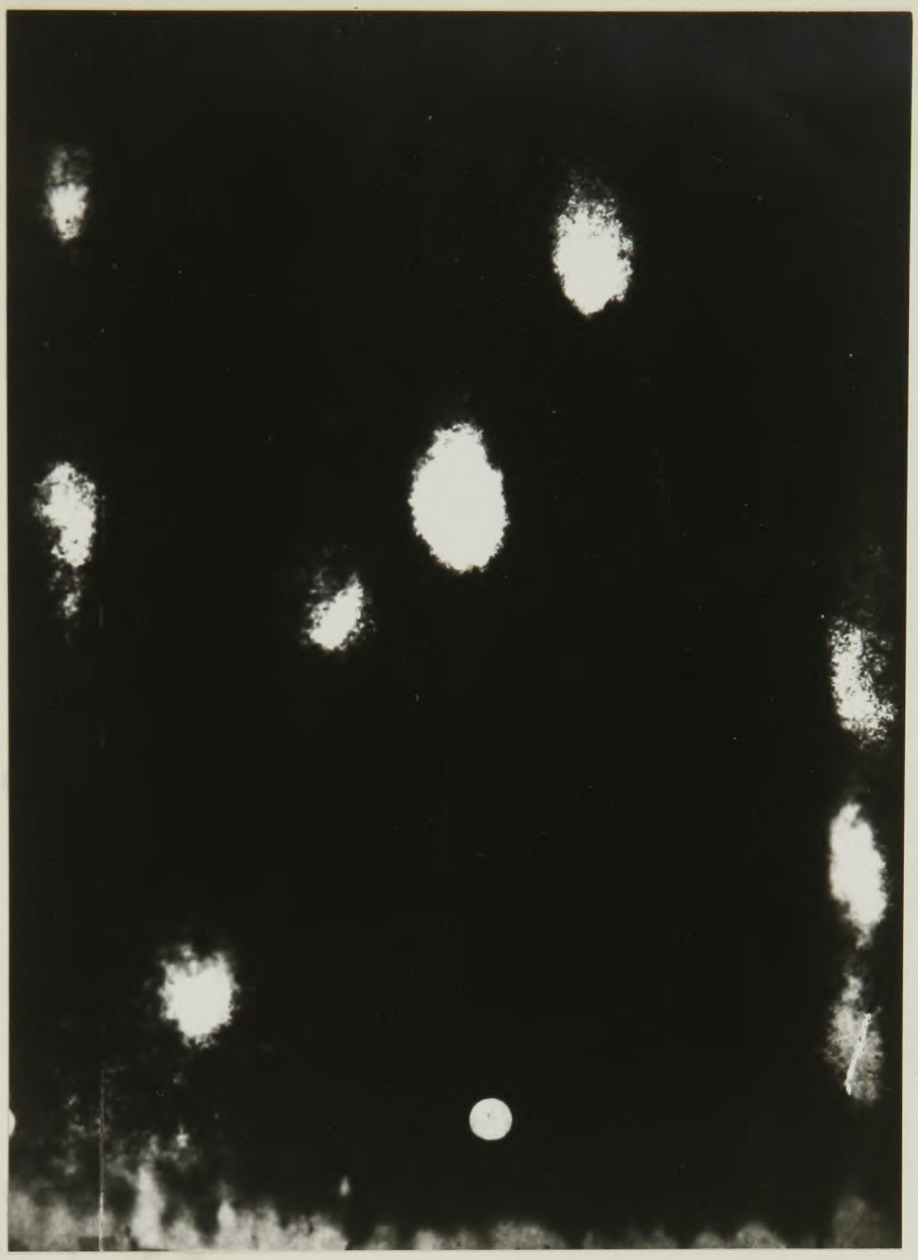
	DNA (1)	MDNA(1)
nitrogen (% dry weight)	14.55	12.20
phosphorus (% dry weight)	8.88	6.40
sulphur (% dry weight)	-	2.52
nitrogen (atoms/4 atoms P)	14.52	16.88
sulphur (atoms/4 atoms P)	-	1.52
guanine (mols/4 atoms P)	0.88	0
adenine (mols/4 atoms P)	1.02	0.03
cytosine (mols/4 atoms P)	0.89	2.72
thymine (mols/4 atoms P)	1.05	4.28
thymine/cytosine (mol.prop.)	1.18	1.58
Extinction coeff. per atom P (at λ_{max})	5830	6640

Table 2.

From theoretical considerations it is expected that the atomic nitrogen/phosphorus ratio should drop upon the preparation of MDNA, since the phosphodiester bonds should remain largely intact and the purines, which are responsible for 67% of the total nitrogen atoms found in the four bases, should have been removed. MDNA(1) shows an increased nitrogen/phosphorus ratio compared with its parent DNA, yet the purines are virtually removed. This again suggests random fission of nucleotide bonds and it has been observed by Dekker, Michelson & Todd (1953) that acid hydrolysates of DNA contain large amounts of

nucleoside diphosphates. Incomplete mercaptalation could lead to oligonucleoside diphosphates large enough to resist dialysis, and indeed the sulphur/phosphorus ratio indicates incomplete mercaptalation. Since two sulphur atoms replace every purine, there should be of the order of 4 atoms of sulphur for every 4 of phosphorus. Finally the thymine/cytosine ratios would not be expected to vary significantly between MDNA(1) and DNA(1), but in this instance there is a considerable variation.

The combined dialysate solutions (fluid outside the membrane) were evaporated at 40° until the volume had dropped to 10 ml., when a white solid separated. This was removed before final evaporation to dryness. Extraction of the dry residue with chloroform and evaporation yielded a yellow syrup. This gave a strong colour with the Dische reagent, contained no phosphorus, and when treated with hot acid evolved the vapour of ethyl mercaptan. It was mainly 2-deoxyribosediethylmercaptal. The dry dialysate residue and the previously separated solid were both chromatographed in a solvent of iso-propanol and 2N hydrochloric acid. Fig.10 represents a photograph of the ultraviolet absorbing components in this chromatogram. The least soluble portion of the residue is seen to consist almost entirely of the two purines guanine and adenine, whilst the remainder consisted of 3 faster running components. When the chromatogram was sprayed with perchloric acid - ammonium molybdate spray (Hanes & Isherwood, 1949) to detect phospho-esters, only the middle one of the three fast running



A. Less soluble portion of MDNA(1) dialysate residue.

B. Thymine.

C. Cytosine.

D. Adenine.

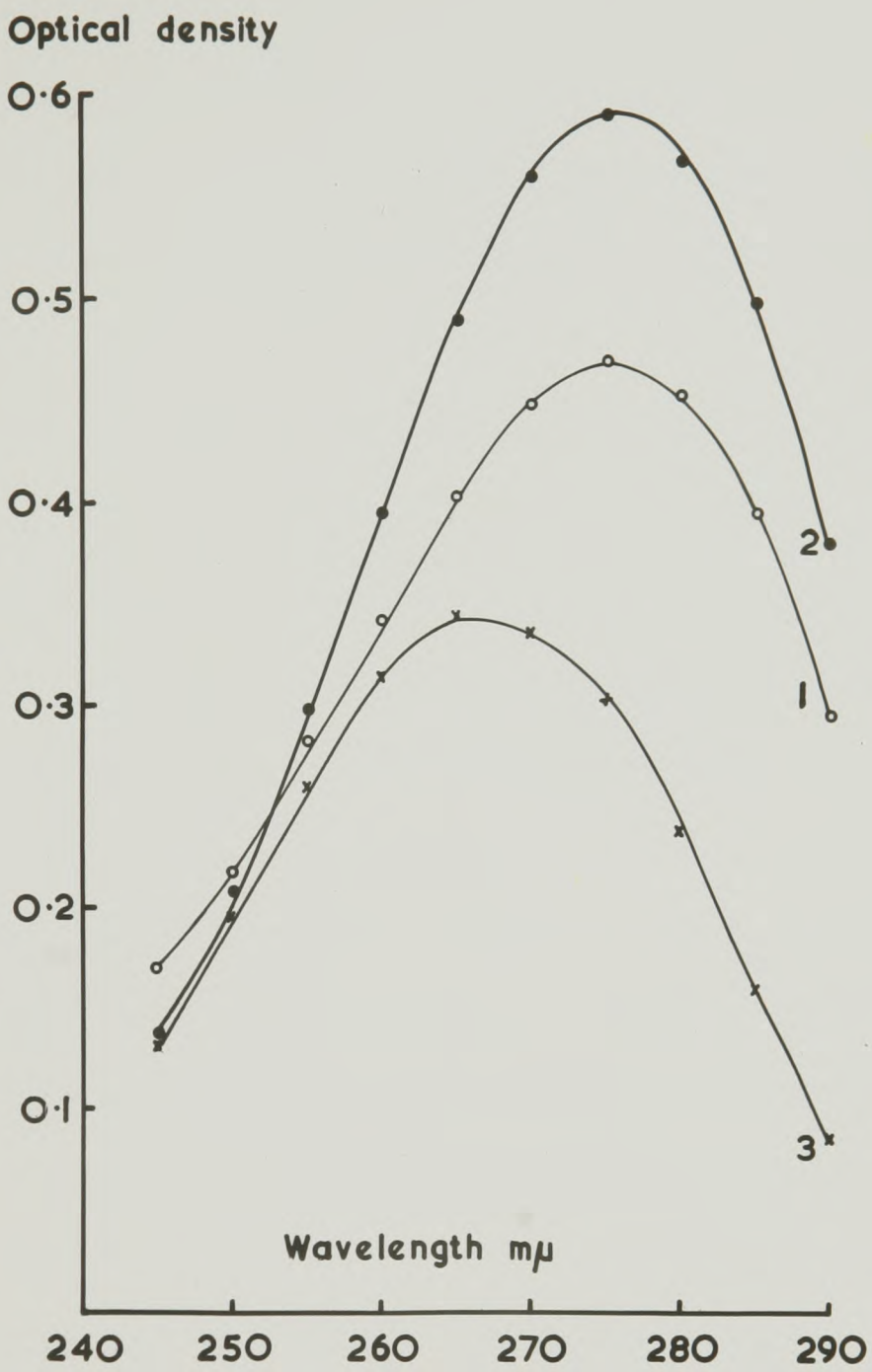
E. Guanine.

F. More soluble portion of MDNA(1) dialysate residue.

Solvent: iso-propanol(138 ml.) HCl(sp.gr.1.18, 31.7 ml.) water(30.5 ml.).

Whatman No. 1 paper. Downward solvent development.

Fig 10. Ultra-violet Chromatography of MDNA(1) Dialysate Residue.



1. = Component 1 in fig. 10

2. = Component 2 in fig. 10

3. = Component 3 in fig. 10

Fig 11. Ultra-violet Absorption of Components of MDNA(1) Dialysate Residue.

components contained phosphorus. In a similar chromatogram, these three spots were cut out, eluted with dilute hydrochloric acid, and their absorption curves plotted. The fastest running component gave a sharp peak at 267 mμ similar to that for thymine, and the other two components gave sharp peaks at 275 mμ similar to that for cytosine (see fig.11).

It may be concluded that under the conditions of preparation of MDNA(1) considerable hydrolysis of the nucleic acid polymer accompanies the hydrolytic cleft of the purines. The presence of mercaptalated deoxyribose and pyrimidine-containing components in large quantities in the dialysate suggests that the conditions used were too vigorous. It was considered that the heterogeneous nature of the reaction was undesirable. Both DNA and hydrochloric acid are relatively insoluble in ethyl mercaptan with the result that vigorous shaking of the reaction vessel is necessary to maintain a finely divided suspension. No means of making the reaction mixture homogeneous was discovered. Solvents such as methanol, ethanol and dioxan in which both ethyl mercaptan and aqueous hydrochloric acid are soluble were unsuccessful since the relative proportions could not be arranged in such a way that the nucleic acid would dissolve. In another series of trial experiments very dilute hydrochloric acid (pH 1.6) was shaken with DNA and ethyl mercaptan until the two layers were interdispersed. These always furnished products deficient in sulphur.

During the course of this work, Jones & Letham (1954) reported high yields of a mercaptalated nucleic acid derivative using thioglycollic acid and anhydrous zinc chloride on DNA. It is uncertain, however, whether under these conditions the thioglycollic acid behaves merely as a mercaptalating agent or as an acylating agent also, forming esters with the DNA or its derivatives.

Further mercaptalation of DNA. Two successful preparations of MDNA were obtained, using a technique very similar to that used for MDNA(1) (see p.71). The essential differences are (i) greater attention to vigorous mechanical shaking especially whilst adding the acid, (ii) the use of less acid, (iii) a shorter reaction time and (iv) neutralisation of acid at the end of the reaction by addition of the calculated amount of sodium bicarbonate, whilst the shaking is continued. MDNA(2) was prepared from DNA(1) and MDNA(3) from DNA(2). Analytical data for these, together with their parent DNA's for comparison, is given in table 3.

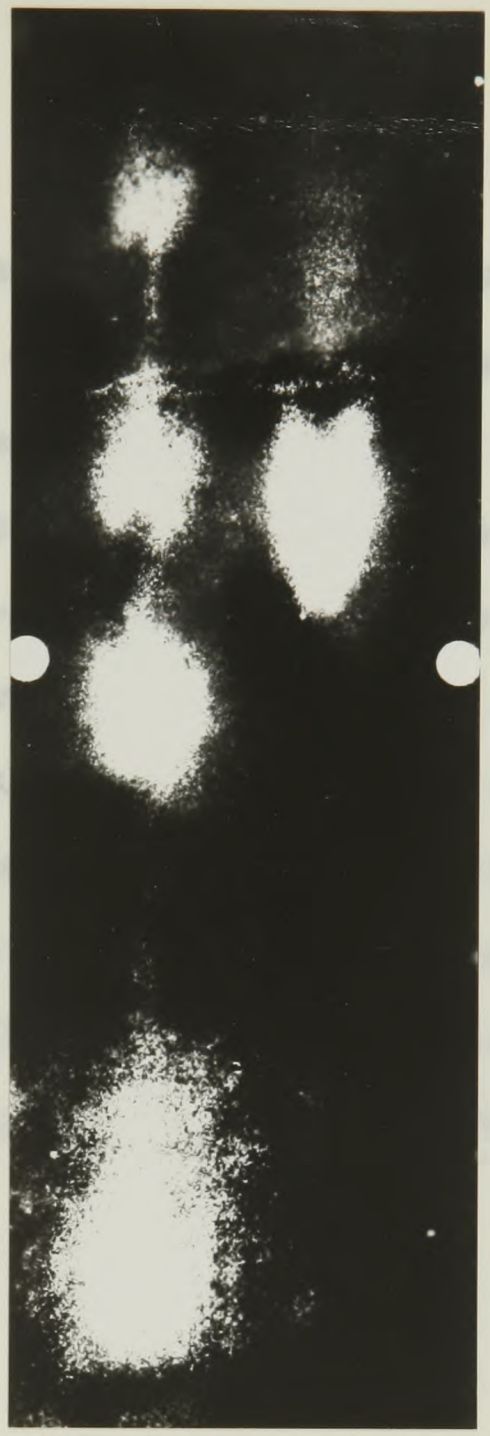
	DNA(1)	MDNA(2)	DNA(2)	MDNA(3)
nitrogen (% dry weight)	14.55	6.28	14.20	6.84
phosphorus (% dry weight)	8.88	8.11	8.55	9.02
sulphur (% dry weight)	-	6.79	-	7.32
nitrogen (atoms/4 atoms P)	14.52	6.88	14.71	6.72
sulphur (atoms/4 atoms P)	-	3.24	-	3.16
guanine (mols/4 atoms P)	0.88	0	0.88	0
adenine (mols/4 atoms P)	1.02	0	1.05	0
cytosine (mols/4 atoms P)	0.89	1.08	0.81	1.19
thymine (mols/4 atoms P)	1.05	1.53	1.03	1.58
thymine/cytosine (mol.prop)	1.18	1.42	1.27	1.33
Extinction coeff. (per atom P) (λ_{max} 259 mμ)	5830	-	5640	-
Extinction coeff. (per atom P) (λ_{max} 270 mμ)	-	4280	-	3600
Dische value (per atom P)	1460	895	1510	917

Table 3.

The dialysed, freeze dried, samples in table 3 represent a 67% and 76% yield respectively for MDNA(2) and MDNA(3), if allowance is made for approximately 20% of the parent DNA's being composed of purines. The dialysates were concentrated to dryness in vacuo, and when chromatographed in iso-propanol and 2N hydrochloric acid as a solvent,

contsined only guanine and adenine as detectable ultraviolet absorbing components (see fig.12). The dialysate residues contained no material extractable with chloroform, and had a very low phosphorus content. For instance, in MDNA(3) only 3.4% of the phosphorus present in the parent DNA became dialysable, and of this 3.0% was organically bound and 0.4% was free phosphate. This residue weighed 1.26 grams of which 0.65 grams is the amount of sodium chloride calculated to be present from neutralising the hydrochloric acid of the reaction, and 0.4 grams were purines.

Examination of table 3 shows close agreement in composition of the two MDNA's. No purines could be detected chromatographically in these compounds after hydrolysis. In view of this the number of nitrogen atoms per 4 atoms of phosphorus appears to be rather high, possibly due to the loss of some phosphorus in the preparation. The sulphur content is in excellent agreement with the calculated value of approximately 4 atoms per 4 atoms of phosphorus. The cytosine and thymine contents have increased somewhat, but the molar ratio of these two bases is virtually unchanged after mercaptalation. It may be concluded that the analysis of MDNA(2) and MDNA(3) fits in well with compounds having the theoretical structure of fig.8. It was not possible to do molecular weight measurements on the MDNA's, but in view of the appearance of some dialysable organically bound phosphorus, they are expected to have a lower molecular weight than their parent DNA's.



Solvent: iso-propanol(138 ml.) HCl (sp.gr.1.18, 31.7 ml.) water (30.5 ml.).

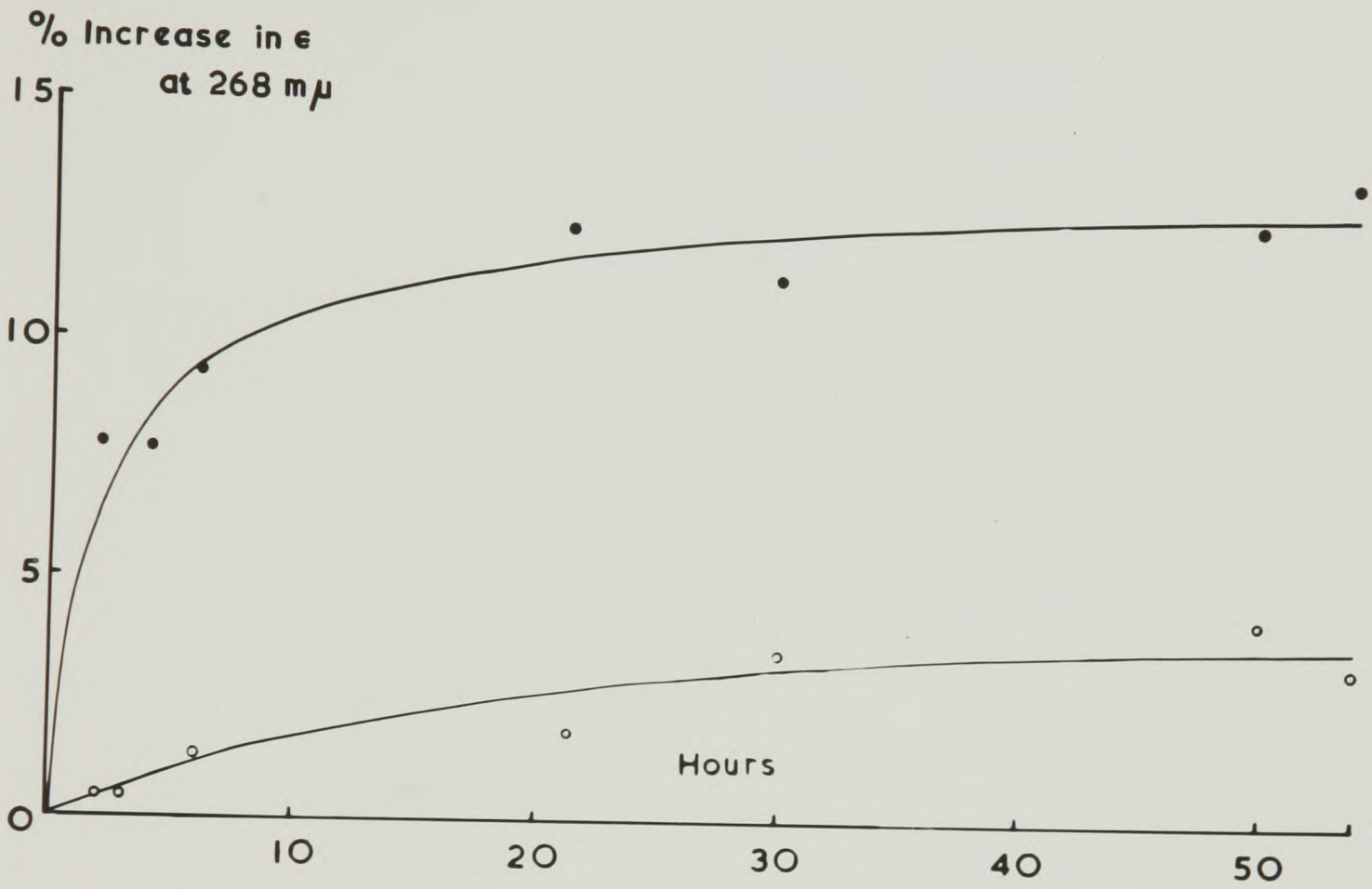
Downward solvent development.

Fig.12. Ultra-violet Chromatography of MDNA(3) Dialysate.

Action of alkali on MDNA. The rate of action of alkali on MDNA may be examined by following the increase in optical density of the solution at 270 mμ. As degradation occurs the optical density rises. It is found (fig.13) that MDNA dissolved in 0.5 N sodium hydroxide solution at 37°C attains its maximum optical density after about 72 hours. This formed the basis of a large-scale experiment (see p.72) in which 0.5 g. of MDNA(3) were treated under these conditions. After neutralisation of the sodium hydroxide with hydrochloric acid the reaction solution was freed from sodium chloride and small molecules by dialysis against distilled water, and finally freeze dried. The product was 0.16 g. of a white amorphous solid. This pyrimidine polynucleotide (PPN) had the composition shown in table 4. Its predecessors MDNA (3) and DNA(2) are also shown.

	INA(2)	MINA(3)	PPN
Nitrogen (% dry weight)	14.20	6.84	7.64
phosphorus (% dry weight)	8.55	9.02	8.41
sulphur (% dry weight)	-	7.32	1.48
nitrogen (atoms/4 atoms P)	14.71	6.72	8.04
sulphur (atoms/4 atoms P)	-	3.16	0.68
guanine (mols/4 atoms P)	0.88	0	0
adenine (mols/4 atoms P)	1.05	0	0
cytosine (mols/4 atoms P)	0.81	1.19	1.34
thymine (mols/4 atoms P)	1.03	1.58	1.88
thymine/cytosine (mol.prop.)	1.27	1.33	1.40
Extinction coeff. (per atom P) (at λ_{max} 259 mμ)	5640	-	-
Extinction coeff. (per atom P) (at λ_{max} 270 mμ)	-	3600	5920
Dische value (per atom P)	1510	917	703

Table 4.



● = MDNA

○ = DNA

Fig. 13. Alkaline Hydrolysis of DNA and MDNA.

Properties of PPN. If the alkaline degradation of MDNA has taken place as envisaged on p.28, then the existence of a non-dialysable PPN shows that tracts of adjacent pyrimidine nucleotides occur. These non-dialysable molecules will be of the types P1 and P2 described on p.30. The retention of sulphur by PPN is consistent with molecules of type P2, and the greater nitrogen/phosphorus ratio for PPN over MDNA favours molecules of type P1.

If a section of a molecule of MDNA is represented by fig.14, in which a favoured tract of pyrimidine nucleotides is terminated on either side by a mercaptalated deoxyribose molecule, then, with some reservations, postulates can be made on how this section will behave on alkaline hydrolysis.

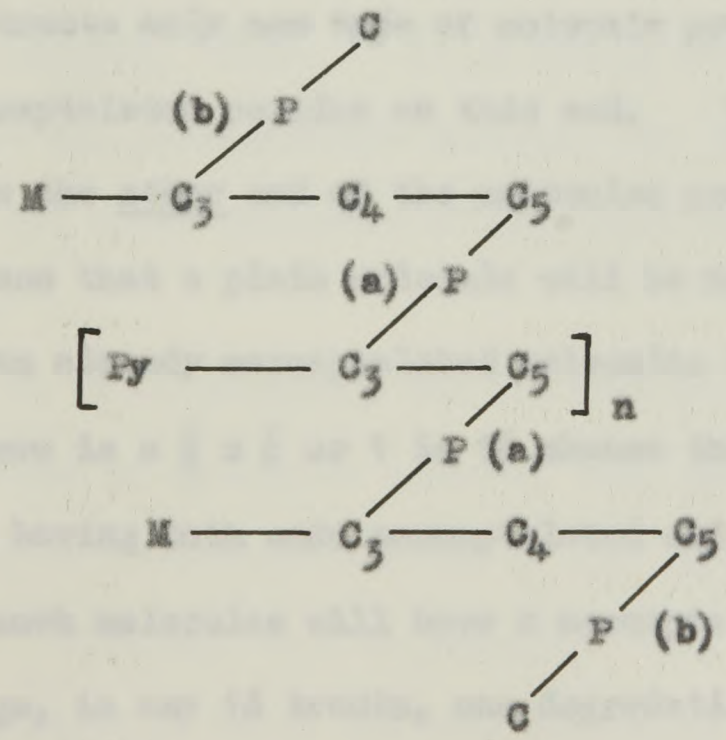


Fig. 14

1. It is likely that there is a 50% chance that either the phosphodiesters marked with "a's" or those marked with "b's" in fig.14 will cyclise first.

2. If the "a" phosphodiesters cyclise first, then the final result of alkaline hydrolysis will be that no mercaptalated sugar will terminate the tract.

3. If the "b" phosphodiesters cyclise first then it is likely that there is a 50% chance that a mercaptalated sugar will terminate the tract.

Considering <u>one</u> end of the molecular species obtained from the hydrolysis of commonly occurring tracts of pyrimidine nucleotides as in fig.14, then in the light of the above postulates, on the average, in any four tracts only one type of molecule produced will bear a terminal mercaptalated residue on this end. The same argument may be applied to the <u>other</u> end of the molecules produced. Hence there is a 3 in 4 chance that a plain molecule will be mercaptalated and a 1 in 4 chance that an already mercaptalated molecules will be mercaptalated. Therefore there is a $\frac{1}{4} \times \frac{1}{4}$ or 1 in 16 chance that a tract will give rise to molecules having both ends mercaptalated and a $\frac{3}{4} \times \frac{1}{4}$ or 3 in 16 chance that such molecules will have a mercaptal on one end only. Hence, on the average, in any 16 tracts, one degradation molecule produced will have both ends mercaptalated, 3 will have one end mercaptalated and 12 will not be mercaptalated. Since each mercaptalated sugar has two

sulphur atoms, 16 tracts will give products having 10 sulphur atoms, but from the analysis of PPN for every 6.8 sulphur atoms there are 32.2 pyrimidine molecules. Hence for every 10 sulphur atoms there will be 47.5 pyrimidine molecules. This allows each tract to have products with 3 pyrimidine molecules, and enables the average molecular weight for PPN to be calculated as approximately 925 for the free acid or 975 for the sodium salt.

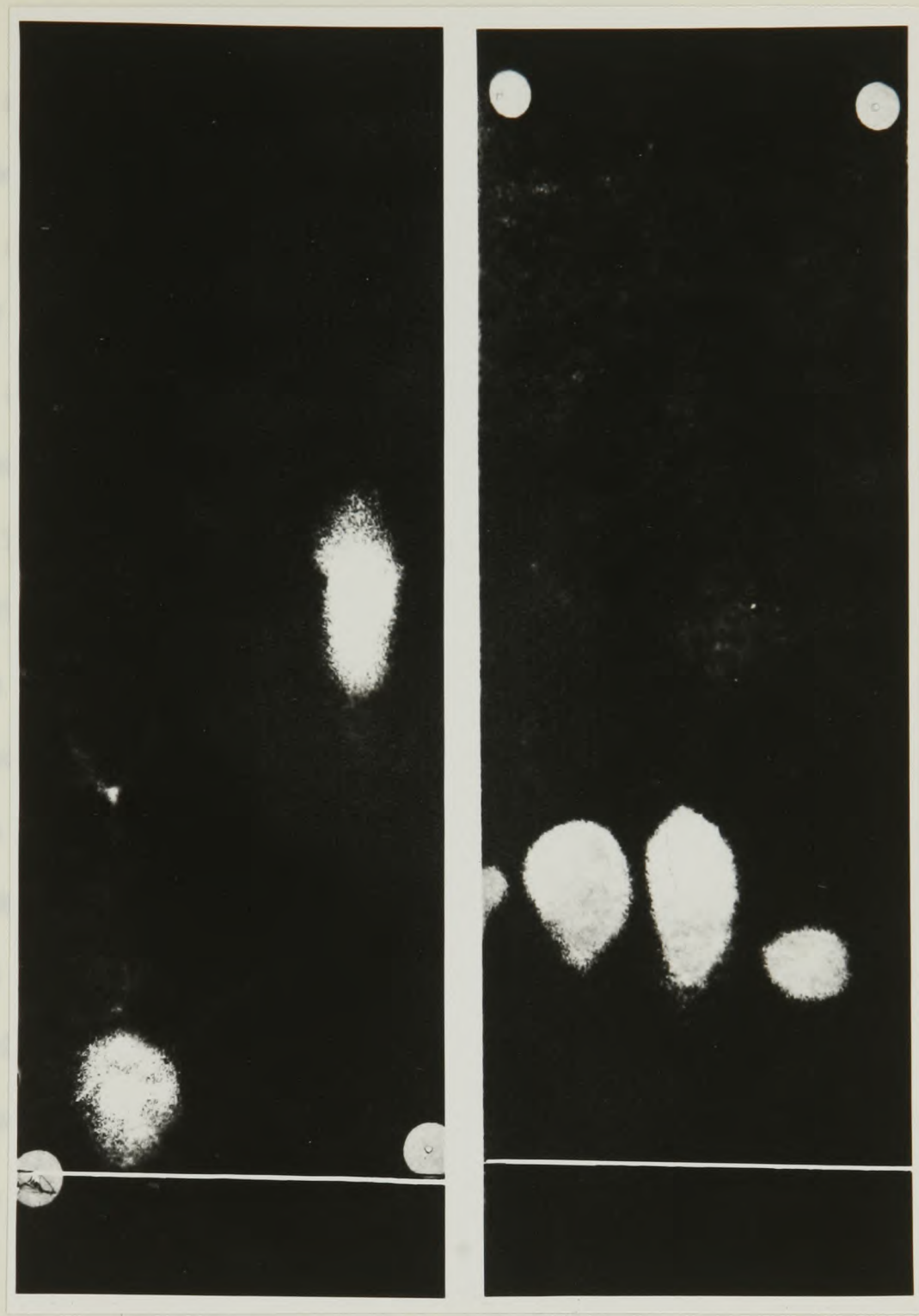
This calculation will be inaccurate if there is an unequal tendency for the "a" and "b" phosphodiesters in fig.14 to cyclise and in the case of the "b" phosphoesters, if there is unequal lability of the vicinal phospho-sugar bonds in the cyclic esters. In similar cases, for instance the alkaline hydrolysis of RNA, there appears to be no evidence on the relative rates of fission of the cyclic ester bonds, though it appears that the P-O bonds are less stable than the C-O bonds (Blumenthal & Herbert, 1945). It is likely however that both statements 1 and 3 on p.50 are approximately correct, and the variations to be expected will not alter the order of magnitude for the average molecular weight of PPN.

If, as to be expected, the DNA from which PPN was prepared is a gross mixture of molecular species, then the structures envisaged above represent an oversimplification of the types of molecules to be found in PPN. Although this is probably true, it is likely that particular sequences of bases are very common in DNA from a single source and the studies given here are intended to discover these. That PPN

does not consist of molecules of vastly differing molecular weight is supported by its behaviour towards paper electrophoresis. Both at pH 3.5 and pH 7.9 the substance moved as a single component (fig.15). In two chromatographic solvents it was immobile during paper chromatography, as are MDNA and DNA.

Attempts were made by Dr Kent to determine experimentally the calculated value for the molecular weight of PPN using osmotic pressure measurements, but without success. A solution of PPN in water was placed in a dialysis membrane which was attached to the lower end of a calibrated capillary tube from a Conway burette. The membrane was immersed in water at a constant temperature of 2°C, but no satisfactory agreement was obtained from a series of experiments.

Properties of PPN dialysate. The dialysate solutions from the preparation of PPN were evaporated to dryness *in vacuo* at 40°C and 0.9 grams of white solid were obtained. This substance gave a smell of ethyl mercaptan when heated with acid, and gave a strong positive Dische test for 2-deoxysugars. It had a phosphorus content of 3.02%, of which 2.94% was organically bound; this meant that 60.3% of the original MDNA phosphorus had become dialysable. If the hydrolysis of MDNA has gone in the expected manner, then the presence of nucleosidic end groups in quantity in PPN would favour the existence of the larger proportion of phosphorus bound to dialysable molecules. The small percentage of inorganic phosphorus in the dialysate indicates that



guanine cytosine | PPN | deoxy- cytidine 5' phosphate | PPN | thymidine diphosphate

0.05M borate buffer pH 7.9 15.4 v/cm. 1.5 hrs.

0.05M acetate buffer pH 3.5 15.4 v/cm. 1.5 hrs.

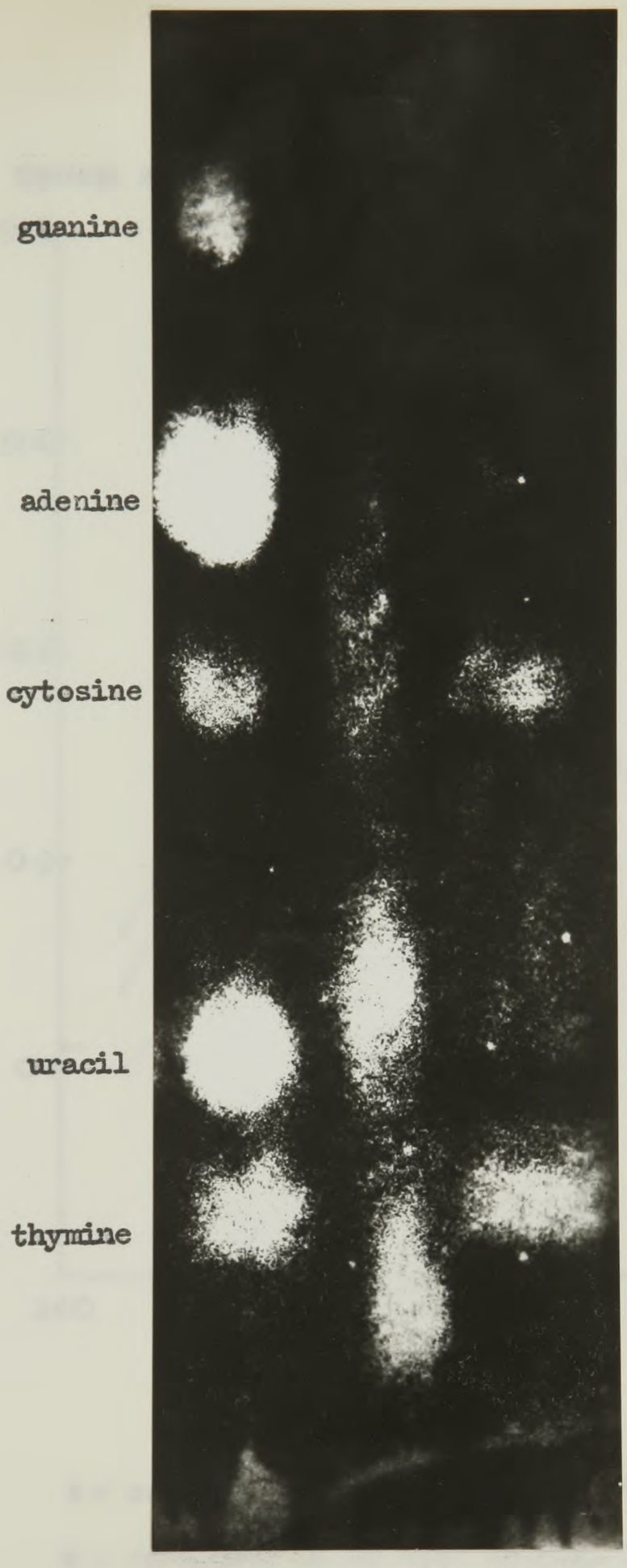
Fig 15. Electrophoresis of PPN.

hydrolysis has not resulted in random splitting of C-O-P bonds to any extent as in the preparation of "thymic acid". Extraction of the dialysate with chloroform yielded no detectable residue. This precludes the presence of mercaptalated deoxyribose.

When examined by paper chromatography in a solvent of iso-propanol and 2N hydrochloric acid, the dialysate revealed four components. Three of these components (R_f 0.49, 0.67 and 0.79) absorbed ultraviolet light as shown in fig.16. When sprayed with perchloric acid - ammonium molybdate solution (Bandurski & Axelrod, 1951) the paper showed three phosphorus containing spots. Two of these were coincident with the ultraviolet absorbing components of R_f 0.67 and 0.79, and the third was at the solvent front. In another chromatogram the ultraviolet absorbing spots were eluted separately with 5 ml of N/10 hydrochloric acid and their absorption characteristics measured (see fig.17), and in a third chromatogram it was discovered that all four spots when cut out gave a positive Dische colouration. These chromatographic results are summarised in table 5.

R_f value	UV absorpn. λ_{max}	Absorbence ratios A_{250}/A_{260}	A_{280}/A_{260}	Base with same absorptn.	P content	Dische test	mercaptan test
0.49	275	0.619	1.33	cytosine	-	+	-
0.67	275	0.604	1.27	cytosine	+	+	+
0.79	265	0.626	0.746	thymine	+	+	+
1.00	-	-	-	-	+	+	+

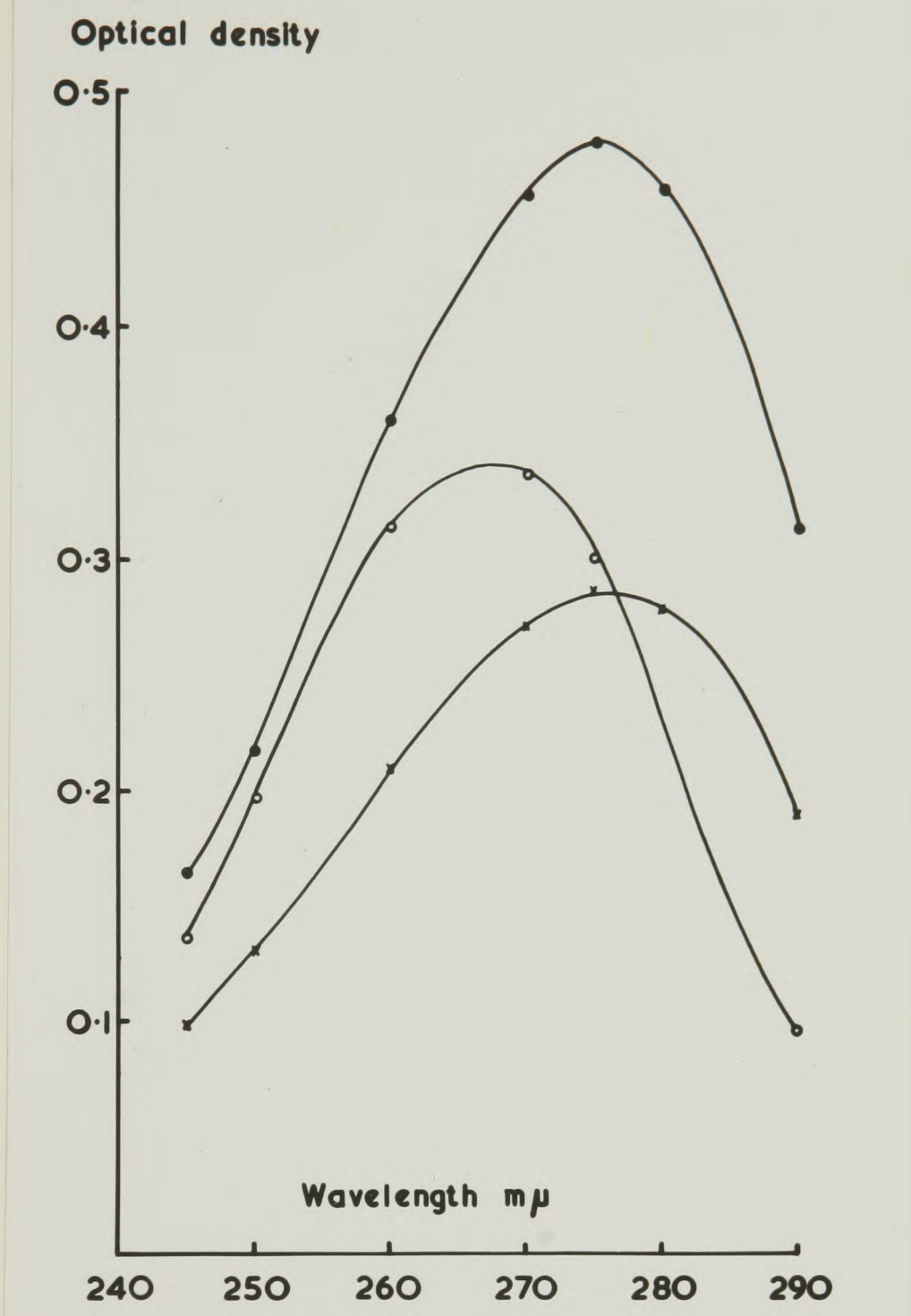
Table 5.



Solvent: iso-propanol(138ml.) HCl (sp.gr.1.18, 31.7 ml.) water(30.5 ml.).

Downward solvent development.

Fig.16. Ultra-violet Chromatography of PPN dialysate.

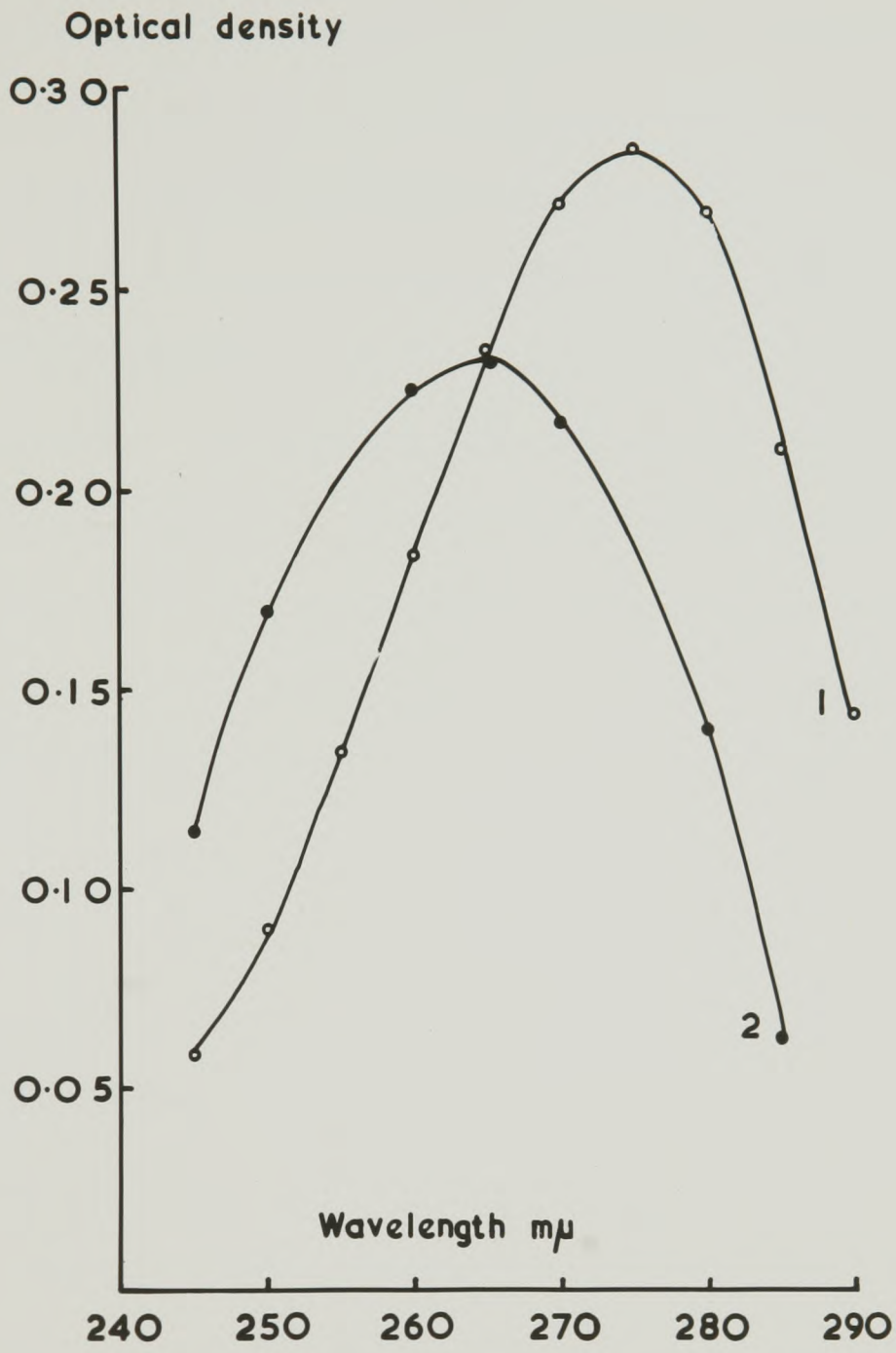


× = component of R_f 0.49 in fig. 16.

● = component of R_f 0.67 in fig. 16.

o = component of R_f 0.79 in fig. 16.

Fig. 17. Ultra-violet Absorption of Components of PPN Dialysate.



o = component chromatographically identical with cytosine.

● = component chromatographically identical with thymine.

Fig. 18. Ultra-violet Absorption of Components of Hydrolysed PPN Dialysate.

When the dialysate was hydrolysed with perchloric acid and chromatographed it gave two phosphorus free spots identical in R_f values and ultraviolet absorption maxima to cytosine and thymine (figs.16 and 18).

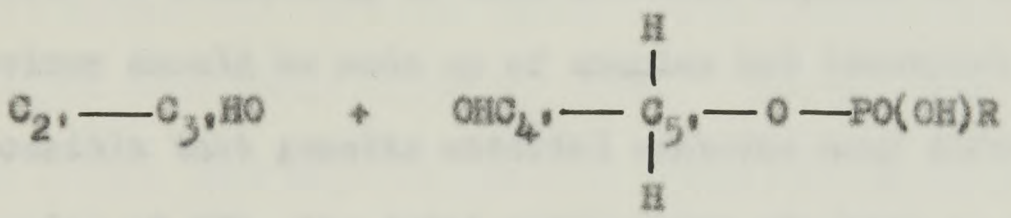
Although the results summarised in table 5 do not provide conclusive evidence for the identification of the components of PFN dialysate they provide important information on the diversity and nature of these components. The ultraviolet absorbing components of R_f 0.67 and 0.79 appear to be pyrimidine nucleosides attached by a phosphoryl link to mercaptalated sugar derivatives, compounds of type P2 and P4 on p.30. From the relatively high R_f values it is expected that these components represent monopyrimidine derivatives. This provides evidence for single pyrimidine nucleotides surrounded by purine nucleotides in the parent DNA. Compounds of type 3 which confirm this conclusion cannot be precluded since deoxycytidine and thymidine have very similar chromatographic mobility to the components under consideration (Wyatt, 1955), and the presence of these simpler compounds would be masked. It is likely that the high ionic charge on pyrimidine dinucleotides with one or two terminal mercaptalated sugar 4' phosphates renders them non-dialysable against distilled water; they do not appear to be in the dialysate. The component of R_f 1.0 may be mercaptalated deoxyribose phosphates, degradation products expected from DNA with adjacent purine nucleotides. The component of R_f 0.49 is not easy to identify. Although its mobility is similar to cytosine the presence of deoxyribose in it suggests that it may be a cytosine deoxyriboside.

Base sequence in Ox spleen DNA. From the study of PPN prepared from ox spleen DNA it is clear that pyrimidine nucleotides occur in tracts surrounded by purine nucleotides in this nucleic acid. On the average three pyrimidines to a tract is favoured. Since this is a statistical mean the presence of pyrimidine polynucleotides of much higher molecular weight must be very limited. There is a predominance of thymine in the tracts of adjacent pyrimidine nucleotides, the ratio thymine/cytosine for PPN being 1.4. This is balanced by a greater variety of cytosine derivatives in the dialysable products from the preparation of PPN. 5-methylcytosine was not determined in these experiments. Examination of PPN dialysate provides evidence for single pyrimidine nucleotides flanked by purine nucleotides, and also adjacent purine nucleotides in ox spleen DNA.

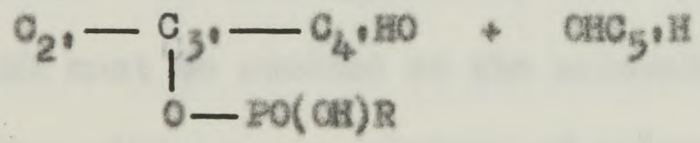
The results from this series of experiments do not easily lend themselves to comparison with similar results obtained from herring-roe DNA (Lucy & Kent, 1953; Kent & Lucy, 1954; Kent, Lucy & Ward, 1955) (see back cover). Mercaptalated nucleic acids from herring-roe are seen to diverge much more widely from the theoretical concept of fig.8. than those from ox spleen; this is evident from the higher base/phosphorus ratios. The loss of phosphorus in this way in the preparation of MDNA is a measure of the extent of uncontrolled degradation that has occurred and if structural conclusions on the parent DNA are to be drawn from the degradation of MDNA it is essential that MDNA itself should be as undegraded as possible. The advances in the technique clearly obvious from the mercaptalation of ox spleen DNA, when still further improved, may provide

interesting comparisons between the sequence of pyrimidine bases found in the DNA's from different sources.

Degradation of PPN. It would be desirable to effect the controlled degradation of PPN in order to identify the components conclusively. In one scheme phosphomonoesterase may be used to remove the end phosphoesters from mercaptalated molecules. These molecules now have vicinal hydroxyls between the 3'4' position or 4'5' position and will be readily oxidised by potassium periodate to the following products (R represents the rest of a PPN molecule):



or



The periodate may also be expected to cleave mercaptal groups from deoxyribose. The remaining pentose fragments attached to PPN may now be removed by alkaline hydrolysis and the resultant "core" investigated by column chromatography.

FRACTIONATION OF OX SPLEEN DNA.

In general, composition of preparations of DNA from a specified biological source show remarkably little deviation, yet there is considerable variation in the proportions of nitrogenous bases found in those from different sources. This has led to the classification of DNA's into the AT types rich in adenine and thymine, and the GC types rich in guanine and cytosine. In the light of the current conception that DNA is an important constituent of the genes it would be surprising if this material capable of such variable behaviour should be made up of complex but identical molecules. It is possible that genetic material contains many different though similar molecules of DNA, the total composition of which is an average value. The constancy of this value may represent the unchanging character of the species. If this hypothesis were true then the absolute base sequence in DNA must be studied at the molecular level. At the present time studies with a gross mixture of molecules will enable only favoured sequences to be recognised. The first attempts at DNA fractionation, based on fractional centrifugation (Wyatt, 1950) and charcoal adsorption (Zamenhof & Chargaff, 1951), were unsuccessful, but in 1953 two similar and successful fractionations were reported (Chargaff, Crampton & Lipshitz, 1953; Brown & Watson, 1953). Both these methods depended on the dissociation of DNA and protein by sodium chloride in solution. In Chargaff's fractionation, the native nucleoprotein from calf thymus

was dissociated by successive extraction with sodium chbride solutions of increasing concentration, whereas Brown & Watson employed artificial "nucleoproteins" formed by adsorption of DNA into histone coated keiselguhr columns. In both cases the results were closely similar; DNA fractions richer in guanine and cytosine were found to be extracted by the sodium chloride solutions of lower ionic strength whilst the adenine and thymine rich fractions were less easily dissociated from their protein.

Fractionation with heparin. During the preparation of ox spleen DNA for the degradation experiments, some of the nucleoprotein was not deproteinised but used for fractionation experiments involving a different principle from those described above. It was discovered by Chargaff & Olson (1937) that heparin forms a highly insoluble complex with protamines, devoid of anticoagulant activity. Further examination of this type of complex showed that the histone protein associated with DNA also formed a similar insoluble white compound with heparin. Making use of such a complex a stepwise precipitation of the protein from the nucleoprotein was envisaged in which it was expected that the more weakly bound DNA-protein links would be broken preferentially. Consequently an experiment was devised in which a number of tubes containing nucleoprotein suspended in 0.14 M saline were shaken with a quantity of the sodium salt of heparin varying from about 0.5 to 3.0 times the weight of nucleoprotein (see p. 74). After centrifugation, the separate supernatants containing nucleic acid and some nucleoprotein were deproteinised, dialysed and freeze dried. The results of a preliminary experiment are shown in Table 6.

Tube No.	1	2	3	4	5	Unfractionated DNA (DNA(2))
nitrogen % dry weight	6.20	3.12	3.06	3.18	2.99	14.90
phosphorus % dry weight	3.6	2.6	2.6	2.2	2.4	8.55
guanine % total bases	23.3	24.7	21.8	20.8	15.6	23.4
adenine % total bases	29.5	33.4	32.6	35.2	37.2	27.9
cytosine % total bases	32.7	16.1	21.5	13.4	14.1	21.5
thymine % total bases	14.5	25.8	24.1	30.6	33.1	27.3
total bases (referred to adenine as unity)	3.39	2.99	3.07	2.84	2.69	3.59
adenine/guanine	1.27	1.35	1.49	1.69	2.38	1.19
thymine/cytosine	0.44	1.60	1.12	2.29	2.34	1.27
$\frac{\text{adenine and thymine}}{\text{guanine and cytosine}}$	0.79	1.45	1.31	1.92	2.37	1.23
adenine/thymine	2.04	1.30	1.35	1.15	1.12	1.02
guanine/cytosine	0.71	1.54	1.02	1.55	1.11	1.09

Table 6.

The value of this procedure was greatly impaired by a water soluble impurity in the heparin. This contaminant appeared in large quantities in the DNA finally obtained from each tube, giving rise to extremely low analyses for nitrogen and phosphorus. The base analyses were also affected since considerable quantities of the DNA were required for

hydrolysis in order to give chromatograms capable of analysis by the method of Wyatt (1951). The difficulty in making these measurements may well account for the consistently low values obtained for guanine and cytosine and the high values for adenine. The technique appeared promising, however, since there was a marked tendency for guanine and cytosine to be richer in the nucleic acids formed from nucleoprotein most easily broken down to form the histone-heparin complex. These results are in keeping with those of Chargaff and Brown & Watson.

The histone-heparin complex. A pure sample of the sodium salt of heparin was procured which did not affect the subsequent nucleic acid analysis. The proportions of histone and heparin in a complex were determined by titration of a suspension of calf thymus gland histone kindly provided by Dr Kent and the chosen sample of heparin in solution. This showed that for every 1 mg of histone, 0.21 mg of heparin was required to prevent an aliquot of the supernatant from giving a positive Sakaguchi test.

Further fractionation of DNA with heparin. Making use of the equivalence value between thymus nucleohistone and heparin, an experiment was devised for the stepwise fractionation of a sample of ox spleen nucleoprotein by the repeated addition of heparin and removal of the DNA displaced. The addition of heparin was so arranged that after five aliquots sufficient had been added to react with all the protein calculated to be present (see p. 75). The results of this experiment are given in Table 7, the fraction numbers increasing with increasing heparin concentration.

Fraction No.	1	2	3	4	5	Unfractionated DNA
nitrogen % dry weight	14.9	14.71	14.82	14.86	14.79	14.86
phosphorus % dry weight	8.47	8.52	8.56	8.63	8.68	8.51
guanine % total bases	27.5	25.8	24.7	24.7	22.0	24.1
adenine % total bases	25.8	25.9	26.8	26.3	26.8	26.5
cytosine % total bases	24.5	22.3	23.5	20.6	20.5	21.5
thymine % total bases	22.3	26.0	25.0	28.3	30.7	27.9
Total bases (referred to adenine as unity)	3.88	3.86	3.73	3.81	3.74	3.77
adenine/guanine	0.94	1.01	1.08	1.07	1.22	1.10
thymine/cytosine	0.91	1.17	1.06	1.37	1.50	1.30
adenine and thymine / guanine and cytosine	0.93	1.08	1.08	1.21	1.35	1.19
adenine/thymine	1.15	1.00	1.07	0.93	0.87	0.95
guanine/cytosine	1.12	1.16	1.05	1.20	1.07	1.12

Table 7.

In this fractionation the nitrogen and phosphorus analyses of DNA recovered after treatment with heparin agree more closely with those of the unfractionated material, unlike the earlier experiments. Base analyses performed on these fractions by the method of Wyatt (1951) were excellently reproducible. The results confirm the trend of base variation shown in table 6, guanine andcytosine rich nucleic acidsbeing more easily displaced from nucleoprotein than those rich in adenine and thymine. This trend is manifested by an increase in the ratios adenine/guanine, thymine/cytosine and(adenine and thymine/guanine and cytosine) in the displaced DNA as the concentration of heparin is increased. These ratios are in keeping with fractionation experiments performed by others (Chargaff et al 1953; Brown & Watson, 1953; Lucy & Butler, 1954, 1955, 1956; Lipshitz & Chargaff, 1956), but although the ratio guanine/cytosine is reasonably constant as expected, there is a marked tendency for the ratio adenine/thymine to decrease in succeeding fractions. This is due to the lesser variation of adenine concentration in the fractions, a property not revealed by other methods of fractionation.

Principles of fractional extraction of DNA. The mechanism of the fractional extraction of DNA from nucleoprotein is not clear at the present time. The bonds between the nucleic acid and a basic protein appear to be electrostatic in character, hence they are dissociated by neutral salt in solution and the nucleic acid is displaced by heparin. There is some disagreement over the mode of separation of the ionic

species with salt solutions, whether it be dependent on a concentration gradient (Chargaff et al, 1953) or a time gradient (Lucy & Butler, 1954) but as with other ionic separations both factors probably play an important part. The bond strengths between a nucleic acid and its conjugate protein are unequal and although this has been shown by the present method and others to be related to the base composition of the nucleic acid it is also likely that the composition of the protein is involved. It has been demonstrated by Stedman & Stedman (1951) and Davison & Butler (1954) that calf thymus histone contains at least two distinct components differing greatly in their content of lysine and arginine, and in a preliminary note Lucy & Butler (1955) were led to conclude that a nucleic acid of high guanine and cytosine content was linked to a histone rich in lysine whilst a nucleic acid with a predominance of adenine and thymine was linked to a histone rich in arginine. Further investigations, however, (Lucy & Butler, 1956) have shown this to be an over-simplification of the picture.

Although the mechanism of fractional extraction of DNA from nucleoprotein is still uncertain, considerable evidence has now been accumulated both by dissociation of the nucleoprotein bonds in a variety of ways and by displacement of the nucleic acid by an electrophilic agent, that DNA from calf thymus and ox spleen is a mixture of molecular species which at the minimum show considerable variations in their composition of nitrogenous bases. Material from fractions 1 and 5 in Table 7 would provide interesting comparisons if subjected to the mercaptalation technique for base sequences described above.

EXPERIMENTAL METHODS AND RESULTS

Elementary analyses for nitrogen phosphorus and sulphur were performed by Drs Weiler and Strauss, Oxford. Nitrogen analyses were also performed by the Kjeldahl procedure and phosphorus by the method of Allen (1940). Moisture content of separate samples of DNA, MDNA and PPN were determined by drying to constant weight at 60°C and the analytical figures corrected for moisture content.

Purine and Pyrimidine analyses were performed by the hydrolytic and paper chromatographic procedure of Wyatt (1951).

Ultraviolet absorption measurements were performed on a Hilger Uvispek spectrophotometer, and a Unicam S.P.500 spectrophotometer, using 1 cm quartz cells.

Dische extinction coefficients were determined by the method of Allerton, Overend & Stacey (1952).

Paper chromatography was carried out on Whatman No.1 paper in glass tanks and glazed baked clay sewer pipes, by the method of descending solvent front. Solvents used were (a) iso-propanol, 138 ml, hydrochloric acid (sp.gr.1.18) 31.7 ml and water 30.5 ml; (b) iso-propanol 70 ml, water 30 ml with 0.35 ml ammonia solution (sp.gr.0.880) for each litre of tank volume, poured into the bottom of the tank. Phosphate esters were detected by perchloric acid, ammonium molybdate spray (Hanes & Isherwood, 1949; Bandurski & Axelrod, 1951). Purines and pyrimidines and their derivatives were detected by ultraviolet photography (Markham & Smith, 1949).

Paper electrophoresis was carried out on Whatman No.1 paper in 0.05M ammonium acetate buffer pH 3.5 and 0.05M sodium borate buffer pH 7.9. Strips of filter paper damped with buffer were marked with spots and placed with their ends in buffer tanks. The rest of the paper was immersed in a cooling tank of petroleum ether (bp 40 - 60°C) and a potential gradient of 15.4 volts/cm was applied to it by carbon electrodes placed in the buffer tanks at a p.d. of 1000 volts. The voltage was maintained for 1 - 2 hours (Markham & Smith 1952).

Preparation of herring-roe DNA. The nucleoprotein was extracted by the method of Pollister & Mirsky (1946). Protein was removed by the method of Sevag, Lackman & Smollens (1938). The nucleic acid was dialysed free from sodium chloride in distilled water and freeze dried. Herring roe (12 g) yielded DNA (0.17 g). (Found: N, 15.7%; P, 7.35%; moisture content 13.75%. Corrected for moisture: N, 18.2%; P, 8.52%).

Preparation of Ox spleen DNA. Fresh frozen minced spleen (150 g) was homogenised in a Waring blender in the cold room for 6 min. with a solution containing 0.14 M sodium chloride and 0.01 M sodium citrate (350 cc) and ice (150 g). The suspension was centrifuged at 4,000 g. for 30 min. at 0°C. The supernatant was discarded and the residue washed twice more with three volumes of physiological saline/citrate solution. The final residue was treated with 3 vols of M sodium chloride. After homogenising for a few seconds, the viscous liquid was clarified by centrifuging at 65,000 g. for 30 min. The clear supernatant was diluted by pouring into 6 vols of water. The insoluble strings of nucleoprotein

were removed, dissolved in M sodium chloride and deproteinised by shaking with chloroform 35 parts and amyl alcohol 10 parts until no denatured protein appeared at the liquid interface. The aqueous layer was dialysed against 5 litres of distilled water four times till free from sodium chloride and freeze dried. Yield from 450 g. spleen: 3 g. DNA(1). (Found: N, 12.57%; P, 7.64%; moisture content 13.65%. Corrected for moisture: N, 14.55%; P, 8.88%). ϵ_p 5830 (λ_{max} 259 mμ). Dische extinction coefficient (per atom P): 1460. DNA (2). (Found: N, 12.97%; P, 7.82%; moisture content, 8.55%. Corrected for moisture: N, 14.20%; P, 8.55). ϵ_p 5640 (λ_{max} 259 mμ). Dische extinction coefficient (per atom P): 1510.

Preparation of MDNA(1). To a glass stoppered flask containing DNA(1) (1 g) and ethyl mercaptan (40 ml) was added hydrochloric acid (sp.gr.1.18) (1 ml). The flask was mechanically shaken for 48 hours at room temperature. Water (100 mls) was added and the aqueous layer removed and neutralised (sodium bicarbonate). Traces of dissolved ethyl mercaptan were removed by shaking with ether. The solution was dialysed twice against distilled water (5 litres) at 2°C and freeze dried. Yield: 48.6 mg, 6.1%. (Found: N, 10.8%; P, 5.65%; S, 2.23%; moisture content, 11.65%. Corrected for moisture: N, 12.2%; P, 6.40%; S, 2.52%). ϵ_p 6640 (λ_{max} 270 mμ).

Dialysable material from preparation of MDNA(1). The combined dialysates (10 l) were evaporated to 10 ml in vacuo at 40°C. The solid was filtered off. The filtrate was evaporated to dryness in vacuo at 40°C. Yield:

1.3 g. The residue was extracted 3 times with dry chloroform (10 ml); after evaporation of the chloroform a yellow syrup (96 mg) remained. The syrup gave a strong smell of ethyl mercaptan with hot 2N hydrochloric acid. The syrup 2.6 mg gave an intense blue colour in the Dische test. No phosphorus could be detected. The remaining dialysate residue was chromatographed in solvent (a). (Fig.10 and Fig.11).

Preparation of MDNA(2). DNA(1) (1.5 g) was finely shredded and placed in a glass-stoppered conical flask with ethyl mercaptan (40 ml). A small drop funnel containing hydrochloric acid (sp.gr. 1.18) (1 ml) was fitted to the flask, and the contents vigorously mechanically shaken. When the DNA and ethyl mercaptan were uniformly mixed the hydrochloric acid was slowly dripped in. Shaking was continued for 19 hours. Sodium bicarbonate (0.93 g) to neutralise exactly the hydrochloric acid was added, and shaking continued for 10 min. longer. Water (100 ml) was added and the aqueous layer removed. Excess ethyl mercaptan was extracted by shaking three times with ether (50 ml). The solution was dialysed twice against distilled water (5 l) at 2°C for 24 hours, and freeze dried. Yield: 0.8 g, 67%. (Found: N, 6.1%; P, 7.88%; S, 6.59%; moisture content, 2.85%. Corrected for moisture: N, 6.28%; P, 8.11%; S, 6.79%). ϵ_p 4280 (λ_{max} 270 mμ). Dische extinction coefficient (per atom P) 895.

Dialysable material from preparation of MDNA(2). The combined dialysates (10 l) were evaporated to dryness in vacuo at 40°C. Yield: 1.1 g. (Found: Total P, 0.5%; inorganic P, 0.08%). The residue was extracted three times with dry chloroform (10 ml); after evaporation of the

chloroform no residue remained. The dialysate residue was chromatographed in solvent (a). An ultraviolet photograph revealed the presence of only guanine and adenine.

Preparation of MDNA(3). DNA(2) (2 g) was treated by the same procedure as described for the preparation of MDNA(2). Yield 1.22 g, 76%. (Found: N, 6.23%; P, 8.22%; S, 6.67%; moisture content 8.85%. Corrected for moisture: N, 6.84%; P, 9.02%; S, 7.32%). ϵ_p 3600 (λ_{max} 270 mμ). Dische extinction coefficient (per atom P) 917.

Dialysable material from preparation of MDNA(3). The combined dialysates (10 l) were evaporated to dryness in vacuo at 40°C. Yield: 1.26 g. (Found: Total P, 0.20%; inorganic P, 0.02%). The residue was extracted three times with dry chloroform (10 ml); after evaporation of the chloroform no residue remained. The dialysate was chromatographed in solvent (a) (fig. 12).

Hydrolysis of MDNA(3). MDNA(3) (1.1 mg) was dissolved in 0.5 N sodium hydroxide solution (25 ml) and maintained at 37°C. The optical density at 268 mμ was measured initially and at intervals of several hours. DNA(2) (1.0 mg) was treated similarly. (fig.13).

Preparation of PPN. MDNA(3) (0.5 g) was dissolved in 0.5 N sodium hydroxide solution (100 ml). The solution was maintained at 37°C for 72 hours. The solution was neutralised with hydrochloric acid (pH meter) and dialysed four times against distilled water (5 l) at 2°C. The dialysed solution was freeze dried. Yield: 0.16 g. (Found: N, 7.17%; P, 7.90%; S, 1.39%; moisture content, 6.10%. Corrected for moisture:

N, 7.65%; P, 8.41%; S, 1.48%). ϵ_p 5920 (λ_{max} 270 mμ). Dische extinction coefficient (per atom P) 703.

Dialysable material from preparation of PPN. The combined dialysates (20 l) were evaporated to dryness in vacuo at 40°C. Yield: 0.9 g. (Found: Total P, 3.02%; inorganic P, 0.08%). The residue was extracted three times with dry chloroform (10 ml); after evaporation of the chloroform no residue remained. The residue when treated with hot 2N hydrochloric acid gave off ethyl mercaptan vapour. The residue (3 mg) gave a strong blue colour after the Dische test. The residue was chromatographed in solvent (a). (Fig.16 and Table 5).

Preparation of ox spleen deoxyribonucleoprotein. Fresh, frozen minced ox spleen (150 g) was homogenised in a Waring blender in the cold room for 6 min. with a solution containing 0.14 M sodium chloride and 0.01 M sodium citrate (350 ml) and ice (150 g). The suspension was centrifuged (4,000 g) for 30 min. at 0°C. The supernatant was discarded and the residue washed twice more with 3 volumes of the physiological saline/citrate solution. The final residue was treated with 3 vols of M sodium chloride. After homogenising for a few seconds the viscous liquid was clarified by centrifuging (65,000 g) for 30 min. The clear supernatant was diluted by pouring into 6 vols of water. The insoluble strings of nucleoprotein were removed with a bent glass rod. Yield: nucleoprotein (DNP(1)), 11.67g. Found: moisture content 87.25%. Yield nucleoprotein (DNP(2)), 10.54 g. Found: moisture content 86.3%.

Fractionation of DNP (1). To 6 glass-stoppered tubes (Table 8) containing nucleoprotein suspended in 0.14 M sodium chloride solution (10 ml) was added sodium heparinate (B.D.H. 97.3 units/mg) dissolved in 0.14 M sodium chloride (10 ml). The tubes were mechanically shaken vigorously (1 hr) at 2°C. The contents were centrifuged at 4000 g. for 15 min. at 2°C. The supernatants were separately shaken with an equal volume of chloroform (35 parts) mixed with amyl alcohol (10 parts) until no protein appeared at the interfaces. The aqueous layers were dialysed free of sodium chloride against distilled water at 2°C and freeze dried. Each sample was analysed for bases by the hydrolytic and paper chromatographic method of Wyatt (1951).

Tube No.	1	2	3	4	5	Unfractionated DNA (DNA(2))
% nitrogen	6.20	3.12	3.06	3.18	2.99	14.90
% phosphorus	3.6	2.6	2.6	2.2	2.4	8.55
Guanine mols/litre x 10^{-5}	1.03	3.05	1.35	1.22	1.18	2.81
adenine mols/litre x 10^{-5}	1.3	4.12	2.02	2.07	2.80	3.35
cytosine mols/litre x 10^{-5}	1.44	1.98	1.33	6.79	1.06	2.58
thymine mols/litre x 10^{-5}	0.64	3.17	1.56	1.80	2.49	3.28

Base concentrations are calculated using Wyatt's millimolecular extinction coefficients and are the average of 3 values.

Table 8.

Titration of calf thymus histone with heparin. Calf thymus histone (moisture content 20.4%) was suspended in separate tubes in 0.14 M sodium chloride solution, and increasing amounts of sodium heparinate (BDH 130 units/mg) dissolved in 0.14 M sodium chloride solution was added to successive tubes. Aliquots from each tube were tested for free histone, using the Sakaguchi reaction.

Tube No.	1	2	3	4	5	6	7	8
Histone (moist weight in mg)	2.1	1.9	2.1	2.0	2.1	2.1	2.0	2.1
Vol of 0.14 M NaCl (ml)	1.3	1.1	0.9	0.7	0.5	0.8	0.75	0.8
Vol of heparin soln. (ml)	0.2	0.4	0.6	0.8	1.0	0.7	0.75	0.65
Wt. of heparin in soln. (mg)	0.1	0.2	0.3	0.4	0.5	0.35	0.375	0.325
Reaction with Sakaguchi	+	+	+	-	-	-	-	+

Table 9.

From Table 9. 1 mg histone is equivalent to 0.16 mg heparin.

Correcting for moisture content of histone, 1 mg histone is equivalent to 0.20 mg heparin.

Further titration of calf thymus histone with heparin. The histone (5 mg) was suspended in 0.14 M sodium chloride solution (5 ml) in a 25 ml flask, and stirred. Sodium heparinate (B.D.H. 130 units/mg) solution (5 mg/ml) in 0.14 M sodium chloride was added slowly from a 2 ml burette. Small aliquots were removed at intervals and tested for free histone by the Sakaguchi test. Found: 5 mg moist histone is equivalent to 1.7 ml heparin solution. Correcting for moisture: 1 mg. dry histone is equivalent to 0.21 mg heparin.

Fractionation of DNP(2). Moist nucleoprotein (9.34 g) was suspended in 0.14 M saline (50 ml). Sodium heparinate (80 mg) (B.D.H. 130 units/mg) was added and the flask mechanically shaken for 30 min at 2°C. The contents were centrifuged (4,000 g. for 15 min.) at 2°C and the supernatant removed. The residue was suspended in 0.14 M saline (50 ml) and shaken with more heparin (80 mg) until five fractionations had been completed. The separate supernatants were deproteinised (chloroform/amyl alcohol), dialysed, and freeze dried.

Fraction No.	1	2	3	4	5	Unfractionated DNA
% nitrogen	13.34	13.18	13.62	12.66	12.57	12.99
% phosphorus	7.58	7.63	7.86	7.35	7.37	7.44
% moisture	10.45	10.40	8.13	14.8	15.04	12.57
Nitrogen % dry weight	14.90	14.71	14.82	14.86	14.79	14.86
phosphorus % dry weight	8.47	8.52	8.56	8.63	8.68	8.51
guanine mols/l x 10^{-5}	3.06	3.21	2.04	2.81	1.54	2.39
adenine mols/l x 10^{-5}	2.87	3.23	2.21	2.99	1.88	2.63
cytosine mols/l x 10^{-5}	2.73	2.77	1.94	2.34	1.44	2.13
thymine mols/l x 10^{-5}	2.49	3.24	2.06	3.21	2.16	2.76

Base concentrations are calculated using Wyatt's millimolecular extinction coefficients and are the average of 3 values

Table 10.

SECTION II

THE RELATION BETWEEN NUCLEIC ACIDS AND SOME ENZYMIC PROCESSES.

Stereochemical specificity is a phenomenon exhibited by many enzyme systems. One such system, which has been the subject of a great deal of study by Rosenthaler (1908, 1909, 1913, 1922), Krieble & Wieland (1921) and others, catalyses the formation of mandelonitrile from benzaldehyde and hydrocyanic acid. Under special conditions, at 0°C, the product obtained from the two symmetrical starting materials is entirely (-)-mandelonitrile. Although most systems do not exhibit absolute stereospecificity, some degree of optical preference is common. An allied phenomenon is the influence of asymmetric compounds on certain enzymic and non-enzymic reactions, so-called asymmetric induction. Erlenmeyer (1930) discovered that glucosamine (2-amino-2-deoxy-D-glucose) was able to influence the specificity of decarboxylation experiments, and Stewart & Lipkin (1939) found that the reduction of β-methylcinnamic acid by glucose yielded optically active β-phenylbutyric acid. As with enzymic reactions the stereospecificity varied with the reaction conditions. Partial asymmetric syntheses of this type are not confined to small molecules since diethylamino cellulose readily takes part in such a reaction (Bredig & Gestner, 1932). The behaviour of enzymes <u>in situ</u>, where they are in an asymmetric environment, may be influenced in a

stereochemical manner by a number of compounds, not least of which are the nucleic acids. It has already been found that nucleic acids inhibit certain dehydrogenases (Greenstein & Chalkley, 1945; Zittle, 1946), and it is considered that extensions of this phenomenon may provide an understanding of the way in which chromosomal components influence cellular functions. Mims, Swendseid & Bird (1947) have shown that the regulating effect of nucleic acids is not confined to reducing enzymes since pteroylglutamic acid conjugase, a hydrolytic enzyme, is strongly inhibited by them. Since pteroylglutamic acid occurs in body tissue mainly in the conjugated form and nucleic acids are universal cellular constituents, the latter may provide a regulating mechanism for the release of free pteroylglutamic acid within the cell. A possible mechanism for the action of nucleic acids on enzymes may be deduced from the results of Ohlmeyer (1950), who showed that the strong inhibition of phosphatase by nucleic acids may be reversed by the addition of protein. This suggested that the nucleic acid conjugates with the enzyme forming a nucleoprotein with greatly reduced catalytic power, and the displacement of the enzyme by another protein releases the enzyme in its original, active form.

In addition to results suggesting the regulation of recognised enzymes by nucleic acids, a preliminary report by Binkley (1952) claims that the enzyme which hydrolyses cysteinylglycine in pig kidney is identical with RNA. Further investigation of this action would lead to a better understanding of the biological role of RNA, particularly in protein synthesis. Binkley himself suggests a mechanism for protein synthesis in which the first stage is the synthesis of dipeptides by RNA.

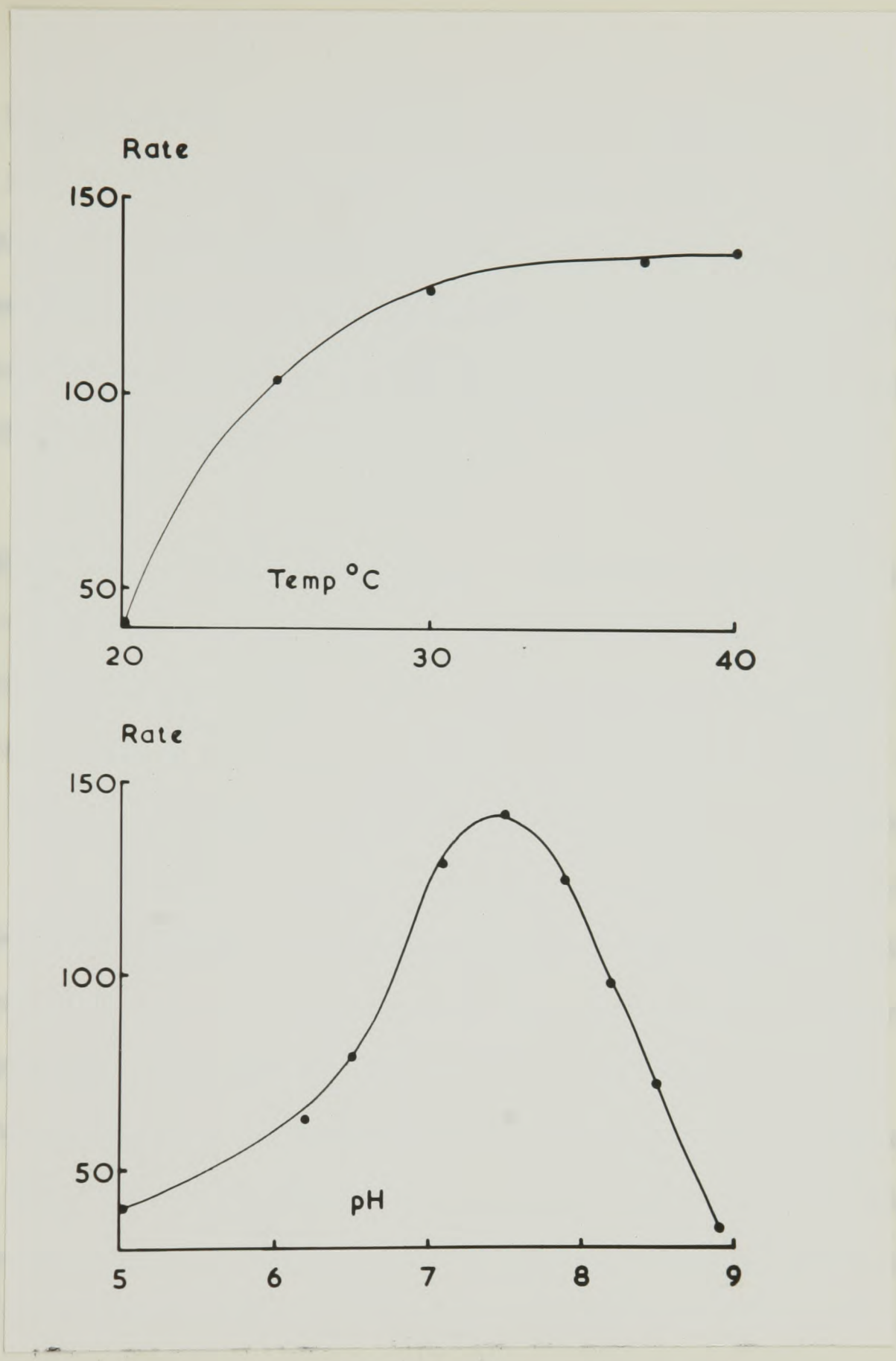
Such a mechanism has very little experimental basis except the finding of Linderstrøm-Lang (1940) that dipeptidases occur in large quantities in protein synthesizing cells. Binkley's enzyme has not been investigated further, except for the single observation by Chantrenne (1952) that cysteinylglycinase from pigeon liver has a sedimentation constant different from RNA. The object of the present work therefore is to study the general effect that nucleic acids have on enzymic processes and to examine the catalytic behaviour of the nucleic acids themselves.

Choice of enzyme system. In order that the effect of nucleic acids on an enzyme system may be studied with a view to detecting possible asymmetric influences, it is desirable that a system should be chosen such that small changes in its behaviour may be readily recorded. In such a system it is necessary to have a soluble substrate, products having high optical activity, and an enzyme pure enough not to cloud the reaction solution. A suitable experimental system was provided by pig liver esterase acting on methyl(±)-mandelate. The substrate was sufficiently soluble in water to allow solutions of sufficient concentration to be prepared, yet like the hydrolysis product, mandelic acid, it was so much more soluble in ether that extraction of reaction products at any stage was relatively easy. Both optical isomers of mandelic acid and both isomers of its ester have very high specific optical rotations, hence a variation in the rate of hydrolysis between (+) and (-) forms could be observed as readily as possible, and the effect that nucleic acids may have on the equilibrium noted.

Purification of enzyme. The esterase which was extracted from pig liver was required in a condition free from extraneous protein and pigmented material. The method of purification was similar to that used by Connors, Pihl, Dounce & Stotz (1950) with horse liver. The process involved six stages. The first was the preparation of an acetone powder which provided a convenient and stable form for storage. The other stages included an ammonium sulphate fractionation, selective heat denaturation, an acetone fractionation, and a copper acetate fractionation (p.108). After dialysis a clear colourless solution was obtained which contained 0.11 mg of solid per ml and 0.015 mg nitrogen per ml. The solution readily hydrolysed methyl mandelate under appropriate conditions.

Method of Assay. The substrate, methyl(±)-mandelate, was prepared from (±)-mandelic acid by esterification and its hydrolysis by the esterase was measured colorimetrically by the method of Hestrin (1949). In this procedure, the rate of disappearance of ester is estimated by the purple colour produced by the ester when it is treated with alkaline hydroxylamine, followed by acidic ferric chloride solution. The intensity of the purple colour is directly proportional to the concentration of ester in solution over a wide range and the ester may be measured quantitatively using a Spekker colorimeter. The enzyme action on this substrate was negligible around room temperatures, and the optimum temperature appeared to be above 40°C. Satisfactory results

were obtained by conducting the reactions at 37°C. The system functioned best at pH 7.5 when tested over the range pH 5.0 to pH 9.0 (see fig.19) and experiments were performed in M/15 phosphate buffer close to the optimum pH. The buffer was sufficiently strong to maintain a steady pH during the liberation of the acidic hydrolysis products. The Michaelis constant (Km) for the system was calculated from a series of experiments in which the substrate concentration was varied whilst the enzymic concentration remained constant. A graph of the reciprocal of the initial rate of hydrolysis plotted against the reciprocal of the substrate concentration gave a straight line from which the limiting velocity and hence Km could be deduced. The value of Km was 7×10^{-4} and the limiting velocity corresponded to a rate of hydrolysis of methyl mandelate of 0.3 millimols/hour. These values are in agreement with those for a similar system studied by Weber & Ammon (1929). Fig.20 represents the results from a typical experiment in which 161.5 mg of methyl mandelate dissolved in M/15 phosphate buffer pH 7.30 and made up to 50 ml was hydrolysed by the addition of 0.2 ml of esterase. Aliquots were removed at intervals and the remaining ester estimated. Aqueous solutions of methyl mandelate undergo slow hydrolysis when maintained at 37°C as indicated by the decrease in ester concentration without the presence of esterase. Optical rotation was observed during the reaction through a 1 decimetre tube surrounded by a water jacket. The tube containing the reaction mixture was kept in the waterbath until a reading was

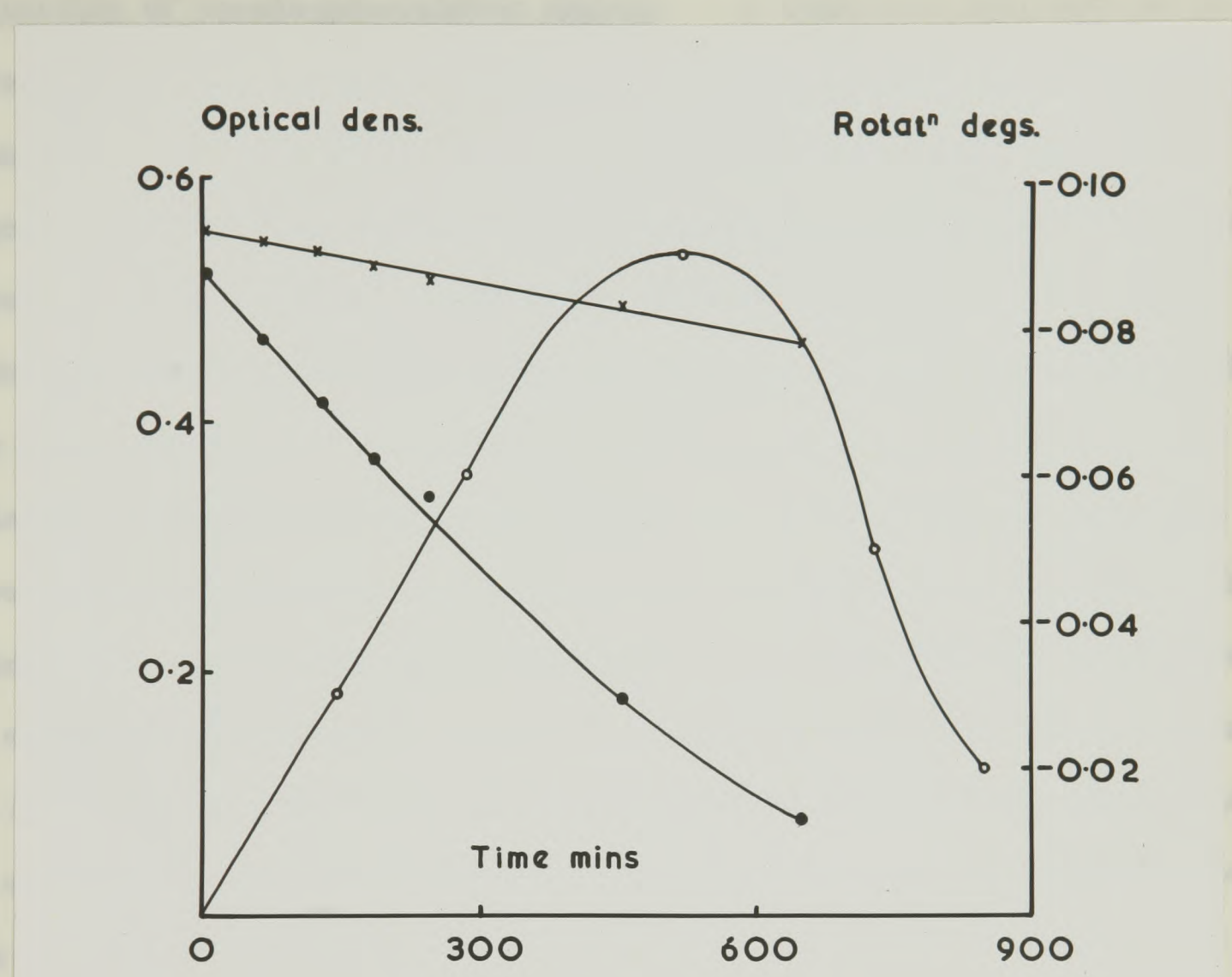


Rate = μmols of methyl mandelate hydrolysed/hour/ml.enzyme.

Fig.19. Temperature and pH Characteristics of Pig Liver Esterase.

taken in the polarimeter whereupon the contents of the tube were maintained close to 37°C by the water jacket. In accordance with the results of Dakin (1903) the solution became optically active during enzyme action but the activity had disappeared by the time all the ester was hydrolysed, thus indicating a difference in affinity of the enzyme for the optical isomers of methyl mandelate.

Inhibition of esterase-mandelate system. With the above pig liver esterase - methyl mandelate system the effect of nucleic acids and their derivatives was investigated. DNA prepared from herring-roe and ox spleen was used and RNA from ox liver prepared by the method of Grinnan & Mosher (1951). None of these preparations showed any effect on the system whatever. The rate of hydrolysis showed no variation, neither did the optical rotation when the concentration of these nucleic acids in the reaction mixture was varied from zero to 1 mg/ml. Greater concentrations were not possible since the opalescence they produced made optical measurement difficult. Adenosine was also without effect on the system. The pH of the reaction was varied down to 4.9 but in no case was there any measurable difference in the rate with or without the presence of nucleic acids. On the other hand, as little as 1 μmol/ml of quinine added to the system at pH 7.0 had a marked inhibitory effect on the rate of hydrolysis and the optical specificity of the enzyme.



Reaction temperature = 37°C.

x = rate of hydrolysis of methyl mandelate only.

● = rate of hydrolysis of methyl mandelate with enzyme

o = optical rotation of reaction mixture.

Fig.20. Hydrolysis of Methyl Mandelate with Pig Liver Esterase.

Inhibition of pepsin-haemoglobin system. A study was then made of the effect of nucleic acids on the rate of peptic hydrolysis of haemoglobin. Pepsin has optimum activity around pH 2.0 and at this pH it rapidly digests denatured haemoglobin at a rate that is nearly independent of the substrate concentration. The hydrolysis was followed by incubating a number of flasks containing enzyme and substrate at the same concentration for differing times. The reaction was terminated by the addition of trichloracetic acid and after filtration the soluble peptides and amino acids were estimated according to their tyrosine and tryptophane content. These amino acids give a blue colour with Folin's reagent which may be measured in a Spekker colorimeter. Samples of herring-roe and ox spleen DNA and ox liver RNA were added to this system and as before no inhibition could be measured in the rate of hydrolysis when the nucleic acid concentration was varied between 0.1 mg and 1 mg. per ml.

General action of nucleic acids on enzymes. Although there are isolated examples of enzymes that are inhibited by nucleic acids and their derivatives, this effect does not appear to be universal and therefore is unlikely to be of general significance. In the case of the esterase-mandelate system, the low Michaelis constant suggests a strong affinity between the enzyme and its substrate; this appears to be stronger than the tendency for the enzyme to form a nucleoprotein with the nucleic acid, which has modified catalytic properties. The hydrolysis of the methyl mandelate follows a zero order reaction except at low ester concentrations which

indicates that the reaction rate is dependent on the rate of breakdown of the enzyme - substrate complex whilst its formation is almost instantaneous. Although nucleic acids do not appear to influence all enzymes amongst those that are influenced, the most interesting so far is cysteinglycinase.

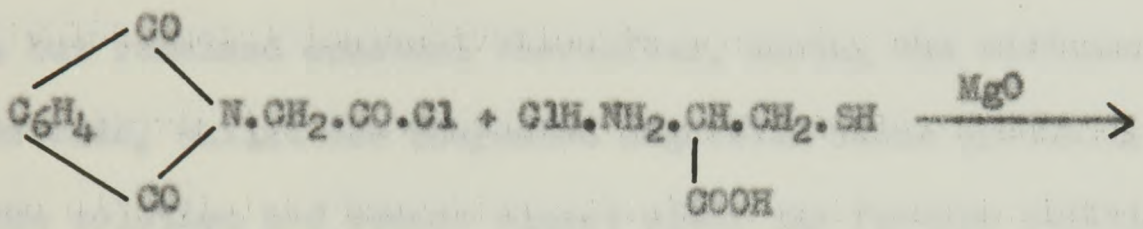
Cysteinylglycinase. When Binkley and his collaborators studied the metabolism of glutathione, they discovered that two enzymes promoted its cleavage to amino acids (Binkley & Nakamura, 1948). The first step was the removal of glutamine by glutathionase followed by the splitting of cysteinylglycine by cysteinylglycinase. Both these enzymes were found in rat and pig kidney although cysteinylglycinase was subsequently found to be extensively distributed in other tissues. The two enzymes could be separated by an ethanol and chloroform fractionation (Olson & Binkley, 1950) whereupon the more labile glutathionase was inactivated and the cysteinylglycinase remained largely unchanged. Carrying the chloroform fractionation still further Binkley (1951, 1952, 1954) subjected cysteinylglycinase to exhaustive denaturation by the method of Sevag, Lackman & Smollens (1938) and recovered a fully active enzyme containing no detectable protein, but having properties similar to a pentose nucleic acid. This remarkable discovery has remained so far unsubstantiated even though the existing reports are preliminary in nature.

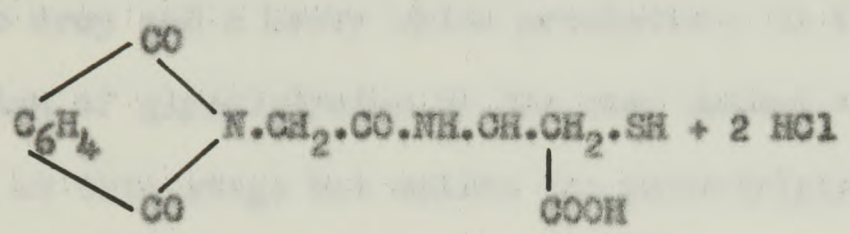
Existing evidence concerning the nature of cysteinylglycinase. From the evidence accumulated by Binkley it appears that cysteinylglycinase is a relatively non-specific dipeptidase. Greatest activity is shown when cysteine or leucine are the initial groups of the dipeptide and lowest activity when glycine is the initial group. Nevertheless glycylcysteine is stated as being a good substrate. The enzyme shows optical specificity and although this specificity is not always made clear it is assumed that maximum activity is shown towards the L-dipeptides. At low pH values, the enzyme is quickly inactivated and the greatest activity is manifested at pH 8. Metal ions appear to be necessary for activation of the enzyme and with cysteine containing substrates, manganese, cobalt or ferrous ions are necessary. The active enzyme contained no protein detectable by biuret and phenol tests, and the activity was not removed by incubation with proteolytic enzymes. When injected into rabbits, the enzyme preparation did not induce the formation of antibodies, and the ultra-violet absorption characteristics were typical of a nucleic acid; also during chromatography and electrophoresis on paper the activity was associated with the pentose and purine-rich fractions. No details of solvents or buffers are given, hence it is not clear how this was accomplished. The activity resisted dialysis, though slow loss by diffusion was reported, but again experimental details unfortunately are not available. These results provide impressive evidence for the polynucleotidic and non-proteinaceous nature of cysteinylglycinase. However, it is not inconceivable that a highly active protein in high

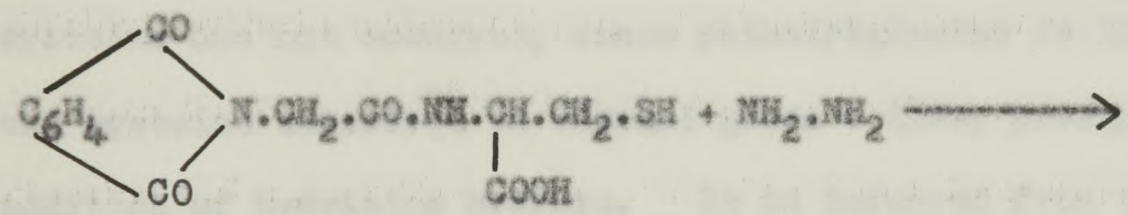
dilution may be responsible for the activity. In contrast to Binkley's findings, Spackman, Smith & Brown (1955) and Smith & Spackman (1955) have isolated a dipeptidase of low specificity from the same source (pig kidney) which, when highly purified, shows the properties of a typical protein. Divalent metal ion activation is necessary and the same pH/activity characteristics are exhibited as with Binkley's enzyme. Furthermore the ultraviolet absorption of this dipeptidase is intense at 280 mμ but shows no inflection around 260 mμ. Such spectral data is in accord with the presence of aromatic amino acids but the authors state that no evidence was found for the presence of nucleic acids.

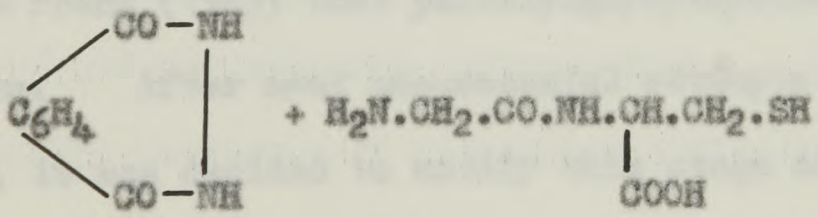
Preparation of substrates. Cysteinylglycine was prepared in solution by Binkley, by acid hydrolysis of glutathione, followed by neutralisation. Attempts to isolate cysteinylglycine proved unsuccessful and the mixture of amino acids, peptides and probably diketopiperazine derivatives were therefore used as a substrate. No data is available on the preparation and hydrolysis of alternative substrates except that glycylcysteine may conveniently be prepared by the method of Sheehan & Frank (1949). Cysteinylglycine is very readily oxidised by atmospheric oxidation and although derivatives have been prepared (Harington & Mead, 1932; du Vigneaud & Miller, 1936), it does not seem to have been isolated in the free state. In view of the desirability of a pure synthetic substrate it was decided to prepare glycyl-L-cysteine. In the method of Sheehan & Frank (1949) the amino group of glycine is protected as the phthalyl derivative, and in the

form of the acid chloride it is condensed with L-cysteine hydrochloride. The protecting phthalyl group is removed by treatment with hydrazine and the product, diglycylcystine, is reduced to glycyl-L-cysteine.









In practice no difficulty was found until the preparation of phthalylglycylcysteine, when many attempts to repeat the observations of Sheehan & Frank (1949) were unsuccessful. At the end of thisreaction a solution of the magnesium salt of phthalylglycylcysteine containing suspended magnesium oxide was acidified with hydrochloric acid. Instead of the

expected colourless solution, a white precipitate formed on addition of acid. After removing the water the white residue could not be dissolved in alcohol for treatment with hydrazine hydrate. Investigation of the acidification stage showed that the pH of the solution dropped rapidly to 4 but remained constant thereafter, during the addition of a large quantity of acid, whilst the suspended magnesium oxide gradually dissolved. When the solution had become almost clear the further addition of acid caused the pH to drop and a heavy white precipitate to form. During a model preparation of glycylglycine by the same method a similar precipitate occurred at this stage but unlike the phthalylglycylcysteine, phthalylglycylglycine was easily crystallised from alcohol, and the preparation was successful. It is suggested that the formation of phthalylglycylcysteine has not occurred, since phthalylglycine is insoluble in cold acid and cysteine dissolved in alcohol gives a heavy precipitate upon the addition of hydrazine hydrate. It is inferred from the observations of Sheehan & Frank (1949) that phthalylglycylcysteine is soluble under these conditions. After many unsuccessful attempts to prepare phthalylglycylcysteine, it was decided to modify this stage of the synthesis.

It was observed by King & Kidd (1949) that the carboxyl group of phthalyl-dipeptides rendered them soluble in sodium carbonate solution. Hydrazine was used to cleave the phthalyl group and on acidification with hydrochloric acid phthalhydrazide was precipitated leaving the dipeptide in solution with sodium chloride. This elegant method was applied to the preparation of glycyl-L-cysteine. Phthalylglycyl chloride was added to a solution of L-cysteine hydrochloride containing three equivalents of sodium

carbonate, and the resultant solution of the sodium salt of phthalylglycyl-cysteine was treated with the calculated quantity of hydrazine hydrate. The filtered aqueous solution of sodium chloride and the expected dipeptide was evaporated, but attempts to separate a compound having the properties of glycylcysteine or diglycylcystine from the residue were unfruitful. Glycyl-cysteine probably contaminated with sulphuric acid has also been prepared by Pirie (1931). Although melting point determinations do not provide reliable data for the characterisation of amino acids and peptides, it is of interest that Pirie reports the melting point of glycylcysteine as 177°C with sintering occurring at 130°C. On the other hand Sheehan & Frank give the melting point of this compound as 91 - 96°C.

Further attempts to prepare glycyl-L-cysteine were then made using an entirely different approach. Wieland & Bokelmann (1952) were able to prepare N-glycylcysteamine hydrochloride by heating glycyl chloride with cysteamine hydrochloride. The resultant S-glycylcysteamine dihydro-chloride was rearranged by sodium hydroxide, followed by hydrochloric acid and yielded N-glycylcysteamine hydrochloride. The applicability of these reactions to the synthesis of glycyl-L-cysteine was tested by the following scheme:

$$ClH.NH_2.CH_2.CO.Cl + HS.CH_2.\underset{\displaystyle COOH}{\underset{|}{CH}}.NH_2.HCl \longrightarrow ClH.NH_2CH_2.CO.S.CH_2.\underset{\displaystyle COOH}{\underset{|}{CH}}.NH.HCl + HCl$$

$$ClH.NH_2.CH_2.CO.S.CH_2.\underset{\displaystyle COOH}{\underset{|}{CH}}.NH_2.HCl \xrightarrow{NaOH} ClH.NH_2.CH_2.CO.NH.\underset{\displaystyle COOH}{\underset{|}{CH}}.CH_2.SH + HCl$$

The preparation of glycyl chloride hydrochloride proved somewhat unpredictable. The chlorination procedure of Fisher (1905) was used, and unless the initial glycine was in a highly divided state and the reaction carried out in a totally enclosed apparatus, the desired product was not obtained (Fischer, 1906). At the same time β-alanyl chloride hydrochloride was prepared by this method with relative ease. Using the same conditions as Wieland & Bokelmann (1952) a sample giving the correct analysis for S-glycylcysteine dihydrochloride was obtained but this resisted all attempts at rearrangement to N-glycylcysteine hydrochloride and only glycine hydrochloride resulted. Under similar conditions S-β-alanylcysteamine dihydrochloride was successfully transformed into N-β-alanylcysteamine hydrochloride, however.

Finally a satisfactory cysteine-containing substrate was prepared from glutathione (Gutcho & Laufer, 1954). After acid hydrolysis of glutathione cysteinylglycine was precipitated by addition of mercuric sulphate. Under these conditions glutamic acid remains in solution. After removal of the mercuric ions with hydrogen sulphide and most of the sulphate ions with barium carbonate a preparation of the dipeptide was obtained which behaved satisfactorily in six solvents when examined by paper chromatography. The product contained some traces of free cysteine, but no free glycine.

As an alternative substrate, L-leucylglycine was prepared according to a method based on the observations of Schumann & Boissonnas (1952). Phthalyl-L-leucylglycine was treated with phenylhydrazine and,

as the phthalyl group was removed, the dipeptide crystallised out. The recrystallised compound had the correct elementary analysis, decomposition temperature and optical rotation and moved as a single component when chromatographed on paper in three different solvents.

Preparation of cysteinylglycinase. The dipeptidase was isolated from pig kidney exactly as described by Binkley (1952) except that the final purification by ion exchange chromatography was omitted. All operations were carried out at room temperature except the deproteinisation with chloroform-octanol, which was performed at 2°. Unless this operation was carried out in the cold room the chloroform evaporated before the deproteinisation was complete. In order to retain enzymic activity it was essential that the pH should not fall below 7, as pointed out by Binkley. The purified active enzyme was contained in approximately 50 mls of colourless solution. No protein could be detected in 1 ml aliquots of the solution as determined by the biuret, Sakaguchi and Folin colour tests. The enzyme solution contained 2.45 mg of solid material per ml and had an ash content of 66.5%. Dipeptidase activity was retained when the enzyme solution was freeze dried. The resulting material had a phosphorus content of 0.43% dry weight, of which 0.34% was inorganic phosphate on a total weight basis and a nitrogen content of the order of 0.2%. If the total nitrogen and organically bound phosphorus in cysteinyl-glycinase is present in the form of polynucleotides, then the ratio of nitrogen/phosphorus is of the correct order, but the enzyme clearly contains

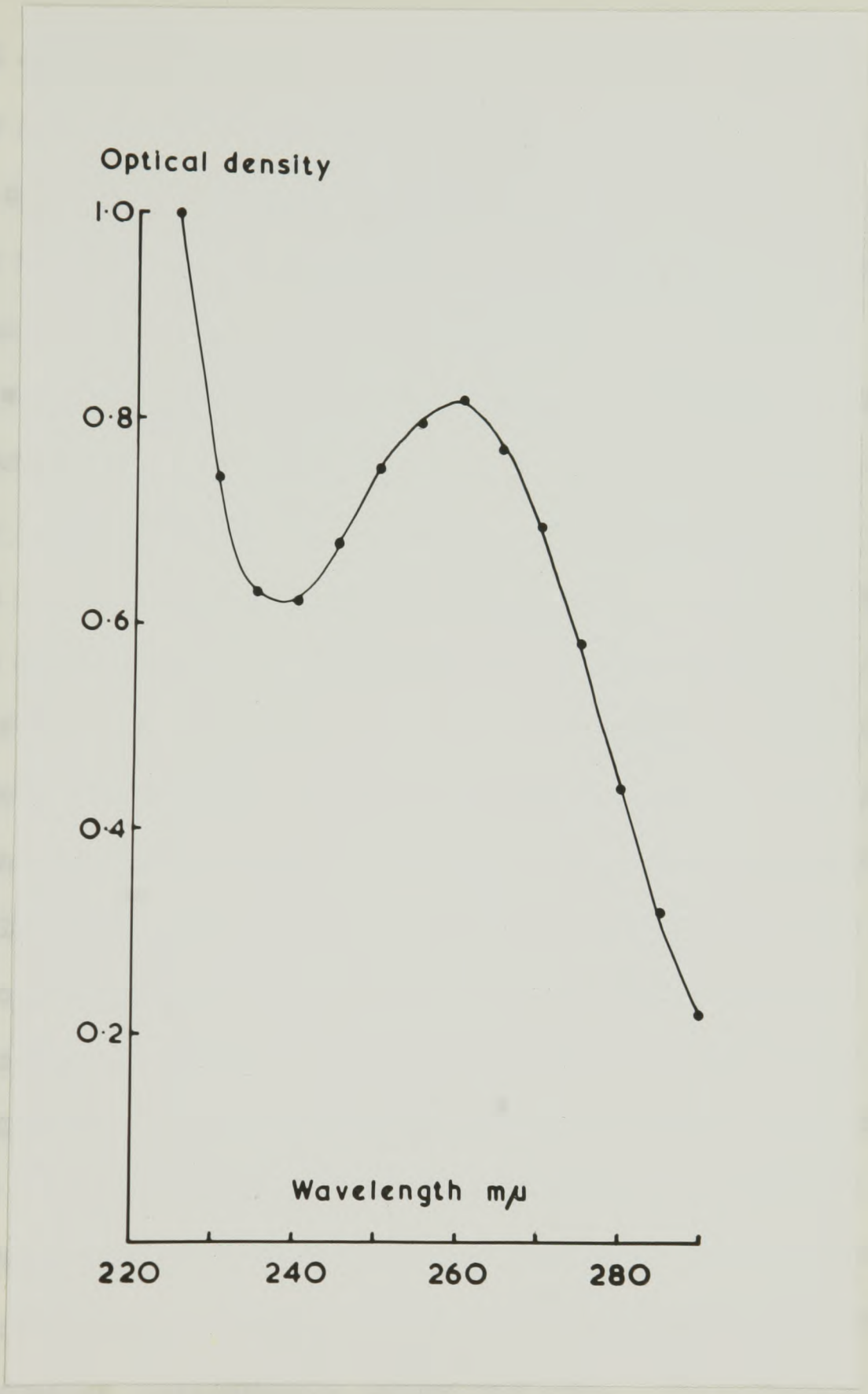


Fig.21. Ultra-violet Absorption of Cysteinylglycinase at pH 7.5.

very small quantities of this material. Binkley does not report absolute values for nitrogen and phosphorus content of his cysteinylglycinase but the ratio of nitrogen/phosphorus is given as 1.32. In the enzyme under discussion no pentose or deoxypentose was detected by the orcinol and Dische colour tests.

Cysteinylglycinase absorbed strongly in the ultraviolet region of the spectrum, having a single smooth sided peak at 260mμ at pH 7.5. No peak or inflection was shown at 280 mμ where aromatic amino acids present in protein would be expected to absorb. Fig.21 shows the absorption curve given by 1 ml of enzyme solution diluted to 4 ml with water. If the peak at 260 mμ were that of a nucleic acid containing approximately 15% nitrogen and 8% phosphorus then the nucleic acid nitrogen and phosphorus would represent approximately 1.25% and 0.67% respectively of the total solid present in solution. This is not in very good agreement with the analysis figures already given.

Dialysis of cysteinylglycinase against distilled water led to loss of activity. When the enzyme was dialysed against distilled water at 2°C for 3 hours no activity could be detected either inside or outside the cellophane even when the external solution was concentrated by freeze drying to about 5 ml. During dialysis the ultraviolet absorbing material remained predominantly within the sac. It is possible that during dialysis the enzyme is parted from a stabilising cofactor. If this be so then the inactivation is irreversible for recombination of the dialysis solutions did not restore the activity.



dipeptidase

guanine
adenine
thymine

hydrolysed
dipeptidase

Solvent: 86% (vol./vol.) aq. n-butanol; Whatman No. 1 paper, descending.

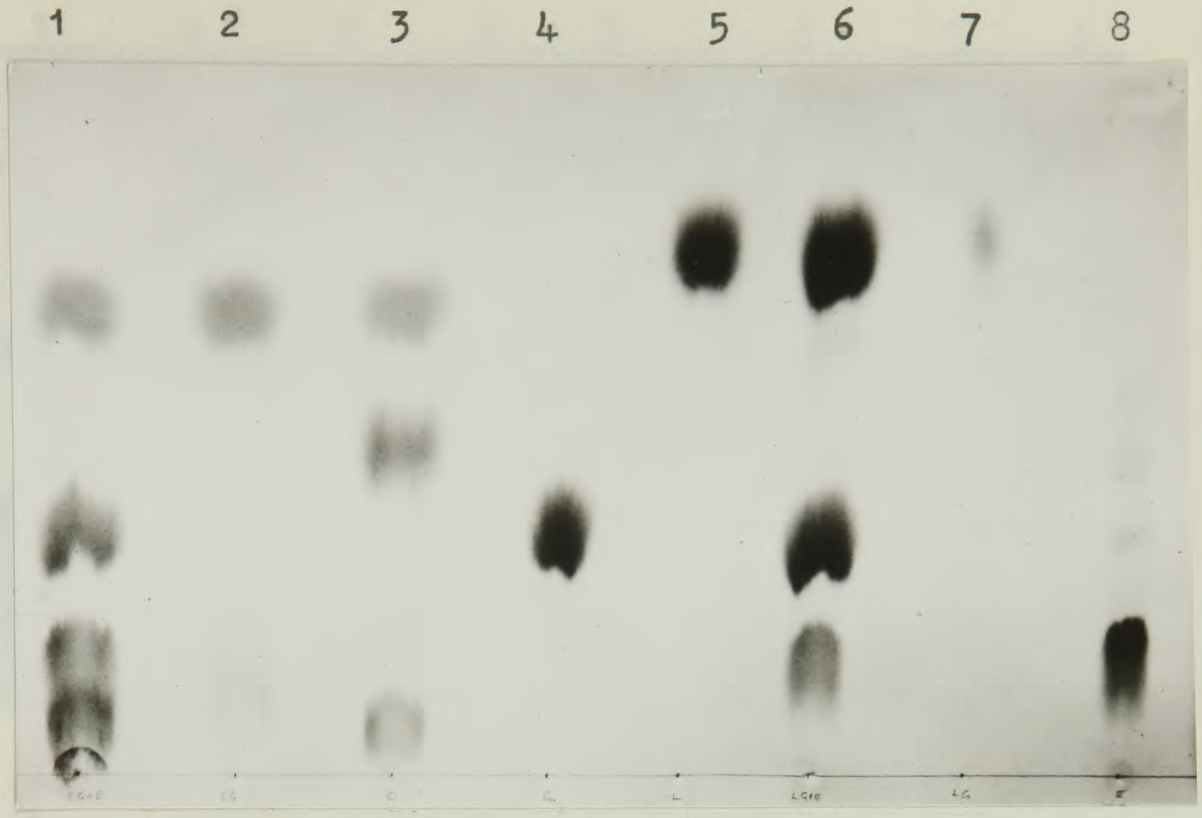
Fig 22. Ultra-violet Chromatography of Dipeptidase.

Paper chromatography of the ultraviolet absorbing material in cysteinylglycinase was difficult because of the small amount present. About 3 mg of solid material was required to give visible ultraviolet absorbing spots when chromatograms were photographed by the method of Markham & Smith (1949). In fig.22 cysteinylglycinase is seen to divide into a series of ultraviolet absorbing components in a solvent of 86% (vol/vol) aqueous n-butanol. These components did not withstand hydrolysis with perchloric acid, which reduced them to the ill-defined streak shown in fig.22. In this chromatographic solvent both RNA and DNA are immobile. It was observed by Binkley that upon paper chromatography complete correlation was obtained between migration of activity and pentose or purine components; however, none of the ultraviolet absorbing components shown in fig.22 when eluted had measurable dipeptidase activity.

Measurement of dipeptidase activity. A chromatographic method for the detection of activity was found convenient. A specimen of enzyme buffered at pH 7.4 was pre-incubated at 37°C with 0.4 millimolar manganous chloride solution for 2 hours. Afterwards a solution containing the substrate brought to 37°C was added and the reaction allowed to proceed for several hours. After the desired reaction time the solutions were transferred to paper chromatograms which after elution were examined for peptides and amino acids by spraying with ninhydrin solution. The percentage hydrolysis was obtained by cutting out the glycine spot formed from hydrolysed dipeptide, eluting the ninhydrin colour

and measuring its intensity in a Spekker colorimeter. The amount of glycine in the spot was calculated from a calibration curve constructed from known quantities of glycine that had been treated under identical conditions. The reproducibility of the calibration curve was greatly affected by the concentration of inorganic ions in the sample to be chromatographed and this influenced the buffer and concentration of divalent metal ions chosen for the enzyme reaction. Of the buffers normally used at pH 7.4, phosphate and "tris" were unacceptable both from their effect upon chromatography and their reaction with manganese ions. The free amino acid histidine was found to be a most useful buffer. At a concentration of 0.01M it had sufficient buffer capacity but was chromatographically unobtrusive and of a sufficiently low R_f value not to interfere with peptide measurements. The manganese chloride concentration was lowered until enzymic activity was retained yet glycine could be quantitatively measured.

Of the three chromatographic solvents investigated the best separations were obtained in ethanol (60 vols), tert-butanol (20 vols), formic acid (5 vols) water (15 vols). When sulphydryl compounds such as cysteine and cysteinylglycine were chromatographed, trailing of the spots was minimised by addition of N-ethylmaleimide just prior to spotting on paper (Hanes, Hird & Isherwood, 1950). Activity of the enzymes is demonstrated in fig.23a and fig.23b, in which both cysteinylglycine and leucylglycine have been hydrolysed. The mobilities of leucine and leucylglycine were identical in the solvents used but hydrolysis can be



cg	cg	cg	g	l	lg	lg	h
g		csh			g		
h		cs			h		
cs							

cg = cysteinylglycine. csh = cysteine.

g = glycine. l = leucine.

h = histidine. lg = leucylglycine.

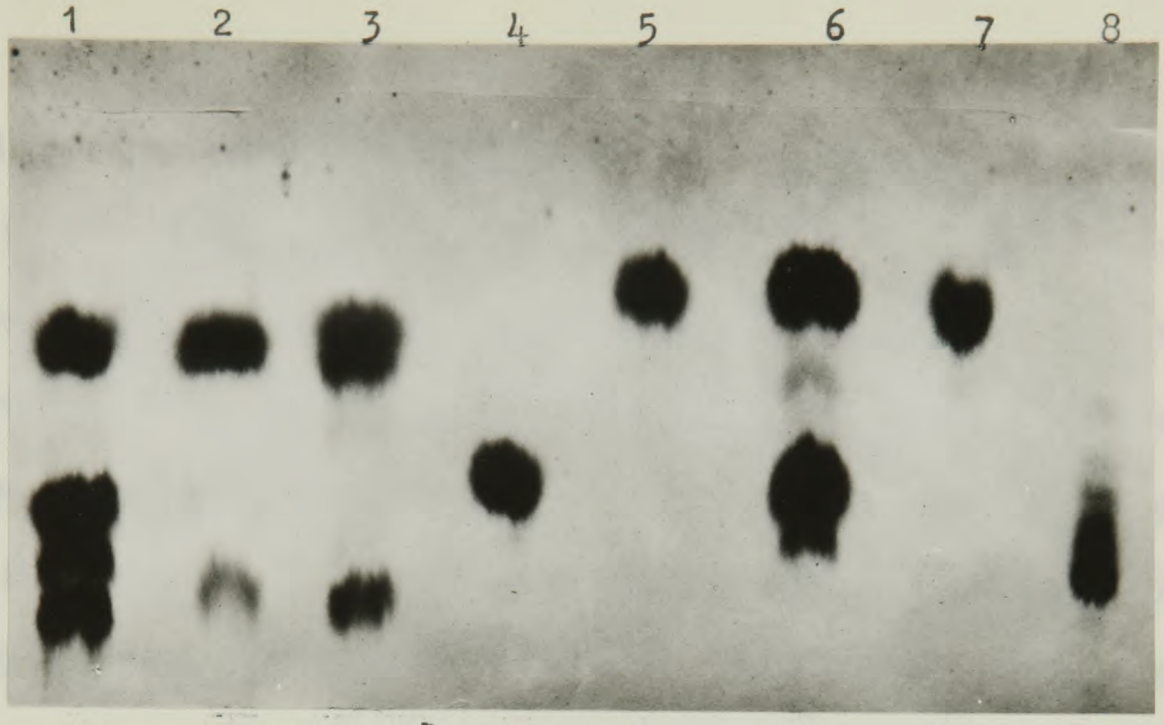
cs = cystine.

1. Cysteinylglycine + enzyme in histidine buffer.
2. Cysteinylglycine.
3. Cysteinylglycine + cysteine.
4. Glycine.
5. Leucine.
6. Leucylglycine + enzyme in histidine buffer.
7. Leucylglycine.
8. Enzyme in histidine buffer.

Buffer: 0.01M histidine pH 7.4. Incubation at 37°C for 5 hours.

Solvent: ethanol(60) tert-butanol(20) formic acid(5) water(15). Ascending.

Fig 23a. Hydrolysis of Dipeptides by Dipeptidase.



1	2	3	4	5	6	7	8
cg g h cs	cg cs	cg+csh cs	g	l	lg g h	lg	h

cg = cysteinylglycine.

g = glycine.

h = histidine.

csh = cysteine.

l = leucine.

lg = leucylglycine.

cs = cystine.

1. Cysteinylglycine + enzyme in histidine buffer.
2. Cysteinylglycine.
3. Cysteinylglycine + cysteine.
4. glycine.
5. Leucine.
6. Leucylglycine + enzyme in histidine buffer.
7. Leucylglycine.
8. Enzyme in histidine buffer.

Buffer: 0.01M histidine pH 7.4. Incubation at 37°C for 5 hours.

Solvent: n-butanol(50) acetic acid(25) water(25). Ascending.

Whatman No. 1 paper.

Fig 23b. Hydrolysis of Dipeptides by Dipeptidase.

detected by the formation of glycine. In view of the greater purity and the better chromatographic properties of leucylglycine, it was chosen as a substrate for the majority of experiments. Measurements of activity towards leucylglycine showed that on the average ½ ml of enzyme containing approximately 2.5 µg of nitrogen produced 1 mg of glycine from 5 mg of dipeptide (50% hydrolysis) in 5 hours. This is not of the same order of activity as the dipeptidase isolated by Binkley.

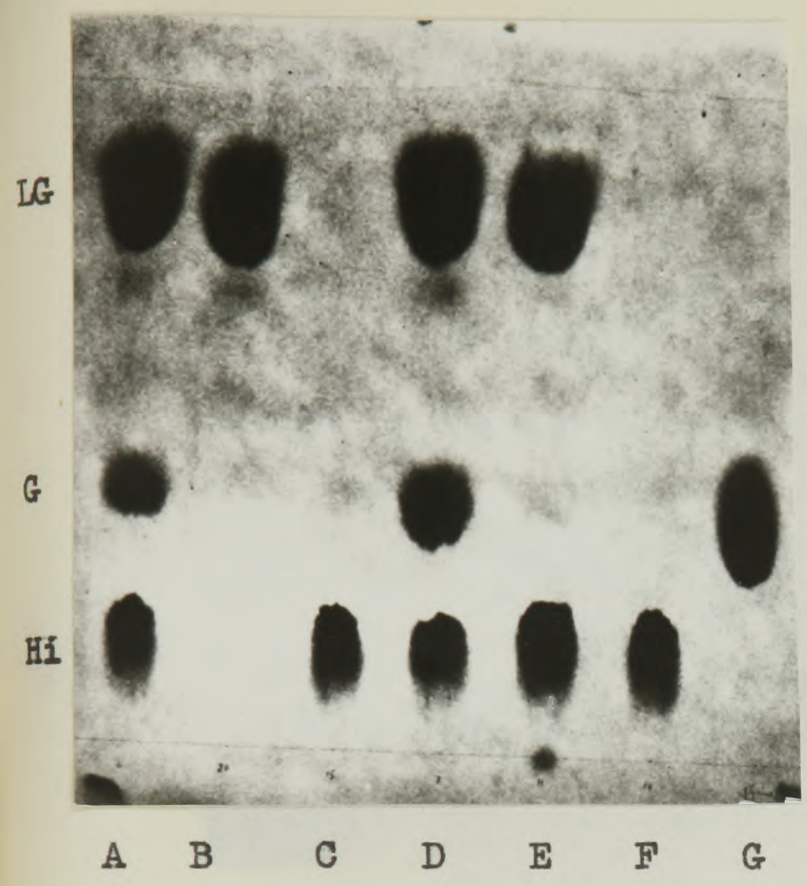
Action of nucleic acid and ion exchange resin on dipeptides. Three samples of high molecular weight, nucleic acids isolated by mild procedures, were tested for dipeptidase activity towards cysteinylglycine and leucylglycine. Two sources of DNA, one from herring-roe and one from ox spleen, and RNA derived from ox liver were used, but in every case no glycine was produced by peptide hydrolysis.

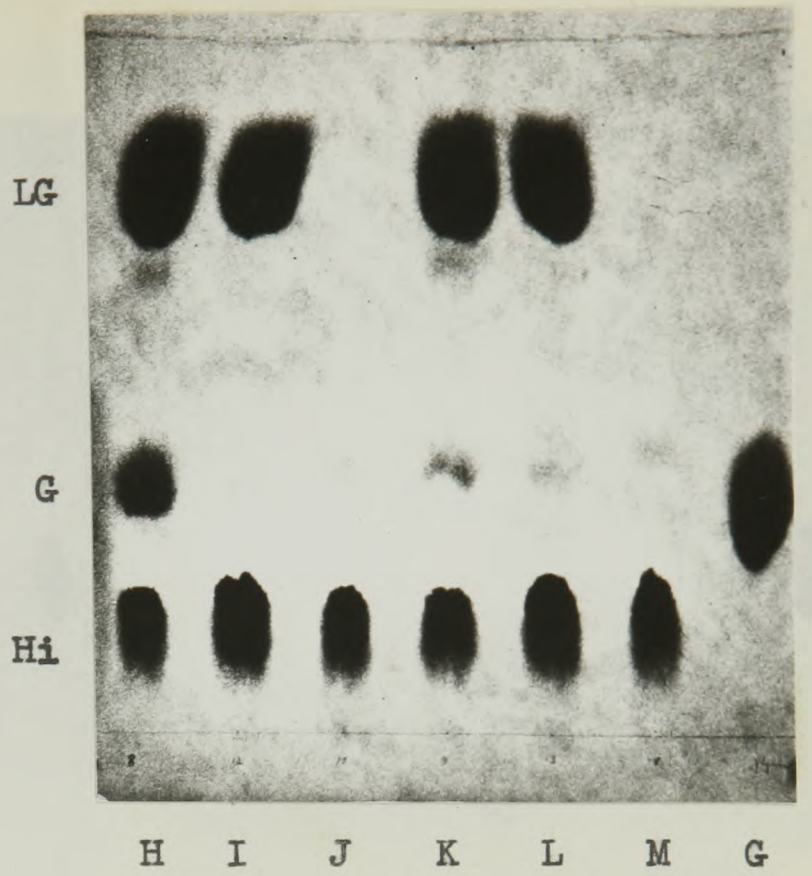
In the final stage of the purification of cysteinylglycinase, Binkley employed a resin column for adsorption and elution of the active material. This stage has been omitted from the present preparations because of the known catalytic properties exhibited by some ion exchange resins. It can be easily demonstrated that deionised water containing traces of dissolved ion-exchange substances is capable of hydrolysing esters such as methylbutyrate and Dowex 50 also catalyses the hydrolysis of peptides (Whitaker & Deatherage, 1955). In view of this, the hydrolytic property of Dowex 1 chloride eluted in the same way as for the purification of cysteinylglycinase was tested against cysteinylglycine and leucylglycine. In neither case however were the dipeptides hydrolysed by such solutions.

The action of ribonuclease and proteolytic enzymes on cysteinylglycinase.

In a series of experiments the digestion of cysteinylglycinase was attempted using crystalline ribonuclease, crystalline trypsin and crude preparations of pepsin and trypsin. All these enzymes were used at a concentration of 0.1 mg/ml reaction mixture and the results of one experiment are shown in fig.24. The dipeptidase after pre-incubation with manganous ions for the normal period, were incubated with the digesting enzyme for 1 hour prior to addition of the substrate. The pH throughout the experiment was maintained at 7.4. Ribonuclease, crude trypsin and crude pepsin were without effect on the dipeptidase but crystalline trypsin reduced its activity by 90%. Although pepsin has little activity at pH 7.4 the other enzymes are within their normal activity range. Under the conditions described ribonucleic acid and proteins digested by ribonuclease and trypsin respectively should have been completely removed. In a similar experiment the dipeptidase was incubated for three hours with crystalline ribonuclease and crystalline trypsin. In this case although ribonuclease was without effect on the active material, as before, peptide hydrolysis was virtually abolished by trypsin (see fig.25).

The activity of cysteinylglycinase was also removed by boiling its solutions. A sample of the enzyme which had been preincubated with manganous ions for two hours was heated in a boiling water bath and the temperature retained at 98°C for two minutes. It was then cooled rapidly to 37°C in an ice bath. Addition of the substrate and incubating for 5 hours produced no hydrolysis, as shown in fig.26.







A.Hydrolysis by untreated enzyme
B.Incubated leucylglycine only.
C.Incubated buffered enzyme.
D.Hydrolysis by RNAase treated enzyme.
E.Buffered leucylglycine with RNAase.
F.Buffered enzyme with RNAase.
G.Glycine
H.Hydrolysis by crude trypsin treated enzyme
I.Buffered leucylglycine with crude trypsin.
J.Buffered enzyme with crude trypsin.
K.Hydrolysis by cryst.trypsin treated enzyme.
L.Buffered leucylglycine with cryst.trypsin.
M.Buffered enzyme with cryst.trypsin.
N.Hydrolysis by crude pepsin treated enzyme.
O.Buffered leucylglycine with crude pepsin.
P.Buffered enzyme with crude pepsin.

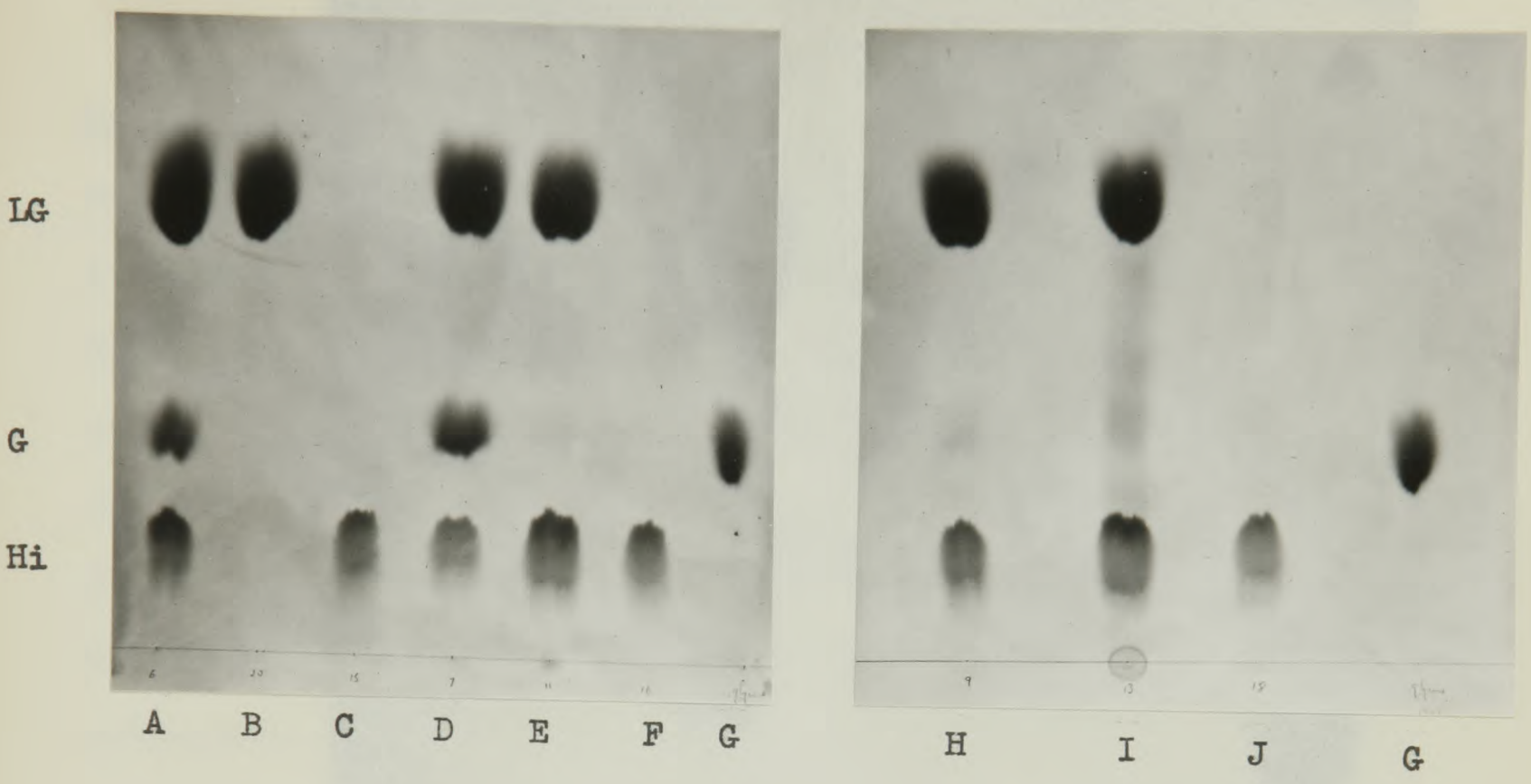
Dipeptidase digested for 1 hr. followed by incubation for 5 hrs. with substrate.

Buffer 0.01M histidine pH 7.4. Temp: 37°C.

Upward solvent development.

Solvent: ethanol(60) tert-butanol(20) formic acid(5) water(15)

Fig.24. Attempted Digestion of Dipeptidase by RNAase and Proteases.



A. Hydrolysis by untreated enzyme.

B. Incubated leucylglycine only.

C. Incubated buffered enzyme.

D. Hydrolysis by RNAase treated enzyme.

E. Buffered leucylglycine with RNAase.

F. Buffered enzyme with RNAase.

G. Glycine.

H. Hydrolysis by crystalline trypsin treated enzyme.

I. Buffered leucylglycine with crystalline trypsin.

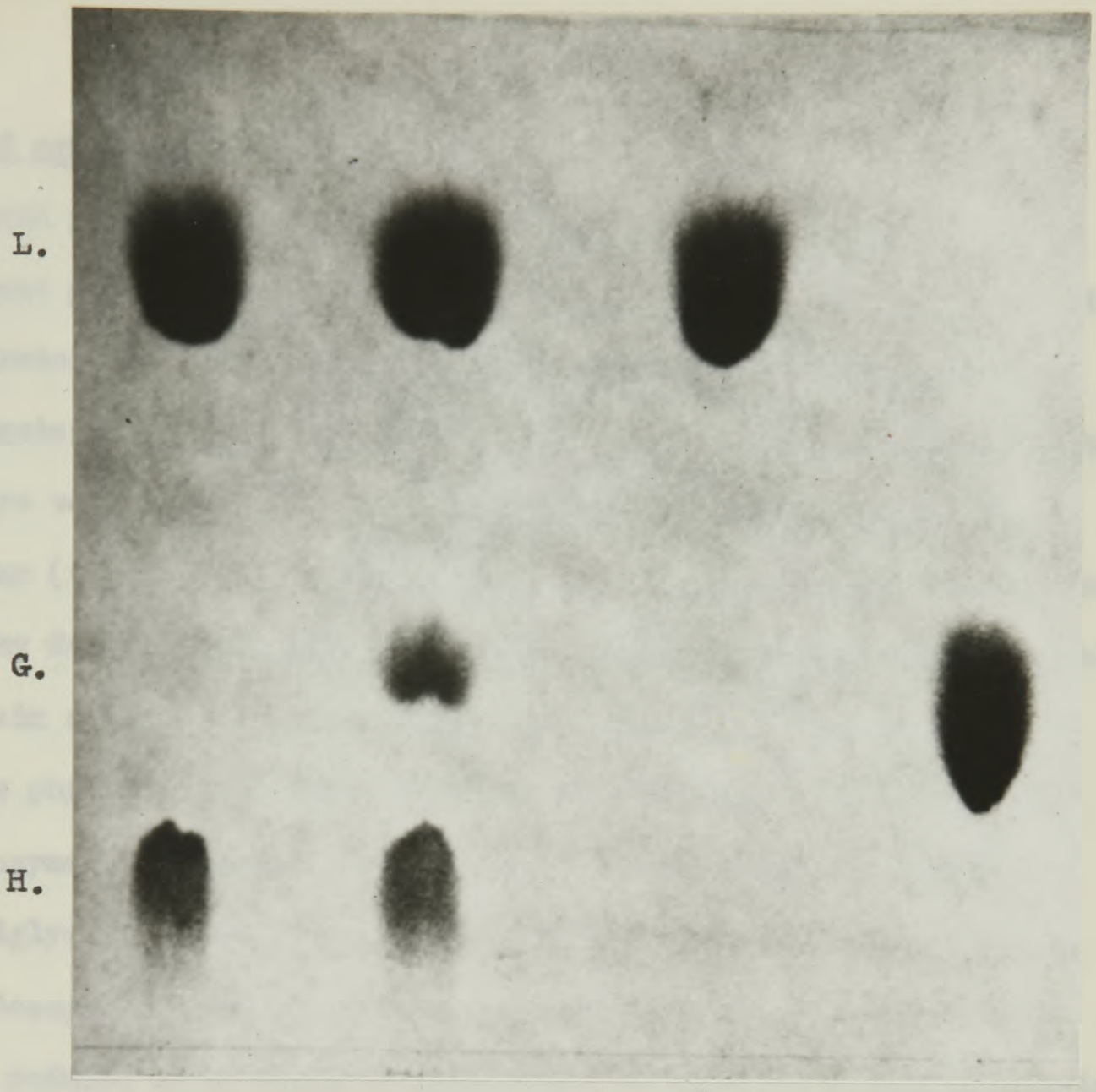
J. Buffered enzyme with crystalline trypsin.

Enzyme digested for 3 hrs. followed by incubation for 5 hrs with substrate.

Buffer: 0.01M histidine pH 7.4. Temperature: 37°C. Ascending solvent.

Solvent: ethanol(60) tert-butanol(20) formic acid(5) water(15).

Fig 25. Digestion of Dipeptidase by RNAase and Trypsin.



1. Boiled dipeptidase incubated with leucylglycine for 5 hours.
2. Dipeptidase incubated with leucylglycine for 5 hours
3. Leucylglycine incubated for 5 hours.
4. Glycine.

Solvent: ethanol(60) tert-butanol(20) formic acid(5) water(15).
Upward solvent development. Whatman No.1 Paper.
L = leucylglycine G = glycine H = histidine

Fig.26. Effect of heat on dipeptidase activity.

Nature of cysteinylglycinase. Although cysteinylglycinase has some exceptional properties, sufficient evidence is available to show that it has many properties in common with other enzymes. The inability to detect protein by chemical tests does not necessarily mean that an enzyme contains no protein, although in general enzymes purified to this extent are very unstable. Willstatter & Bamann (1928) and Kraut & Rubenbauer (1928) have purified gastric and hepatic esterases until they contain no detectable protein, but their preparations are non-dialysable and contain carbon, hydrogen, oxygen and nitrogen. More modern and sensitive physical analytical techniques have revealed that even the purest enzymes yet studied are proteins. The relative ability of cysteinylglycinase to resist proteolytic enzymes is also not unique. Euler & Josephson (1924) have reported that purified yeast saccharase was only reduced in activity by 3.4% after 37 hours of peptic hyrolysis and was unaffected by 20 hours of tryptic hydrolysis. Although cysteinylglycinase is resistant to treatment that would denature most proteins and its method of purification resembles the preparation of a nucleic acid, its analysis and properties are not consistent with a high molecular weight polynucleotide. Furthermore the nucleic acids examined do not have dipeptidase activity under the same conditions as cysteinylglycinase. The similarity of pH stability and divalent metal ion activation between cysteinylglycinase and leucine aminopeptidase prepared by Spackman, Smith & Brown (1955), which is a protein, also favours the view that cysteinylglycinase is a protein. It is concluded therefore that the present enzyme is unlikely to be a nucleic acid.

EXPERIMENTAL METHODS & RESULTS.

Preparation of pig liver esterase. 1. Minced pig liver (1,350 g.) was stirred with ice-cold acetone (8 l.) for 1 hour. The solid was strained through a Büchner funnel and suspended and stirred twice more with ice-cold acetone (8 l.) for 1 hour. The liver was filtered off through muslin and dried at room temperature. When dry it was powdered in a pestle and mortar and stored at 5°C. Yield 280 g.

2. The acetone powder (100 g.) was stirred with water(1 l.) for 9½ hours at 2°C. The solid material was removed by centrifugation (2,000 g.) and the turbid solution clarified by ultracentrifugation (35,000g.). The active supernatant was a clear brown liquid.

3. The supernatant (860 ml) was added to saturated ammonium sulphate solution (860 ml) raised to pH 7.5 (ammonia) at 5°. After 18 hours the precipitate was removed after centrifugation (4,000 g.). Solid ammonium sulphate (94 g.) was dissolved in the supernatant (940 ml) and the solution maintained at 5°C for 12 hours. The white precipitate was removed after centrifugation (4,000 g.) and dissolved in 0.15 M phosphate buffer (150 ml) at pH 7.6.

4. The solution (150 ml) was raised to 60°C for 5 min. then rapidly cooled to 5°C. The denatured protein was removed by centrifugation and discarded.

5. The cold supernatant (150 ml) was added to acetone (50 ml) at 5°C. The acetone solution (25%) was left for 12 hours. The precipitate was discarded and the supernatant (180 ml) added to acetone (65 ml). After

8 hours at 5°C the acetone solution (45%) was centrifuged and the supernatant discarded. The precipitate was extracted with water (40 ml) at 5°C for 5 hours and the solution clarified by centrifugation (4,000 g.).

6. To the supernatant (35 ml) was added cupric acetate (450 mg) with stirring. The solution was kept at 5°C for 4 hours. The precipitate was removed and discarded. The clear supernatant was dialysed against distilled water (5 l.) for 24 hours, and stored at 5°C. Yield 32 ml., nitrogen content 0.015 mg/ml, dissolved solids 0.11 mg/ml.

Preparation of methyl (±)-mandelate. (±)-mandelic acid (10.5 g.) was dissolved and refluxed for 3 hours with 1% methanolic hydrogen chloride (120 ml). The acid was neutralised with lead carbonate and the filtered liquid evaporated. The solid was crystallised and recrystallised from methanol and ether. Yield 10g., m.p. 49-50°C.

Assay of esterase activity. In a typical experiment methyl mandelate (323 mg) was dissolved in phosphate buffer (0.15M) and made up to 100 ml. The solution was divided equally between two flasks. To flask (1) was added esterase (0.2 ml) while to a third flask (flask 3) esterase (0.2 ml) wasadded to phosphate buffer pH 7.3 (50 ml). The flasks were incubated at 37°C. A water jacketted polarimeter tube (1 dm) was filled from flask (1) and incubated at 37°C. After suitable intervals the residual ester was measured. To an aliquot of the contents of flask (1), flask (2) and flask (3) (1 ml) was added water (1 ml), alkaline hydroxylamine (2 ml), hydrochloric acid (2 ml), ferric chloride (1 ml); the purple colour produced from flasks (1) and (2) was measured against flask (3) in a Spekker colorimeter using 1 cm cells and Ilford filter 605 (Hestrin 1949) (fig.20).

opt.dens. flask (1)	opt.dens. flask (2)	time mins.	opt.rot. degrees	time mins.
0.521	0.557	0	0	60
0.471	0.553	65	-0.03	135
0.422	0.545	130	-0.05	190
0.368	0.528	185	-0.06	285
0.340	0.521	245	-0.09	520
0.183	0.505	455	-0.05	730
0.078	0.488	650	-0.02	850
0	0.450	1200	0	1230

Table 11.

Optimum pH for esterase activity. The enzyme was incubated with methyl mandelate as above and the rate of hydrolysis measured. The pH was maintained with borate buffer (0.15M) in each experiment but varied for different experiments from pH 8.9 to pH 5.0. Below pH 7.0 the pH was depressed with manitol (3.3%). The rate of hydrolysis was measured in μ mols of methylmandelate hydrolysed/hr/ml enzyme (fig.19). These results are recorded in Table 12, below.

pH.	rate of hydrolysis.
5.0	40
6.2	63
6.5	79
7.1	129
7.5	142
7.9	125
8.2	98
8.5	72
8.9	35

Table 12.

Optimum temperature for esterase activity. The rate of hydrolysis (mols/hr/ml enzyme) of methyl mandelate in 0.15M phosphate buffer pH 7.5 was measured at constant temperatures ranging from 20°C - 40°C (fig.19).

Temperature	rate of hydrolysis
20	40
25	104
30	127
37	135
40	137

Table 13.

Preparation of ox liver RNA. Fresh ox liver (1½ lb.) was frozen in acetone/dry ice and minced in the cold. 100 g. aliquots were homogenised in a Waring blender with 0.14M sodium chloride solution (250 ml) at 2°C for 10 secs. The combined aliquots (1800 ml) were mechanically stirred for 30 min at 2°C. The suspension was centrifuged for 30 min at 4,000 g. and 2°C. To the supernatant (1250 ml) was added guanidine hydrochloride (480 g.) and the pH raised to 6.5 with N sodium hydroxide solution. The suspension was heated to 38°C in warm water and incubated for 1 hr, then lowered to 2°C in an ice bath. The precipitate was removed by centrifugation (30,000 g.) and washed with guanidine hydrochloride solution (4 M) and twice with sodium chloride solution (0.14 M). The precipitate was suspended in 0.14 M sodium chloride solution (35 ml) in a boiling tube and the temperature raised to 90°C within 1 min and maintained at 90°C for 2 mins, then chilled to 2°C in an ice bath. The suspension was shaken with an equal volume of a mixture of chloroform (35 parts) and amyl alcohol (10 parts) until no more protein appeared at the aqueous/organic liquid interface. The opalescent aqueous solution was dialysed against distilled water at 2°C until free of sodium chloride, then freeze dried. Yield: 2.4 g. (Found: N, 14.6%; P, 7.6%; moisture content 11.5%. Corrected for moisture content: N, 16.5%; P, 8.6%). (Grinnan & Mosher, 1951).

Inhibition of esterase/mandelate system. The rate of hydrolysis of methyl mandelate with esterase was compared in tubes containing 0, 0.1, 0.5 and 1 mg./ml. of the following nucleic acids separately: herring roe DNA (p.69), ox spleen DNA (p.69) and ox liver RNA (p.112).

Inhibition of pepsin/haemoglobin system. Flasks were incubated at 37°C with the following: (1) dialysed horse haemoglobin (2 ml), 0.1N hydrochloric acid (3 ml), 0.1% solution of crystalline pepsin (1 ml), ox spleen DNA (6 mg); (2) dialysed horse haemoglobin (2 ml), 0.1N hydrochloric acid (3 ml), 0.1% solution of crystalline pepsin (1 ml); (3) dialysed horse haemoglobin (2 ml), 0.1N hydrochloric acid (3 ml), water (1 ml); (4) water (2 ml), 0.1N hydrochloric acid (3 ml), 0.1% solution of crystalline pepsin (1 ml). After 5 mins 4% trichloracetic acid (10 ml) was added to each flask and the contents filtered. To each filtrate (3 ml) was added 0.1M sodium hydroxide solution (20 ml) and Folin's reagent (1 ml) at 37°C. The solutions were incubated for 10 mins and the blue colour estimated from flasks (1) (2) and (3) against flask (4) in a Spekker colorimeter using 1 cm cells and Ilford 606 filter. The procedure was repeated, but with 10 mins, 15 mins and 20 mins elapsing before adding trichloracetic acid.

opt.dens. flask (1)	opt.dens. flask (2)	opt.dens. flask (3)	time mins.
0.686	0.679	0.800	5
0.465	0.465	0.796	10
0.313	0.327	0.785	15
0.195	0.172	0.810	20

Table 14.

Preparation of phthalylglycine. (Dreschel, 1883) Phthalic anhydride (50 g.) was melted by heating in a flask, powdered glycine (25 g.) was added, and the yellow solution formed cooled. The solid was recrystallised from hot water. Yield 65 g. (94.3%) m.p. 193-4°C. (Found: C, 53.8%; H, 3.20%; N, 6.2%; $C_{10}H_7O_4N$ requires C, 53.8%; H, 3.14%; N, 6.3%).

Preparation of phthalylglycyl chloride. To dry benzene (200 ml) was added phthalylglycine (20.5 g) and phosphorus pentachloride (20.8 g). The mixture was heated at 60°C for 2 hours and the benzene evaporated. The solid was crystallised twice from dry benzene. Yield 18.3 g (82%) mp. 83°C.

Attempted preparation of glycyl-L-cysteine hydrochloride. Phthalylglycyl chloride (2.24 g) dissolved in dioxane (50 ml) was added dropwise to a stirred cold (5°C) mixture of L-cysteine hydrochloride (1.59g), magnesium oxide (0.95 g) and water (100 ml), after which the mixture was made acid (HCl) to litmus, and concentrated to dryness in vacuo at 0°C. The residue was suspended in ethanol (200 ml) and 90% hydrazine hydrate solution (1.5 ml) was added. The white suspension was refluxed for 1 hour, then evaporated to dryness in vacuo and the residue treated with 1.25N hydrochloric acid (20 ml) for 15 mins. The suspension was filtered and washed with water (50 ml). The filtrate was evaporated to an amorphous white powder insoluble in glacial acetic acid. The powder when dissolved in water gave a strong nitroprusside test but only a weak ninhydrin test. The experiment was repeated eight times with slight modifications.

Preparation of phthalylglycylglycine. Phthalylglycyl chloride (4.47g) dissolved in dioxane (25 ml) was added dropwise to a stirred cold (5°C) mixture of glycine (1.5 g), magnesium oxide (1.25 g) and water (75 ml). The temperature was raised to room temperature and the suspension made acid (HCl) to litmus and concentrated to dryness in vacuo. The residue was recrystallised from alcohol. Yield 4.1 g (85%) mp.230-231°C.

Preparation of glycylglycine. Phthalylglycylglycine (2.62 g) was suspended in ethanol (40 ml) and 90% hydrazine hydrate solution (1.5 ml) was added. The white suspension was refluxed for 1 hour, then evaporated to dryness in vacuo and the residue treated with 2N hydrochloric acid (25 ml) for 10 min at 50°C. The cooled suspension was filtered and the filtrate evaporated to dryness then dissolved in 100 ml water. The free amino acid was obtained by passing the solution through a column of Amberlite IRA 400 resin. The eluate was concentrated to 20 ml and alcohol was added until crystallisation occurred. Yield 1.0 g. (81.3%), m.p.210° decomp. (Found: C, 36.3%; H, 6.15%; N, 21.4%, $C_4H_8O_3N$ requires C, 36.4%; H, 6.06%; N, 21.2%).

Attempted preparation of glycyl-L-cysteine hydrochloride. Phthalyl-glycyl chloride (2.24 g) dissolved in dioxane (50 ml) was added dropwise to a stirred cold (5°C) solution of L-cysteine hydrochloride (1.59 g), and sodium carbonate (1.35 g) in water (100 ml). After warming the solution to room temperature sodium carbonate (1 g) was added, followed by 90% hydrazine hydrate solution (0.65 ml). After 2 days the solution was acidified (HCl) when a precipitate slowly formed, which was

filtered off (m.p. 334-337°C). The filtrate was evaporated to dryness under nitrogen at reduced pressure and the residue extracted with 5% ethanolic hydrogen chloride. No crystals were obtained. The amorphous white powder formed upon evaporation was chromatographed in n-butanol (50 parts), acetic acid (25 parts) water (25 parts), but no ninhydrin staining spot was detected.

Preparation of glycyl chloride hydrochloride. Glycine was prepared in a finely divided state by dissolving in a little warm water and precipitating with excess alcohol. The filtered compound was dried at 100°C and passed through a fine sieve (300 mesh). To the powdered glycine (10 g) was added freshly distilled acetyl chloride (200 ml). The suspension was vigorously stirred through a mercury seal in a dry apparatus open only to the atmosphere through drying tubes and powdered phosphorus pentachloride (31 g) was added in two portions. The apparatus was cooled to 2°C for the first 20 mins, then raised to room temperature for 1 hour. Phosphorus pentachloride (3 g) was then added and stirring continued for 4 hours more. The crystalline precipitate was removed by filtration, washed with acetylchloride and then dry petroleum ether. The fuming unstable product was used immediately.

Preparation of β-alanyl chloride hydrochloride. The preparation was similar to that for glycyl chloride hydrochloride. No special recrystallisation of β-alanine was required.

Preparation of S-β-alanylcysteamine dihydrochloride. Cysteamine hydrochloride (2 g) and fresh β-alanylchloride hydrochloride (3.5 g) were mixed in a mortar in a drying oven at 70°C. The mixture became oily and gave

off hydrogen chloride. After 30 mins the temperature was raised to 85°C and the mixture stirred until it solidified. After cooling the unchanged material was extracted with cold absolute alcohol and the residue dissolved in a little 2N hydrochloric acid. Upon addition of acetone the product crystallised as colourless needles. Yield 2.5 g. (71%) m.p. 175-178°C.

Preparation of S-glycyl-L-cysteine dihydrochloride. L-cysteine hydrochloride (3.25 g) and freshly prepared glycyl chloride hydrochloride (4 g) were mixed in a mortar in a drying oven at 70°C. The mixture became yellow and oily and gave off hydrogen chloride. After 30 mins the temperature was raised to 85°C and the mixture stirred until it solidified. After cooling, the unchanged material was extracted with cold absolute alcohol and the residue dissolved in a little 2N hydrochloric acid. Upon addition of acetone the product crystallised as colour-less needles. Yield 4.0 g. (77%), m.p. 173-175°C. (Found: C, 23.81%; H, 4.85%; N, 11,5%; $C_5H_{12}O_3N_2Cl_2S$ requires C, 23.90%; H, 4.78%; N, 11.16%).

Preparation of N-β-alanylcysteamine hydrochloride. S-β-alanylcysteamine dihydrochloride (3.4 g) was dissolved in 2N sodium hydroxide solution (8 ml) then made acid with 2N hydrochloric acid (2 ml). The solution was evaporated in a nitrogen filled dessicator and the residue extracted with hot absolute alcohol. To the combined extracts dry ether was added until slight turbidity occurred and after centrifugation the clear supernatant was evaporated and crystallised from iso-propanol. Yield 2 g (70.5%), m.p.125-6°C.

Attempted preparation of N-glycyl-L-cysteine hydrochloride. S-glycylcysteine dihydrochloride (25.1 mg) was dissolved in N/10 sodium hydroxide solution (1 ml) and N/10 hydrochloric acid (1 ml) was added. The solution was dried in a nitrogen filled desiccator and the residue extracted with hot absolute alcohol. The combined extracts were evaporated and crystallised from acetone/water. The product was identical with glycine hydrochloride mp. 176-178°C.

S-glycylcysteine hydrochloride (20 mg) was dissolved in a solution of sodium methoxide (0.71 ml) containing 6.05 g./l. and absolute methanol (10 ml). The solution was refluxed over nitrogen for 1 hour and left overnight. The solution was evaporated to dryness and the residue crystallised from acetone/water. The crystals were identical with glycine hydrochloride.

Preparation of phthalyl-L-leucylglycine. Phthalyl-L-leucyl chloride (5.6 g) prepared as for the method of phthalylglycyl chloride, dissolved in dioxane (50 ml), was added dropwise to a stirred cold (5°C) mixture of glycine hydrochloride (1.5 g), magnesium oxide (1.25 g.) and water (100ml). The mixture was raised to room temperature and made acid (HCl) to litmus and concentrated to dryness in vacuo. The residue was crystallised from alcohol. Yield 5.1 g. (80%).

Preparation of L-leucylglycine. A solution of phthalyl-L-leucylglycine (625 mg.), tri-n-butylamine (0.5 ml) and freshly distilled phenylhydrazine (0.4 ml) in ethanol (3 ml) was refluxed for 15 mins, during which crystals precipitated. Methylethylketone (10 ml) was added and the mixture refluxed for 15 mins more. After cooling glacial acetic acid (0.2 ml)

was added and the crystals filtered off, washed with methylethylketone and recrystallised from ethanol/water. Yield 285 mg (77%) m.p. 240°C. (Decomp.). $[\alpha]_D^{21}$ + 82.4° in water (C, 0.6). (Found: C, 51,32%; H, 8.53%; N, 14.7%, $C_8H_{16}O_3N$ requires C, 51.05%; H, 8.51%; N, 14.89%).

Preparation of L-cysteinylglycine. Glutathione (1 g) was dissolved in 0.6 N sulphuric acid (14 ml) and heated in a boiling water bath for 85 mins, then ice (26 g) was added. Sodium carbonate was added until pH 1.4 was reached, then mercuric sulphate (1.8 g) was added, and the white precipitate filtered off using "Hyflo super-cel". The precipitate was washed with water (2 l) until the filtrate was at pH 4.0. The precipitate was suspended in water (100 ml) and hydrogen sulphide bubbled through. The black precipitate was filtered off and the residual hydrogen sulphide removed with charcoal followed by nitrogen. Barium carbonate was added until the pH rose to 3.5, the precipitate was filtered off and the filtrate evaporated in vacuo. The residue was treated with alcohol (20 ml) and the suspension was removed by centrifugation. The suspension was dried in a desiccator.

Extraction and purification of cysteinylglycinase. Freshly excised pig kidney (200 g) was treated by the following method; at each stage the pH was kept above 7.0 by the addition of sodium bicarbonate.

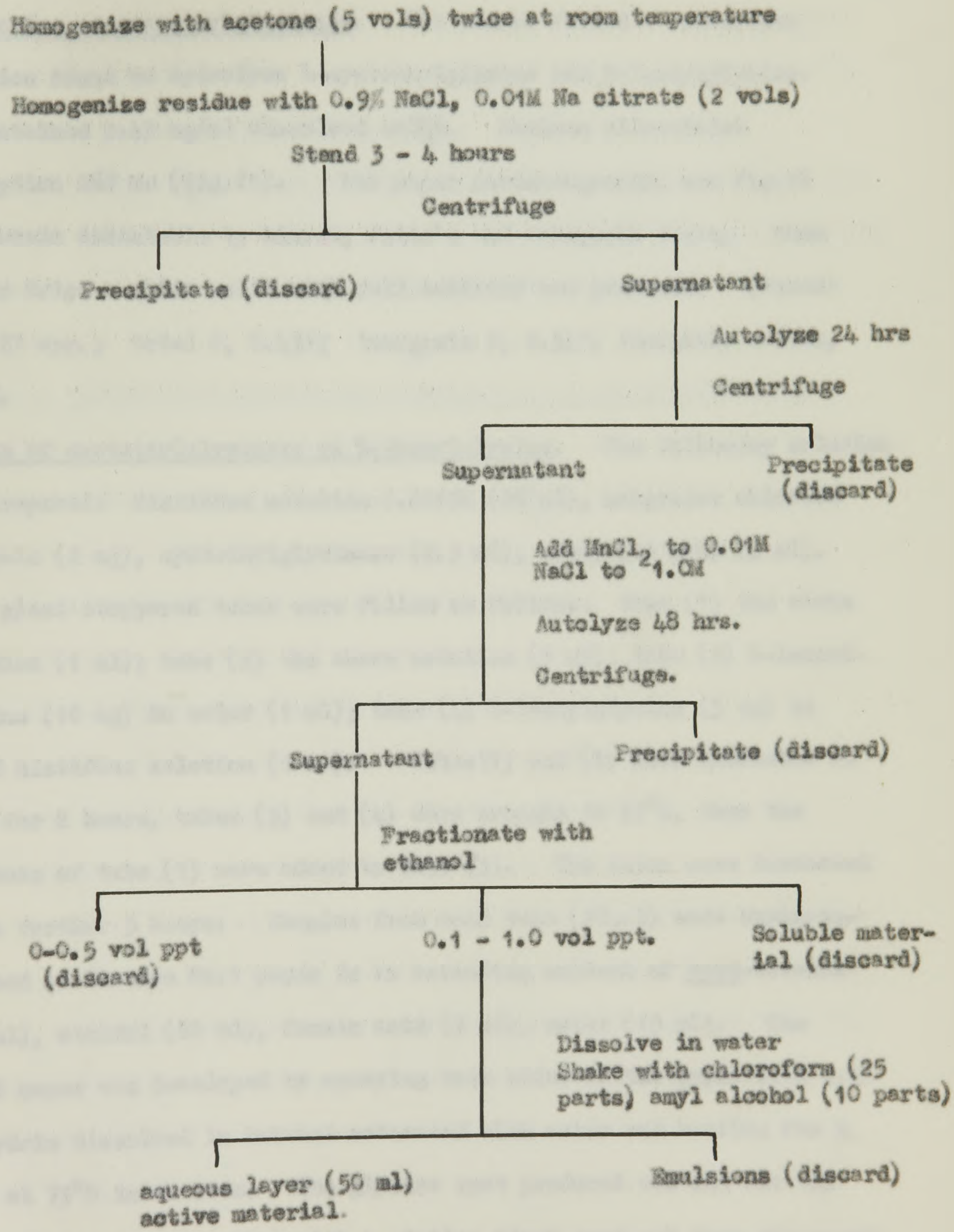

Homogenize with acetone (5 vols) twice at room temperature
Homogenize residue with 0.9% NaCl, 0.01M Na citrate (2 vols)
Stand 3 - 4 hours
Centrifuge
Precipitate (discard)
Supernatant
Autolyze 24 hrs
Centrifuge
Supernatant
Precipitate (discard)
Add $MnCl_2$ to 0.01M
NaCl to 1.0M
Autolyze 48 hrs.
Centrifuge.
Supernatant
Precipitate (discard)
Fractionate with ethanol
0-0.5 vol ppt (discard)
0.1 - 1.0 vol ppt.
Soluble material (discard)
Dissolve in water
Shake with chloroform (25 parts) amyl alcohol (10 parts)
aqueous layer (50 ml) active material.
Emulsions (discard)

Fig.27.

Properties of cysteinylglycinase. The enzyme formed a colourless solution found to hydrolyse L-cysteinylglycine and L-leucylglycine. It contained 2.45 mg/ml dissolved solid. Maximum ultraviolet absorption 260 mμ (fig.21). For paper chromatography, see fig.22 No protein detectable by biuret, Folin's and Sakaguchi tests. When freeze dried a white solid with full activity was produced. (Found: N, 0.2% app.; total P, 0.43%; inorganic P, 0.34%, inorganic solids, 66.5%.

Action of cysteinylglycinase on L-leucylglycine. The following solution was prepared: Histidine solution 0.0125M (20 ml), manganese chloride crystals (2 mg), cysteinylglycinase (2.5 ml), water (to make 25 ml). Four glass stoppered tubes were filled as follows: tube (1) the above solution (1 ml); tube (2) the above solution (1 ml); tube (3) L-leucylglycine (10 mg) in water (1 ml); tube (4) L-leucylglycine (5 mg) in 0.01M histidine solution (1 ml). Tubes (1) and (2) were incubated at 37°C for 2 hours, tubes (3) and (4) were brought to 37°C, then the contents of tube (1) were added to tube (3). The tubes were incubated for a further 5 hours. Samples from each tube (20 μl) were chromatographed on Whatman No.1 paper in an ascending solvent of tert-butanol (20 ml), ethanol (60 ml), formic acid (5 ml), water (15 ml). The dried paper was developed by spraying both sides of the paper with 0.4% ninhydrin dissolved in butanol saturated with water and heating for 5 mins at 75°C in an oven. The glycine spot produced was cut out and eluted with water together with a similar blank spot cut from the paper.

The eluted colour was measured in a Spekker colorimeter using 0.5 cm cell and Ilford filter 605. The quantity of glycine in the spot was found from a standard calibration curve.

Action of cysteinylglycinase on L-cysteinylglycine. The procedure was similar to that for L-leucylglycine, but after 20µl samples had been put on chromatogram papers 0.15 M N-ethylmaleimide solution (20 µl) was superposed.

Action of proteolytic enzymes and ribonuclease on cysteinylglycinase.

The following solutions were prepared:

A. Histidine (151 mg) in water (30 ml)

B. Manganese chloride crystals (80 mg) in water (100 ml)

C. L-leucylglycine (45 mg) in water (4.5 ml)

D. Crude pepsin (22.3 mg) in water (22.3 ml)

E. Crude trypsin (8.9 mg) in water (8.9 ml)

F. Crystalline ribonuclease (2.3 mg) in water (2.3 ml)

G. Crystalline trypsin (2.0 mg) in water (2.0 ml)

Twenty glass stoppered tubes were prepared as follows:

(1) A (1 ml), B (0.5 ml), cysteinylglycinase (0.5 ml) water(2 ml)

(2) A (1 ml) B (0.5 ml), cysteinylglycinase (0.5 ml) water (2 ml)

(3) A (1 ml), B (0.5 ml), cysteinylglycinase (0.5 ml) water (2 ml)

(4) A (1 ml), B (0.5 ml), cysteinylglycinase (0.5 ml) water (2 ml)

(5) A (1 ml), B (0.5 ml) cysteinylglycinase (0.5 ml) water (2 ml)

(6) C (0.5 ml)

(7) C (0.5 ml)

(8) C (0.5 ml)

(9) C (0.5 ml)

(10) C (0.5 ml)

(11) C (0.5 ml), F (1 ml), 0.02M Histidine solution (0.5 ml)

(12) C (0.5 ml), E (1 ml), 0.02M Histidine solution (0.5 ml)

(13) C (0.5 ml), G (1 ml), 0.02M Histidine solution (0.5 ml)

(14) C (0.5 ml), D (1 ml), 0.02M Histidine solution (0.5 ml)

(15) left empty

(16) left empty

(17) left empty

(18) left empty

(19) left empty

(20) L-leucylglycine (5 mg) in 0.01M Histidine solution (0.5 ml)

Tubes 1 - 5 were incubated at 37°C for 2 hours.

Then to tube (1) was added water (1 ml),

to tube (2) F (1 ml)

tube (3) E (1 ml)

tube (4) G (1 ml)

tube (5) D (1 ml)

Then ½ ml of tube (1) was added to tube (15),

½ ml of tube (2) to tube (16)

½ ml of tube (3) to tube (17)

½ ml of tube (4) to tube (18)

½ ml of tube (5) to tube (19).

4 ml of the remaining contents of tubes 1 - 5 were discarded. Tubes 1 - 5 and 15 - 19 were incubated for 1 hour The remaining tubes were brought to 37°C, then tubes 1 - 5 were added to 6 - 10 respectively and the remaining liquid-containing tubes incubated at 37°Cfor 5 hours. The contents of each tube were chromatographed as above (fig.25).

Action of heat on cysteinylglycinase. Two glass-stoppered tubes containing solutions as in tube (1) in the previous experiment were incubated at 37°C for 2 hours. One tube was placed in a boiling water bath and maintained at 98°C for 2 mins then cooled to 37°C rapidly in a water bath. The contents of both tubes were incubated with L-leucylglycine as substrate and afterwards chromatographed as previously described (fig.26).

Attempted dialysis of cysteinylglycinase. Cysteinylglycinase (5 ml) was dialysed for 3 hours at 2°C against distilled water (500 ml). The contents of the cellophane sac had the same unchanged ultraviolet absorption peak but were enzymically inactive. The outside fluid was also inactive, and remained so when evaporated in the frozen state to 5 ml. To the evaporated dialysate (1 ml) was added dialysed solution (1 ml) and the enzymic activity tested. No enzymic activity was present.

Action of nucleic acids on L-leucylglycine and L-cysteinylglycine. The hydrolysis of L-leucylglycine and L-cysteinylglycine was attempted, using the following nucleic acid solutions (1 mg/ml) instead of cysteinylglycinase: herring-roe DNA, ox spleen DNA, and ox liver RNA. The experiment was carried out as described for the action of cysteinylglycinase on L-leucylglycine.

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ABSTRACT.

In part I, the distribution of pyrimidine bases in ox spleen deoxyribonucleic acid (DNA) have been investigated by treating the freshly prepared nucleic acid with hydrochloric acid in the presence of ethyl mercaptan. By this means the purine bases were cleaved and a polymeric mercaptalated deoxyribonucleic acid (MDNA) was obtained in which diethylmercaptal groups replaced the purines. The vicinal hydroxyl groups in acyclic mercaptalated residues rendered MDNA labile to alkaline scission. The controlled degradation of MDNA by alkali yielded a pyrimidine polynucleotide (PPN), mercaptalated monopyrimidine nucleosides and 2-deoxyribosediethylmercaptal phosphates. The examination of these products suggested that in the parent nucleic acid, pyrimidine nucleotides occurred together in tracts. Evidence was found for pyrimidine trinucleotides in which the average ratio thymine/cytosine was 1.4. In addition to adjacent pyrimidine nucleotides and possible adjacent purine nucleotides, evidence also indicated the existence of single pyrimidine nucleotides flanked by purine nucleotides.

The fractionation of ox spleen deoxyribonucleic acid into adenine and thymine rich fractions and guanine and cytosine rich fractions was accomplished. The procedure involved the stepwise displacement of protein from the nucleoprotein with heparin, based on the highly insoluble complex formed between nucleo~~protein~~ histone and heparin. The displacement is

accompanied by the liberation of nucleic acid fractions. The guanine and cytosine rich fractions of the nucleic acid were more easily displaced from nucleoprotein than were the adenine and thymine rich fractions. The ratios(adenine and thymine/guanine and cytosine) varied progressively within the range 0.93 to 1.35 for individual fractions.

In part II, the influence of nucleic acids on certain enzyme systems was investigated. The effect of herring roe and ox spleen deoxyribonucleic acids and ox liver ribonucleic acid on the rate of action of two systems and the stereospecificity of one system was examined, but no effect was detected. The systems studied were the hydrolysis of methyl(±)-mandelate using pig liver esterase and the digestion of horse haemoglobin using pepsin.

The reported polynucleotidic nature of cysteinylglycinase from pig kidney (Binkley, F. (1952) Exp.Cell Res. Suppl.2 p.145) was re-investigated. An active dipeptidase which withstood exhaustive de-proteinisation by mixtures of chloroform and amyl alcohol was prepared from pig kidney. This preparation gave negative tests for proteins (biuret, Folin's and Sakaguchi). The enzyme could be freeze dried without loss of activity and had a strong absorption peak at 260 mμ, but in its analytical composition and paper chromatographic behaviour it did not resemble a polynucleotide. The enzyme required activation by manganous ions and effected the hydrolysis of L-cysteinylglycine and L-leucylglycine. Activity was abolished when the enzyme was dialysed against distilled

water and when it was heated to 98°C. The enzyme was unaffected by ribonuclease but was slowly inactivated by treatment with crystalline trypsin. Herring roe and ox spleen deoxyribonucleic acids and ox liver ribonucleic acid were tested for enzymic activity towards L-cysteinylglycine and L-leucylglycine but no hydrolysis of the dipeptides was observed. Comparison of the behaviour of the present enzyme with that of known dipeptidases suggests that the two are at least closely similar. Evidence is presented which indicates that the former is unlikely to be of a nucleic acid character.

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Distribution of Pyrimidine Bases in Herring-Roe Deoxyribonucleic Acid

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Although considerable evidence is now available for the 3′:5′-polynucleotidic structure of deoxyribonucleic acid (DNA), little is known of the sequence of the purine and pyrimidine groups. Partial degradation of DNA, achieved with deoxyribonuclease, results in a variety of products, including di- and tri-nucleotides (Smith & Markham, 1952*a*, *b*; Sinsheimer, 1954), some of which bear both purine and pyrimidine bases. The validity of such products as representing unaltered sequential fragments of the polynucleotide chain is, however, ambiguous, owing to the possibility of enzymic resynthesis or rearrangement. It is desirable therefore that chemical methods should be developed in order to study the problem.

It has been shown that in dilute aqueous acids purines alone are liberated from deoxyribonucleic acids, yielding strongly reducing products, which are still of large size and which have been called 'apurinic acids' (Tamm, Hodes & Chargaff, 1952). The sole nitrogenous constituents of these products are reported to be pyrimidines (cf. Lucy & Kent, 1953) present in the same ratio as in the parent DNA. Apurinic acids have, in general, much smaller molecular weights than the parent DNA, probably because of the hydrolysis of some of the internucleotidic linkages.

Unlike DNA, apurinic acids are degraded in mild alkaline solutions (Tamm, Shapiro, Lipshitz & Chargaff, 1953). The nature of the degradation is, however, uncertain, since several reactions may be involved. In addition to the cleavage of ester phosphate bonds of the purine-free sugar residues, random breakdown of the sugar itself cannot be discounted. The latter ambiguity may be circumvented and the purine-linked sugars 'marked' by making use of the earlier observation (Kent, 1950) that treatment of deoxyribonucleic acid with phenylmethanethiol in acidic conditions resulted in liberation of purines and the 'blocking', as mercaptals, of the reducing-sugar groups thus formed. The resulting sulphur-containing residue resembled the apurinic acids in being alkali-labile. In this case, however, D-2-deoxyribose dibenzylmercaptal was isolated as a crystalline derivative. A modification of the method has been successfully used (MacDonald & Knight, 1953) to identify the purine-linked sugar in the ribonucleic acid of tobacco mosaic virus.

In the present work, herring-roe deoxyribonucleic acid was treated with ethanethiol in the presence of hydrochloric acid (Kent & Lucy, 1954) and the non-diffusible derivative, mercaptalated deoxyribonucleic acid (MDNA), degraded with alkali to a mixture of stable pyrimidine polynucleotides (PPN).

Our results, of which preliminary reports have already been published (Lucy & Kent, 1953; Kent & Lucy, 1954), have been criticized by Jones & Letham (1954) on the ground of their inability to prepare MDNA from DNA. Although the yields of MDNA (on a weight basis) are in general only about 10% of the initial DNA, we believe that the method furnishes a suitable procedure for the examination of sequences of adjacent pyrimidine nucleotides in DNA. Available evidence indicates that only purines are liberated in the mercaptalation reaction and that pyrimidine polynucleotides are stable under the conditions of mercaptalation. Jones & Letham (1954) claim to have obtained higher yields of mercaptalated derivative by the action of thioglycollic acid on DNA in the presence of anhydrous zinc chloride. Under these conditions, however, it is uncertain whether the mercapto acid behaves solely as a thiol or whether it forms esters with the nucleic acid or its derivatives.

METHODS AND MATERIALS

Elementary analyses for P, N and S were performed by Drs Strauss and Weiler, Oxford. Freeze-dried specimens of DNA, MDNA and PPN were analysed and the results corrected for the moisture content, which was determined separately (Lee & Peacocke, 1951). Phosphorus was also determined by the method of Allen (1940).

Ultraviolet-absorption measurements were made on aqueous solutions with Beckman and Hilger Uvispek spectrophotometers, with 1 cm. quartz cells.

Purine and pyrimidine analyses were performed by the hydrolytic and the paper-chromatographic procedures described by Wyatt (1951).

Dische extinction coefficients were determined by the method of Allerton, Overend & Stacey (1952).

Paper-electrophoretic separations were carried out on Whatman no. 1 paper in 0·2M sodium acetate buffer (containing 0·05M sodium chloride) (pH 5) or in 0·2M sodium

borate buffer (pH 8), (Pasternak & Kent, 1952), with potential gradient 12 v/cm.

Commercial deoxyribonucleic acid (from herring roe) was supplied by L. Light and Co. Ltd., Colnbrook, Bucks.

Fibrous herring-roe deoxyribonucleic acid was prepared by the method of Daly, Allfrey & Mirsky (1950).

Paper partition chromatography. Chromatograms were prepared on Whatman no. 1 paper by downward development in the following solvent systems: (*a*) *n*-butanol–ethanol–water (4:1:5 by vol.); (*b*) phenol saturated with water; (*c*) *iso*propanol (138 ml.)–HCl (sp.gr. 1·18, 31·7 ml.)–water (30·5 ml.); (*d*) water-poor phase of ethyl acetate (100 ml.)–pyridine (45 ml.)–water (10 ml.). Spraying reagents used were aniline hydrogen phthalate (Partridge, 1949) for reducing sugars, perchloric acid and ammonium molybdate (Hanes & Isherwood, 1949) for phosphate esters, and I_2 vapour (Greenway, Kent & Whitehouse, 1953) for mercaptals. Ultraviolet photography was used to detect purines, pyrimidines and their derivatives (Markham & Smith, 1949).

EXPERIMENTAL

Mercaptalation of DNA. (i) Ethanethiol (15 ml.) was added to fibrous DNA (0·49 g.) in a glass-stoppered flask. Hydrochloric acid (sp.gr. 1·18, 0·5 ml.) was then added and the mixture was shaken for 14 hr. at 18°. The reaction mixture was diluted with water (45 ml.) and the unchanged thiol removed by extraction with ether. The aqueous layer was then brought to pH 7 by addition of $NaHCO_3$ and was dialysed in cellophan against distilled water until chloride-free. During dialysis a white solid separated within the sac and was found to be chiefly a mixture of crystalline adenine and guanine. Freeze-drying of the contents of the sac furnished 45 mg. of a white amorphous solid, MDNA (1), containing: N, 9·90; P, 9·62; S, 7·8%.

(ii) MDNA (2) was prepared from commercial herring-roe DNA (6 g.). Ethanethiol (45 ml.) and HCl (sp.gr. 1·18, 12 ml.) were employed and the product was isolated as in the preparation of MDNA (1). The product contained: N, 8·43; P, 7·46; S, 5·9%.

Attempted mercaptalation of apurinic acid. Apurinic acid (0·7 g.) prepared from herring-roe DNA by the method of Tamm *et al.* (1952) was suspended in ethanethiol (30 ml.) saturated with dry HCl. After 1·5 hr. at 0, when solid had dissolved, ether (25 ml.) was added and sufficient dilute $NaHCO_3$ solution to bring the pH to 7. The aqueous layer was re-extracted with ether, then dialysed at 0° in cellophan for 94 hr. against distilled water. Freeze-drying of the dialysed solution yielded a partially mercaptalated derivative (47 mg. Found: N, 4·59; P, 8·01; S, 4·8%).

Diffusible products arising during mercaptalation. In the course of preparation of MDNA (1), the material was dialysed in cellophan against distilled water (3·72 l. for 92 hr.). The external solution was concentrated under reduced pressure below 30° to 10 ml. The resulting concentrate was examined by paper chromatography. The results are summarized in Table 1.

Properties of MDNA (1). Paper-chromatographic examination of this material in butanol–ethanol–water revealed the presence of a major constituent (R_F 0–0·1) and two minor substances (R_F 0·26 and 0·32). The substance (R_F 0·26) contained P and S, and when eluted in 0·1 N-HCl was found to have $\lambda_{max.}$ 275 mμ. The second minor constituent (R_F 0·32) also contained P and had an absorption maximum at $\lambda_{max.}$ 275 mμ. but was sulphur-free.

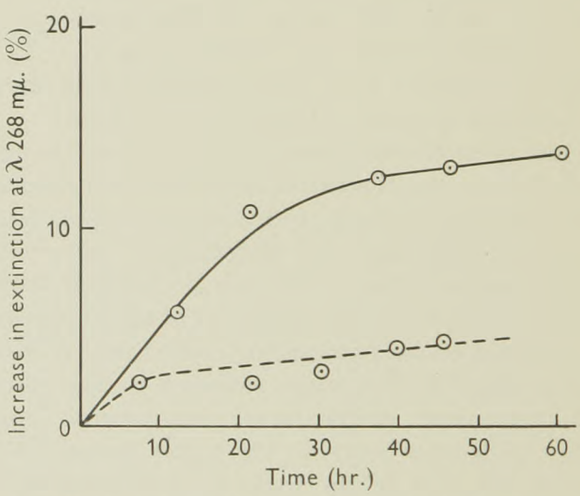


Fig. 1. Alkaline degradation of mercaptalated deoxyribonucleic acid (MDNA). - - - -, Deoxyribonucleic acid; ——, MDNA (2).

Table 1. *Diffusible products formed during preparation of MDNA* (1)

Solvent	R_F: Detection of components: Ultraviolet absorption	Iodine vapour	Perchloric acid–molybdate	Authentic compounds
*iso*Propanol (138 ml.)–hydrochloric acid (sp.gr. 1·18, 31·7 ml.)–water (30·5 ml.)	0·33	—	—	0·31 Guanine
	0·49	—	—	0·43 Adenine
	0·71	—	—	0·90 Deoxycytidine-5′ phosphate
	0·88	—	0·90	—
	1·0	—	0·97	0·97 Thymidine-3′:5′ diphosphate
n-Butanol–acetic acid–water (4:1:5 v/v)	0–0·11	0·09–0·11	—	—
	0·27	0·19†	—	—
	0·36	—	—	—
	0·45	—	—	—
	0·61	0·88*	—	0·9 Deoxyribose diethylmercaptal (?)

* + + Dische test, P-free. † + + Dische test, contained P.

Alkaline degradation of mercaptalated deoxyribonucleic acid (MDNA). (i) In a typical experiment, the rate of degradation was followed spectroscopically. MDNA (2) (0·9 mg.) was dissolved in 0·5 N-NaOH (20 ml.) and solution maintained at 33·5°. The change in light absorption at 268 mμ. was measured at intervals (Fig. 1). A similar quantity of fibrous DNA was treated simultaneously under identical conditions.

(ii) Mercaptalated nucleic acid, MDNA (2) (92 mg.), was treated with 0·01 N-NaOH (6 ml.) for 237 hr. at 18°, and dialysed twice in cellophan against distilled water (90 ml., each time for 48 hr.). The solution (19·3 ml.), adjusted to neutrality, when freeze-dried yielded a white amorphous product (PPN). (Found: N, 6·91; P, 8·31; S, 3·3%.)

Diffusible products formed during the alkaline degradation of MDNA. Examination of the external solutions from the dialysis of PPN in (ii) above failed to reveal any inorganic phosphate. The two solutions contained phosphoric esters (23·5 and 7 μg. P/ml. respectively) corresponding to 48% of the combined P of the MDNA. A portion of the first external solution (25 ml.), neutralized by being shaken with the acidic form of Amberlite IR 100 (H) (Rohm and Haas Co., Philadelphia 5, Pa., U.S.A.), was concentrated to 1 ml. and examined by paper chromatography in ethyl acetate–pyridine–water. Two components (R_F 0·03 and 0·13) were detectable in ultraviolet light, on treatment with iodine vapour and by spraying with perchloric acid and ammonium molybdate (Bandurski & Axelrod, 1951). The ultraviolet-absorbing character of these components was shown, by hydrolysis in 70% (w/w) $HClO_4$ and paper chromatography, to be due to cytosine and thymine residues. A further component, R_F 0·31, which contained no N or P but which on elution gave a positive Dische reaction and positive tests for S, was present.

Properties of PPN. The substance was chromatographically immobile in butanol–ethanol–water. When examined by paper electrophoresis at pH 7·93 (0·2 M sodium borate buffer containing 0·05 M-NaCl) and at pH 5 (0·2 M sodium acetate buffer containing 0·05 M-NaCl), under a potential gradient of 12 v/cm., the substance moved as a single component.

Retreatment of isolated PPN with ethanethiol and HCl under the conditions of preparation of MDNA was without action on PPN.

Attempted desulphuration and methylation of MDNA. MDNA (117 mg.), dissolved in 50% (v/v) aqueous methanol (30 ml.), was warmed at 60° with 1 g. of Raney nickel (Mozingo, 1941) for 5 hr. After removal of the catalyst the solution was concentrated at 40° under reduced pressure. A solid (60 mg.) was obtained which contained S, 1·63%. The initial MDNA contained S, 5·9%. The product was methylated under the conditions described by Anderson, Barker, Gulland & Lock (1952), absolute methanol (15 ml.), methyl iodide (2 ml.) and dry Ag_2O (2 g.) being used. The product was found to have an apparent methoxyl content (Zeisel) of 13·7%. The methylated product was treated with 0·1 N-NaOH (3 ml.) at 100° for 4 hr. After neutralization with 0·1 N-HCl, the solution was concentrated to dryness. The product had OMe 6·35% (corrected for the presence of NaCl) and was free from traces of organically bound sulphur.

RESULTS

The mercaptalated nucleic acid (MDNA) was an amorphous solid, highly soluble in water, and it gave a negative Fehling's reaction. The ratio of contained thymine to cytosine was little different from that of the parent nucleic acid. The analytical composition of DNA and MDNA (1) is shown in Table 2.

In a model experiment each of the four bases was treated with ethanethiol and hydrochloric acid under the same conditions as those prevailing in the preparation of MDNA. The bases were recovered, as indicated by R_F values and by ultraviolet-absorption characteristics.

Towards the Dische diphenylamine reagent (Deriaz, Overend, Stacey, Teece & Wiggins, 1949), MDNA (1) gave less coloration than an equivalent amount of the parent DNA, the respective values being 940 and 1850 (Spekker units/atom P) (Allerton *et al.* 1952). This change is consistent with the replacement of the readily hydrolysed purine groups by $(-S.C_2H_5)_2$ in MDNA. This is further emphasized by the changes in spectral properties. Whereas fibrous DNA has an extinction of 6476 (ϵ_p extinction/atom P) at $\lambda_{max.}$ 259 mμ., MDNA (1) gives a value of 4555 (ϵ_p) at $\lambda_{max.}$ 270 mμ.

In a preliminary examination in the Svedberg ultracentrifuge it was found that the sedimentation

Table 2. *Composition of fibrous-herring-roe DNA, MDNA* (1), *MDNA* (2) *and PPN*

Bases*	DNA	MDNA (1)	MDNA (2)	PPN
(Moles/4 atoms of P)				
Adenine	1·00	0·03	0·0	0·0
Guanine	0·77	0·11	0·16	0·0
Cytosine	0·70	1·44	1·93	1·19
Thymine	1·01	2·02	2·73	1·90
Thymine/cytosine (mol.prop.)	1·45	1·40	1·45	1·60
Nitrogen (atoms/4 atoms of P)	12·92	9·10	10·0	7·36
Sulphur (atoms/4 atoms of P)	0·0	3·17	3·07	1·56
Dische value (per atom of P)	1850	940	1140	990
(ϵ_p) ($\lambda_{max.}$ 259 mμ.)	6476	—	—	—
(ϵ_p) ($\lambda_{max.}$ 270 mμ.)	—	4555	4630	5032

* 5-Methylcytosine was not determined in these preparations.

constant of MDNA (1) is considerably less than that of the parent DNA. Paper electrophoresis of MDNA (1) suggested the presence of at least two ultraviolet-absorbing components.

MDNA (2) was readily degraded in alkaline conditions and the degradation was accompanied by a marked increase in optical density at 268 mμ. and liberation of at least two diffusible products having the properties of deoxycytidine phosphates. Inorganic phosphate was detected in only trace amounts and no substance having reducing-sugar properties was liberated. Model experiments with D-arabinose diethyl mercaptal showed that such derivatives were stable under the alkaline conditions employed in these investigations. Under the conditions used by Brown, Magrath & Todd (1952) for the alkaline degradation of ribonucleotides, it was found that the ϵ_p values of MDNA increased more rapidly than those of a specimen of DNA similarly treated (Fig. 1). In this reaction 48 % of the phosphorus was liberated as ester phosphate, the non-diffusible product (PPN) having the composition shown in Table 2. Both in chromatographic and paper-electrophoretic studies, PPN behaved as a single component.

DISCUSSION

The experimental results indicate that mercaptanolysis of deoxyribonucleic acid is achieved through the purine-linked sugars and that the reaction takes the course shown in Fig. 2.

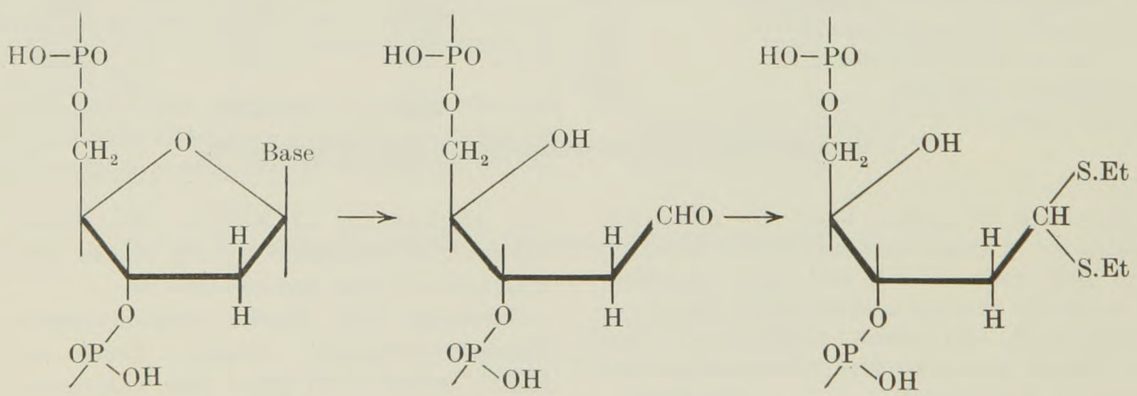


Fig. 2. Mercaptalation of purine deoxyribonucleotidic residues.

This is supported by the following arguments: (*a*) The diminished Dische coloration shown by MDNA compared with DNA. (*b*) DNA has ϵ_p 6476 at $\lambda_{max.}$ 259 mμ., whereas the derived MDNA has ϵ_p 4555 at $\lambda_{max.}$ 270 mμ. [cf. ϵ_p 4578 at $\lambda_{max.}$ 270 mμ. for herring-roe apurinic acid and ϵ_p 4600 at $\lambda_{max.}$ 268 mμ. for calf-thymus apurinic acid (Tamm *et al.* 1952)]. (*c*) The reaction (Fig. 2) requires that for each purine displaced from the nucleic acid 2 atoms of sulphur enter. The analytical composition (Table 2) shows that in mercaptalation 1·62 moles of adenine + guanine (per 4 atoms of P) have been removed and that 3·17 atoms of sulphur are present in the product, i.e. 1·58 sugar residues have been converted into diethylmercaptals. The ratio is therefore 1:2:1. No evidence has yet been found of interaction in any other way between deoxyribonucleic acid components and ethanethiol.

Mercaptalation, under the conditions described, undoubtedly results in products of considerably decreased molecular weight. The reaction is accompanied by liberation of purines, some diffusible nucleotides and deoxyribose derivatives. These appear to include thymidine-3′:5′ diphosphate and deoxycytidine-5′ phosphate, and will be described in a later communication. Pyrimidines and inorganic phosphate are, however, released in not more than trace amounts.

The effect of mercaptalation is to convert every formerly purine-linked sugar into stable acyclic derivatives. Every such sugar residue is endowed with an additional hydroxyl group at $C_{(4)}$, i.e. that which was formerly held in the ring structure. This hydroxyl group is in the α position to the phosphate esters at $C_{(5)}$ and $C_{(3)}$, and a situation similar to that in ribose nucleic acids exists. It has been pointed out (Brown & Todd, 1952) that such α-hydroxy secondary phosphate esters are alkali-labile through the agency of an intermediate cyclic phosphate-ester form, cleavage of which leads to two isomeric primary phosphates. In the case of MDNA, cyclic phosphates may be formed between the 4′:5′- and 3′:4′-positions, yielding on subsequent hydrolysis the 3′:4′- or 5′-phosphate esters.

The experimental results show that MDNA is indeed easily degraded by dilute alkali with elimination of 48 % of its phosphorus as esters, but without the liberation of inorganic phosphate. The non-diffusible residue (PPN) shows properties

consistent with the loss during hydrolysis of pyrimidine-free constituents by (*a*) increase in ϵ_p from 4630 to 5032 without shift in $\lambda_{max.}$ 270 mμ., (*b*) decrease in sulphur content of the product PPN, and (*c*) increase in the N/P ratio.

No free pyrimidines are formed in the reaction. Examination of the diffusible products by chromatography (after removal of the interfering inorganic substances by means of an ion-exchange resin) gave evidence of the presence of both thymine and cytidine nucleotides.

The presence of sugar hydroxyl groups in MDNA is further demonstrated by the fact that, after partial reductive desulphuration with Raney nickel (Mozingo, 1941), the material can be methylated under the same conditions as ribose nucleic acid and its derivatives. The apparent methoxyl content (13·7 %) is unlikely to be an accurate value, however, owing to the presence of residual thioethyl groups which interfere in the Zeisel determination. It is of interest, however, that this value is decreased to 6·35 % by alkaline hydrolysis, a means by which fortuitous methyl phosphoric esters are hydrolysed and the remaining sulphur is expelled. The resulting methylated derivative, unlike MDNA, is stable and does not undergo the 'elimination' reaction in alkaline conditions to form analogues of PPN.

It will be noticed that alkaline degradation of MDNA does not result in complete expulsion of the mercaptalated deoxyribose residues. The retention of sulphur (1·56 atoms/4 atoms of P) is, however, not inconsistent with the 'elimination' mechanism of alkaline breakdown of nucleotides, since products may result, e.g. 4′-phosphate, in which migration has resulted in the blocking of the hydroxyl group requisite for cyclic phosphate-ester formation. Such a product should be stable to alkaline reagents, the retained mercaptalated residue constituting an end-group. Until this has been further investigated, ambiguity persists in the interpretation of the results. It can, however, be concluded that herring-roe deoxyribonucleic acid is not entirely composed of alternating purine and pyrimidine nucleotides and that regions of adjacent pyrimidine nucleotides occur.

In the minimal arrangement, PPN can be regarded as a trinucleotide bearing a mercaptalated-sugar end-unit. In such a substance two deoxycytosine nucleotides (C) are adjacent to one thymidine nucleotide (T) in the form TCT or CCT. Throughout this investigation, bases in DNA have been expressed for convenience in terms of 4 atoms of phosphorus, though neither in this nor in the majority of other reported results are 4 bases present for 4 atoms of phosphorus. In PPN, however, the correspondence is close, 3·87 moles of 1′-substituents (thymine + cytosine + $\frac{1}{2}$ atomic sulphur content) being present for every 4 atoms of phosphorus.

The results indicate that mercaptalation provides a route for the separation of consecutive pyrimidine nucleotides from DNA without possible ambiguities of enzymic rearrangements. These pyrimidine polynucleotides will be discussed further in a subsequent publication.

SUMMARY

1. Herring-roe deoxyribonucleic acid has been partly degraded to pyrimidine polynucleotides by non-enzymic methods.

2. The purines were selectively removed and the liberated reducing sugars were blocked in the acyclic form as ethylmercaptals. The thymine/cytosine ratio of the product differed little from that of the original DNA.

3. The mercaptalated product was hydrolysed by dilute alkali to yield diffusible organo-phosphorus compounds, and a non-diffusible purine-free substance containing thymine and cytosine.

4. The degradation is consistent with the 'elimination' mechanism of nucleotide breakdown.

5. The results suggest that there are regions in DNA where at least three pyrimidine nucleotides are linked together.

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