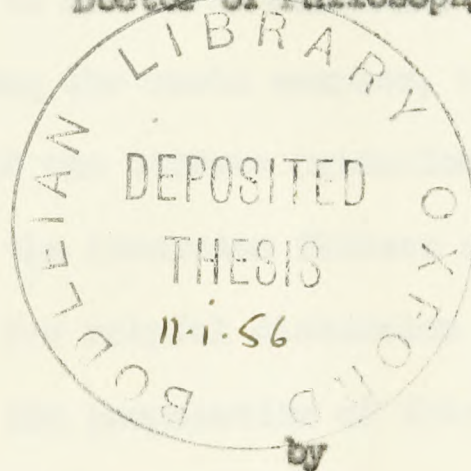


- (1) AUXILIARY COFACTORS IN THE METABOLISM OF CITRATE BY YEAST
(11) BIOCHEMICAL STUDIES OF INTESTINAL MUCINS

A thesis presented in candidature for the degree

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ABSTRACT.

PART I

STUDIES OF SOME AUXILIARY COFACTORS IN
THE METABOLISM OF CITRATE BY YEAST

INTRODUCTION

In 1784, Scheele reported the discovery and isolation from lemon juice of a new organic acid, which he called citric acid. For very many years it was considered that citric acid occurred solely in plant species but following Henkel's discovery of it in milk (1888), it was subsequently detected in a large number of animal tissues and in many microorganisms (Thunberg, 1953).

In animal tissues, citric acid is now known to be synthesised and rapidly metabolised in the tricarboxylic acid cycle. This enzyme sequence (fig.1) first described by Krebs and Johnson in 1937, has been fully elucidated in recent years (reviewed by Ochoa, 1954, Krebs, 1954). Since it may be regarded as both a terminal stage in the oxidation of diverse metabolites, and also a means of supplying compounds which serve as intermediates for the synthesis of fatty acids, steroids, amino acids, porphyrins, etc., the cycle appears to represent an economy of the chemical mechanisms requisite for cell function (Krebs, 1953). It has been generally assumed that the tricarboxylic acid cycle is the fundamental pathway whereby both acetate and citrate are metabolised in all those tissues in which formation of citric acid has been detected. However, it has been realised recently that in microorganisms at least, the cycle may play only a minor role, compared with other pathways of terminal respiration. Some of the alternative mechanisms propounded, which include the functioning of a dicarboxylic acid cycle (fig. 2), have been reviewed by Ajl (1951) and Barron (1952).

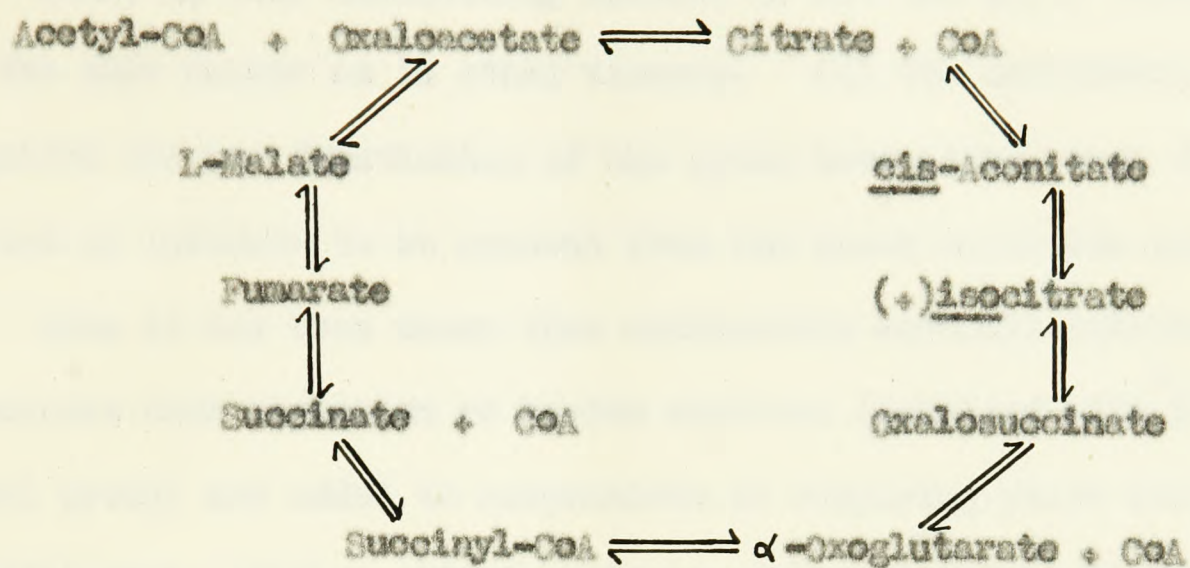


Fig. 1. The tricarboxylic acid cycle.

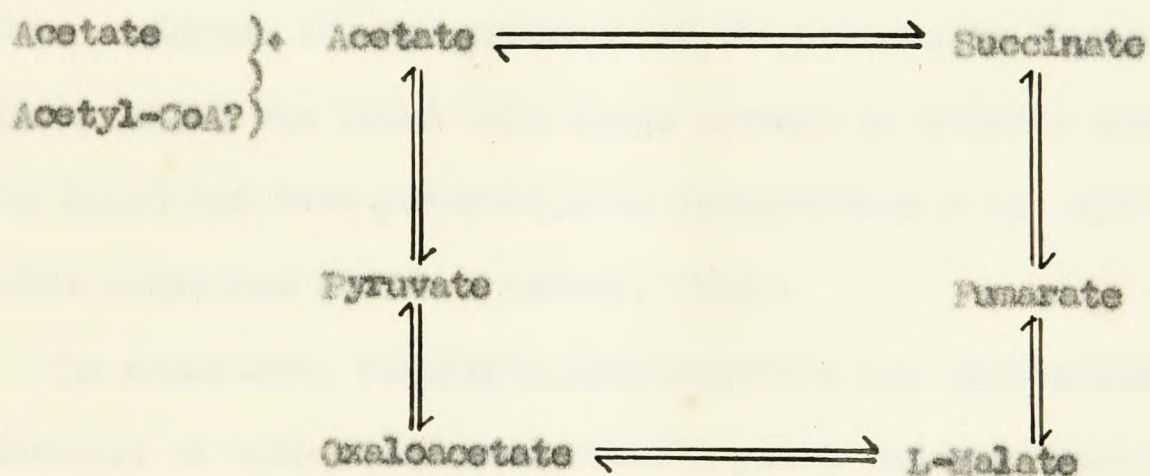


Fig. 2. The dicarboxylic cycle.

Role of the tricarboxylic acid cycle in yeast.

There is some controversy whether or not the cycle functions in yeast in the same manner as in other tissues. All the individual enzyme systems required for the functioning of the cycle have either been definitely reported or inferred to be present from the known metabolic activity of yeast.

Thus it has been shown that radioactive carboxyl-labelled citrate accumulates when magnesium or barium acetates (labelled with ^{14}C in the carboxyl group) are added to suspensions of respiring yeast cells (Weinhouse + Millington, 1947). Citric acid is synthesised in cell-free extracts of yeast incubated with acetate and oxaloacetate (Novelli + Lipmann, 1950) and the condensing enzyme responsible for this synthesis has been partially isolated from two strains of baker's yeast (Ochoa, Stern + Schneider, 1951). Further evidence for the presence of the condensing enzyme has been given by Foulkes (1952) who found that large amounts of citrate accumulated in whole cells which had been poisoned with fluoracetate - one effect of which is to inhibit aconitase (Buffa + Peters, 1950).

The aconitase, isocitric dehydrogenase and oxalosuccinic decarboxylase activities of both commercial baker's yeast and pure cultures of Saccharomyces cerevisiae have been amply demonstrated (Weinhouse + Millington, 1947. Foulkes, 1951 a, Kornberg + Prier^c, 1951, Hirsch, 1952) and further confirmed in the present studies. Kornberg and Prier^c (1951) found that there are two distinguishable systems in baker's and brewer's yeast catalysing the oxidative decarboxylation of (+)-isocitrate to α -oxoglutarate. One of these enzyme systems, which specifically requires the presence of triphosphopyridine nucleotide (TPN, Co II), catalyses the decarboxylation of oxalosuccinate and generally appears to resemble the system in animal tissues

described by Ochoa (1954). The second dehydrogenase system specifically requires diphosphopyridine nucleotide (DPN, CoI) as the coenzyme. It fails to catalyse the reductive carboxylation of α -oxoglutarate and does not appear to involve oxalosuccinate as an intermediate. As a further distinction from the TPN isocitric dehydrogenase, there is a specific and absolute requirement for adenosine-5-phosphate.

The occurrence of the further stages of the cycle has been deduced from observations that under certain conditions, preparations of yeast cells can oxidise succinate to fumarate and malate to oxaloacetate (Lynen + Nequillah, 1939) and the finding of fumarase in both brewer's and baker's yeast (Jacobsohn, 1931, Hirsch, 1952). The presence of α -oxoglutarate dehydrogenase (decarboxylase) has only been inferred from very indirect evidence. Foulkes (1951a) showed that oxoglutarate disappeared when incubated with cell-free extracts of baker's yeast and that this disappearance was neither accelerated by co-carboxylase nor inhibited by arsenite or lewisite, as are the corresponding systems in mammalian tissues (Peters, 1952). The possibility that oxoglutarate was removed by reductive amination to form glutamic acid, carboxylation to synthesise oxalosuccinic acid or by some other pathway not leading to succinate formation, cannot be discounted on this evidence. Nossal (1954a) has noted that the oxidation of oxoglutarate in poorly respiring cell-free yeast extracts is accompanied by a low yield of succinate and the production of an unidentified acid, 'tricarballic acid'. Some experiments leading to the formation of deuterium-labelled succinate (and citrate) when intact yeast cells are incubated with deuterio-acetate (CD_3COCD) and non-isotopic fumarate (Lynen, 1947. cf. Sonderhoff + Thomas, 1937) are considered to support the assumption that

succinate arises from acetate in this organism through the stages of the cycle, including that of oxoglutarate dehydrogenase. These observations are equally explicable however by the possible condensation between two molecules of acetate or related C_2 compounds to yield succinate, as was first proposed by Thunberg (1920) and Knoop (1923) and found experimentally in Aerobacter (Slade + Werkman, 1943) and the mould, Rhizopus nigricans (Poster + Carson, 1950). Weinhouse and Millington (1947) found that the C^{14} - content in the carboxyl groups of C_4 acids isolated from yeast, following incubation with acetate labelled with C^{14} in the carboxyl group, was greater (57%) than the maximum (50%) calculated assuming all the radioactive C_4 acids are produced from the radioactive acetate via the stages of the tricarboxylic acid cycle. This would indicate that not all the C_4 acids in yeast are indeed derived from the cycle but that another mechanism also exists for their formation from acetate.

Thus all the enzyme systems required for the functioning of the cycle (save possibly α -oxoglutarate dehydrogenase) are present in yeast cells, but the experimental evidence does not exclude the simultaneous functioning of a dicarboxylic acid cycle as a pathway for the terminal oxidation of acetate. The qualitative evidence presented above, by itself does not justify the assumption that the tricarboxylic acid cycle is the main terminal respiratory in yeast. Such data as are given in the literature suggest that the rates of oxidation of isocitrate to α -oxoglutarate, succinate to fumarate and malate to oxaloacetate are lower by at least 50% than either the rates of oxidation of acetate or of overall respiration (Krebs, 1954). The fact that these stages of the cycle are very slow argues against the cycle being the major energy-yielding mechanism of the cells in the strains of yeast studied.

Furthermore, it has been found that the addition of citrate, succinate or malate to suspensions of baker's yeast fails to significantly increase the rate of respiration (Hirsch, 1952, Krebs, 1952), even at acidic pH values when the free acids might be expected to penetrate whole cells and therefore be metabolised more readily than their ionic forms (Barron, Ardao + Hearon, 1950).

Krebs, Gúvin and Eggleston (1952) have adduced some experimental evidence which suggested that the tricarboxylic acid cycle does not function in yeast as the main or only pathway for the oxidation of C₂ fragments ('active' acetate, etc.). Cells rendered freely permeable to di- and tri-carboxylic acids by freezing at -40° were found to possess aconitase and fumarase activity, yet lost their ability to metabolise citrate and fumarate, whilst acetate was still oxidised to carbon dioxide. The addition of malonate to these cold-treated cells was without effect on the oxidation of acetate but completely inhibited succinate oxidation. It would therefore appear that succinic dehydrogenase activity is not essential to acetate oxidation in frozen cells.

Some further experiments were reported in which C¹⁴-labelled acetate was simultaneously oxidised together with various non-isotopic intermediates of the cycle by suspensions of cold-treated yeast cells. These intermediates were partially recovered at the end of the experiments and found to be without radioactivity, whilst the respired carbondioxide was radioactive. It was therefore concluded that in the cold-treated cells, which respire at about one-third the rate of untreated yeast, acetate is oxidised to carbondioxide by a pathway which is independent of both the di- and tri-carboxylic acid cycles. However, in the one experiment in which the intra-cellular

succinate of fresh yeast cells oxidising C¹⁴-acetate was examined, it was found to be radioactive. The recent work of Saz, Swim and Kramitz (1954) shows that the non-incorporation of acetate into various added non-isotopic intermediates can no longer be taken as conclusive evidence against the active participation of these intermediates in any pathway of acetate oxidation.

In contrast to these findings of Krebs, Gurin and Eggleston, Nossal (1954b) has found that radioactive citrate, α -oxoglutarate, succinate and fumarate can be recovered following incubation of C¹⁴-acetate and oxaloacetate with the granular extracts obtained from rapidly disintegrated cells. The rate of malate oxidation by these cells was very slow though the actual activity of the malic dehydrogenase present was high. Since the radioactivity of the respiratory carbon dioxide was also found to be low, these findings were interpreted as indicating that acetate is oxidised to carbon dioxide in these cell-free extracts solely through the functioning of the tricarboxylic acid cycle. Any alternative pathway(s) for acetate catabolism in whole cells was presumed to be destroyed by the conditions of cell-fragmentation.

Though the sum of all this evidence would indicate that the component reactions of the tricarboxylic acid cycle do occur in yeast, yet the precise function of the cycle still remains in doubt. It appears that the cycle plays only a limited role as a mechanism for the provision of energy to the cell with the synthesis of high energy phosphate bonds, since the overall rate of oxidation of acetate through the cycle is so low. Kilkenny and Hinshelwood (1951) arrived at a similar conclusion following a study of the rates of adaptation and growth of S. cerevisiae when glucose in the growth

medium was replaced by various intermediates of the tricarboxylic acid cycle. As Krebs has pointed out (1952), in growing microorganisms there are always two major sets of chemical changes. In addition to the energy providing mechanisms of the cell, there are the synthetic processes connected with growth. As far as the turnover of carbon is concerned, these two classes of reaction must be of the same order of magnitude during the active growth phase at least. So the individual reactions of the tricarboxylic acid cycle in yeast perhaps serve to provide intermediates for these synthetic processes.

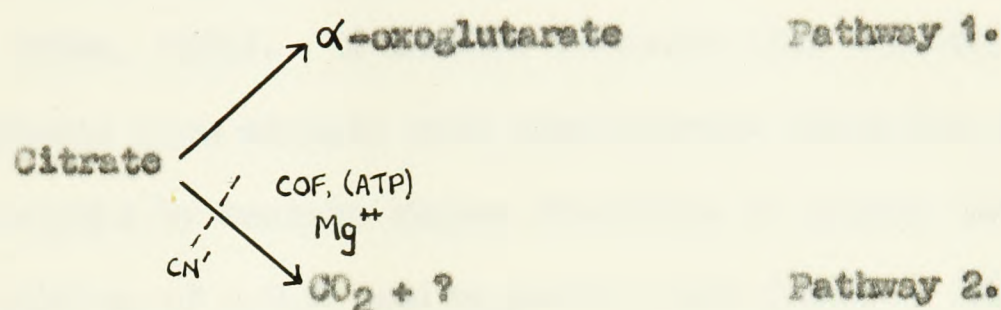
Whether or not the cycle functions to provide such intermediates or energy for the endogeneous processes of the cell, it is clear that the following individual stages constitute one pathway for the oxidation and overall metabolism of citrate in yeast.



Alternative pathways for citrate metabolism.

Foulkes (1951b, 1953b) has postulated the existence of a second pathway for the metabolism of citrate in cell-free extracts of yeast. This is chiefly distinguished from the tricarboxylic acid pathway, depicted above, by the non-accumulation of α -oxoglutarate in the presence of semicarbazide, and by the requirement of a cofactor present in boiled yeast extracts - termed the Citrate Oxidation Factor (COF). The second pathway was associated with the production of one mole of carbon dioxide for each mole of citrate metabolised and was believed to involve an oxidative degradation of the citrate, since oxygen was required and the system was cyanide-sensitive. In the presence of low concentrations of partially purified COF, addition of adenosine triphosphate (ATP) significantly increased the rate of citrate oxidation. Magnesium ions were also thought to be required for the function-

ing of this second pathway. These findings and the nature of the two pathways were summarised as follows:



(after Foulkes, 1952. p.127)

The products of the second pathway were not identified but it was shown that they were neither α or β ketoacids, lactic acid nor a steam-volatile acid. This second pathway could be observed in whole yeast cells rendered permeable to citrate, and Foulkes further reported that citrate is metabolised by the two pathways at roughly equivalent rates, their sum accounting for more than 60% of the pyruvate oxidised by the intact cells.

In certain microorganisms adapted to growth in a citrate medium, a further pathway of citrate metabolism has been detected which involves the anaerobic dissimilation of citrate to acetate and oxaloacetate. The adaptive enzyme involved, citridesmolase, is found in Aerobacter aerogenes (Dagley + Dawes, 1953. Dagley, 1954) and Streptococcus faecalis (Gillespie + Gunsalus, 1953. Dagley 1954) and has been extracted and purified. The dissimilation reaction requires the presence of either magnesium or manganese ions but is not dependent on the presence of Co-A, indicating that this cleavage of the citrate molecule is not a simple reversal of the condensing reaction whereby citrate is synthesised from oxaloacetate and acetyl-CoA. The fact that citridesmolase is only detected in organisms grown in the presence of citrate suggests that in the normal strains of these microorganisms

the tricarboxylic acid cycle does not represent a significant pathway for terminal respiration. The simple reversal of the condensing reaction, leading to the synthesis of acetyl-CoA from citrate has been demonstrated with crystalline preparations of the condensing enzyme from pig heart (Stern et al., 1951. Ochoa, 1954). A similar reaction also leading to the synthesis of acetyl-CoA from citrate with simultaneous formation of oxaloacetate, is catalysed by certain enzyme fractions of pigeon liver but also requires the addition of A.T.P. and magnesium ions (Srere + Lipmann, 1953). Thus in the requirement of magnesium ions, these modes of dissimilation of citrate to yield oxaloacetate and either acetate or acetyl-CoA appear to resemble Foulkes' pathway 2.

At least one other pathway for the oxidation of citrate in microorganisms has been reported. Campbell, Smith and Eagles (1953) observed that 'sonic' extracts of Pseudomonas aeruginosa failed to convert citrate to α -oxoglutarate under aerobic conditions. These extracts metabolised citrate and cis-aconitate (but not isocitrate) to yield succinic and glyoxalic acids.

This would appear to represent a major pathway for citrate metabolism in this organism, under anaerobic conditions at least, since one mole of succinate was formed for each mole of citrate oxidised. It is of potential interest as indicating possible routes for the biosynthesis of glycine and/or oxalic acid.

Where two or more alternative pathways of metabolism are found amongst the chemical activities of a microorganism, one at least may represent an adaptive response to nutritional control, i.e., a nutrient is supplied in far greater excess than is normally metabolised by the energy-yielding processes of the cell. The dissimilation of citrate under the influence of citridismolase appears to be of this type. The two other modes of citrate

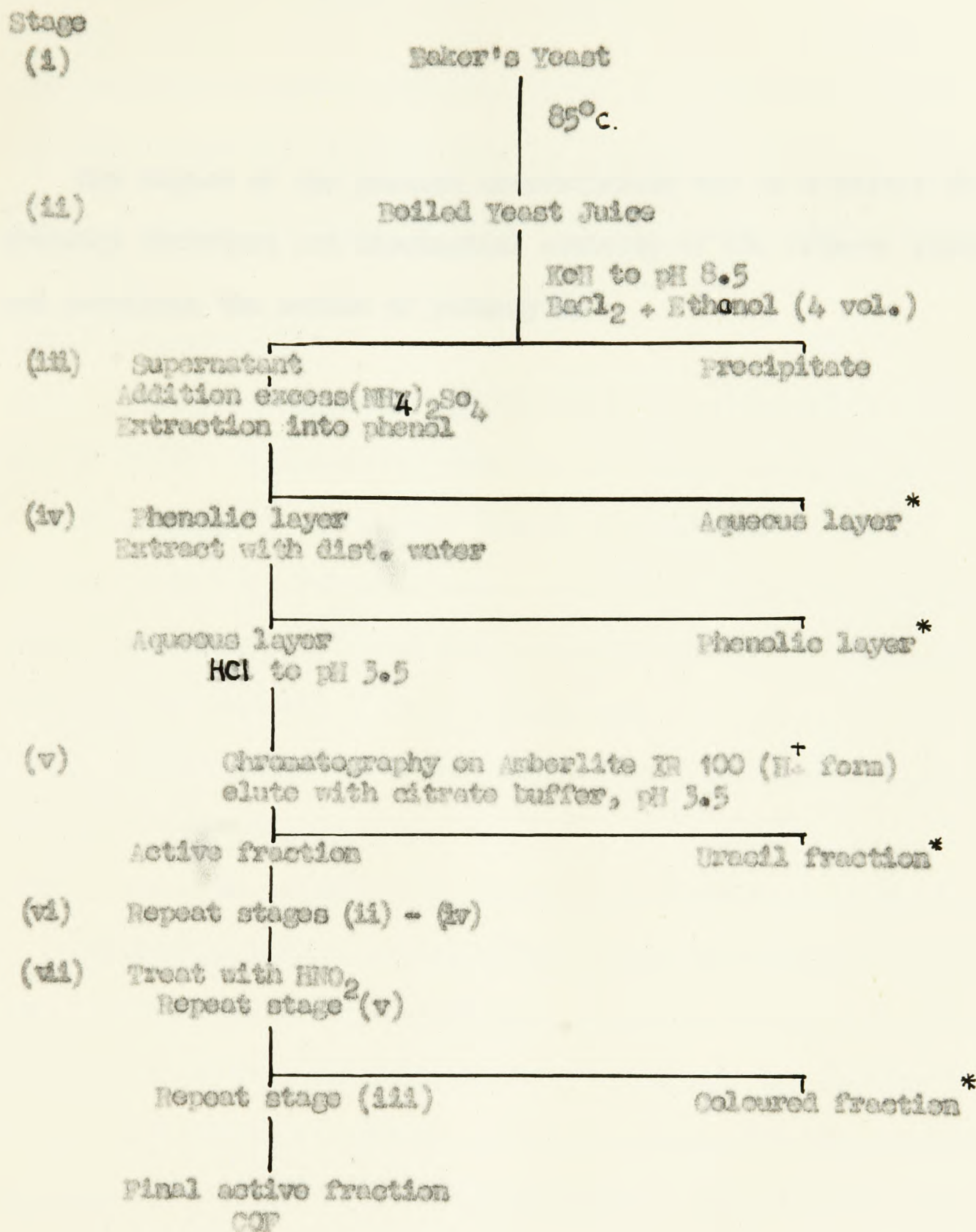
oxidation which have been described i.e., that in Pseudomonas and Foulkes' 'pathway 2' in yeast, are non-adaptive and are therefore presumably determined by the hereditary properties or the genetic apparatus of the cell.

The Citrate Oxidation Factor (COF)

Foulkes (1952. 1953a) devoted much attention to fractionating the factor (COF) present in boiled yeast juice which stimulates the metabolism of citrate by pathway 2. This factor is detected and assayed by the increased citrate oxidation when solutions of the factor are added to dialysed cell-free extracts of yeast. As the assay system is neither reproducible nor stable, no absolute qualitative assay is possible but comparative qualitative information can be obtained when graded dilutions of various active fractions are added to the same enzyme preparation.

Sufficient purification of the factor was obtained by a seven stage fractionation of boiled yeast juice (fig. 3) to allow certain deductions to be made concerning its chemical nature. The factor was reported to be unstable to freeze-drying (lyophilising) and heating with acid or alkali. Since it gave a positive ninhydrin reaction and was retained on passage through acidic ion exchange resins, COF was inferred to be an amino compound. Deamination with nitrous acid followed by removal of excess nitrite with ammonium sulphamate was without effect on COF activity. The COF activity did not appear to be associated with significant amounts of phosphate or pentose sugars.

The factor was also found to be present in the boiled extracts of a number of vertebrate tissues (liver, heart, kidney). COF was shown to be non-identical with many known cofactors - pyridine nucleotides, lipoic acid, thiamine, biotin, coenzyme A, etc.



* Signifies this fraction inactive

Fig. 3. Fractionation of the citrate oxidation factor from whole yeast.

The object of the present investigation was to continue the study of the chemical structure and biochemical activity of the citrate oxidation factor and elucidate the nature of pathway 2.

METHODS AND MATERIALS

A.R. Reagents and glass distilled water were used throughout.

All yeast used was purchased locally (Distillers Co. Ltd) and stored at 20°C. No yeast was used which had aged more than 3 days from the date of purchase.

Preparations of COF:

(i) A large scale preparation of the factor from 21 cwt. baker's yeast was undertaken by Mr. R.C. Codnor at the M.R.C. Antibiotics Research Station, Clevedon. Only the first four stages of the fractionating given in fig. 3. (p.12) were performed. The final volume of concentrate was 4.5 litres, pH7.6. COF activity could be detected using 0.1ml. of a hundred-fold dilution of this preparation.

(ii) A considerably purified preparation, V280, prepared by Foulkes, was left in our keeping, pH 5.4.

(iii) Ammonium sulphate-free preparations were obtained as follows:

Weighted quantities of fresh whole yeast (200-300 gm.) were washed twice with isotonic saline and centrifuged, suspended in water (1:1) and heated to boiling for 5 min. with stirring and the mixture then rapidly cooled and centrifuged at 200g. The yellow turbid extract was separated and the debris extracted with water and recentrifuged. The combined extracts were concentrated, after measurement of the pH (ca 7.0), by 'freeze drying'. Concentration to bulk below 5 ml. was allowed only in the presence of phosphate buffers (0.1M, pH = 5.5 or 7.0). The material obtained by exhaustive drying was very deliquescent but retained COF activity. The use of 'anti-foams' was rigorously avoided.

The active concentrate was a yellow gum containing nucleotides, citric acid, aminoacids and ammonium salts. For the purposes of assay, it was dissolved in 20 ml. water.

Preparation of Enzyme for assay system.

Freshly bought yeast was washed twice with 0.85% NaCl and the washings separated by centrifugation at 200 g. The discoloured top layer of the yeast after centrifuging was removed mechanically and discarded.

5-6 gms. of washed yeast were transferred to a Mickle cup and suspended in 4 ml. 0.9% KCl. 8-10 gms. of Ballotini 14 beads were added and the cup closed with a boiled rubber bung. Two loaded cups were shaken for 20 min. at 0° on the Mickle (1948) apparatus (an electrically-maintained tuning fork carrying two metal cups), operating at optimum amplitude. The suspension of disintegrated yeast cells was then centrifuged at 1200 g. for 20 min.

The efficiency of these conditions for cell rupture was determined by microscopic examination for whole yeast cells, using the Gram stain with crystal violet and counterstaining with iodine in KI. The efficiency of disintegration could also be roughly gauged by the stratified appearance of the sedimented cell debris after centrifugation.

The supernatant extracts were combined and dialysed in cellophane to remove COF present. Only those extracts were taken which were pale yellow and turbid, clearer solutions being without activity. Dialysis in cellophane was maintained for 4½ hr ± 10 min. against 0.1M phosphate buffer pH 7.1, the buffer being mechanically stirred. This period of dialysis was rather critical (see p. 51). All these operations were conducted in the cold room. The dialysed enzyme preparations had a negligible content of citrate.

Assay of the COF Activity.

The following solutions (in order of mixing) were used:

1 ml. phosphate buffer (0.1M, pH 7.0) containing either 2.94 mg. (10 μ moles) or 5.88 mg. (20 μ moles) of tri-sodium citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$)

0.1ml., 0.5% (w/v) Mg SO_4

0.1 or 0.2 ml. other reagents (CN^- , semicarbazide, etc.), neutralised to pH 7

0.1 or 0.2 ml. COF-containing extracts

1 ml. freshly prepared enzyme.

The mixture was incubated at 37° for 40 min. - without shaking in the earlier experiments and with shaking on an oscillating frame in the later work. The incubation was terminated by addition of either 1 ml. 25% (w/v) trichloroacetic acid or 0.75 ml. 97% ethanol. Large-scale incubations were conducted using either the same volume ratios of the reagent or in appropriate cases by increasing the citrate in buffer: enzyme ratio to 3:2.

0.2 or 0.4 ml. of the centrifuged denatured incubation mixtures was withdrawn for the citrate analyses (performed in duplicate) and 1.0 ml aliquots for determination of ammonium ion present.

Analytical methods.

a) The citrate analyses were performed by the colorimetric method of Pucher, Sherman and Vickery (1936) as modified by Buffon and Peters (1950). In principle, the citric acid is oxidatively brominated to yield penta-bromacetone (Stahre reaction) which is extracted into petroleum ether and then estimated by the yellow coloration produced on shaking with solutions of sodium sulphide. The volumes taken for analysis (0.2 ml. or 0.4 ml.

accordingly as 20 or 10 μ moles citrate were originally present) were heated with 1 ml. 15% (v/v) sulphuric acid on a boiling water bath for 5 min. to destroy any 'pseudo citrates' present.

b) Ammonium ion concentration was determined on steam distillation of liberated ammonia in a Markham still into 5 ml. boric acid mixture, (0.5% boric acid in 20% ethanol and containing the indicators, phenol red, bromocresol green and methyl red), as is used in the latter stage of micro-Kjeldahl nitrogen determinations. The distilled ammonia has then titrated directly against 0.0108 N. sulphuric acid. A borate buffer (0.2 M., pH 8.55) was used in place of caustic alkali to liberate the ammonia. Under these conditions no detectable ammonia was liberated from urea and other amides (asparagine, glutamine), amines (e.g., glucosamine) or imino compounds (arginine, creatine). 1 ml. of the solution under test was placed in the still together with 10 ml. of the borate buffer and 3 drops of tributyl citrate added to suppress foaming. A small blank titre (= 0.03 ml.) was regularly obtained for a distilled water blank and all titre values were subsequently corrected by this. The period of distillation was 5 min.

Titre of 1 ml. was equivalent to 10.8 μ moles ammonia in the analytical sample.

c) Aminoacids were detected and estimated following their separation by descending paper chromatography, using the original method and apparatus of Consden, Gordon and Martin (1944). Freshly prepared butanol: acetic acid: water mixtures (4:1:5) or phenol, saturated with water and made alkaline with ammonia, were employed as the solvent systems. Chromatograms developed in phenol were washed in ether before drying and spraying with ninhydrin.

The aminoacids were detected by spraying with ninhydrin (0.3% solution in ethanol) and heating for 5 mins. at 105° or 10 min. at 80°. For estimation of approximate amounts of the individual acids, the relevant developed zones were eluted with 1 ml. water for 2 hrs., ^{the eluate} centrifuged, and the optical densities of the ninhydrin colorations measured with a Spekker photoelectric-absorptiometer using Ilford 606 filters (maxm. transmission at 570 mμ). These were compared with the colorations developed from several known standards of the given aminoacid run simultaneously on the same chromatogram.

pH measurements were made with a Cambridge pH-meter at room temperature.

The pH values of incubating mixtures were determined at the conclusion of the 40 min. incubation and prior to the addition of trichloroacetic acid or ethanol.

RESULTS

Nomenclature used (after Foulkes):

Pathway 1 refers to the enzymic oxidation of citrate in the absence of added cofactor(s) to yield α -oxoglutarate

Pathway 2 refers to the enzymic removal of citrate, specially activated by boiled yeast juice. α -Oxoglutarate is not an end product.

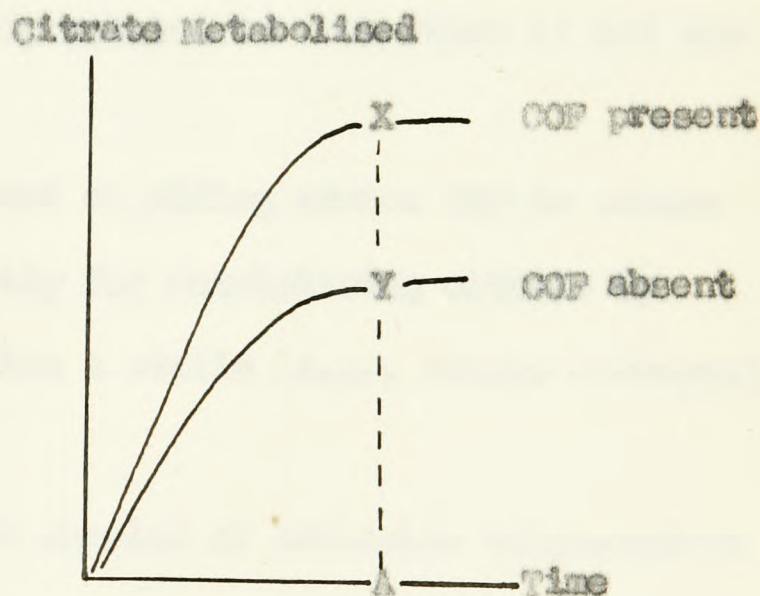
COF refers to a substance (or substances) present in boiled yeast extracts which exhibits the property of stimulating citrate disappearance in the assay system.

DPN, TPN refers to the di- and triphosphopyridine nucleotides respectively.

Activity of the Assay System.

The COF activity of samples added to the assay system is essentially measured by the difference in the amounts of citrate oxidised both in the presence and the absence of the factor (COF).

i.e. AY is a measure of citrate metabolism via pathway 1.



AX is a measure of citrate metabolism by both pathways 1 and 2, the difference, XY representing a measure of the COF activity.

When AX is greater than or equal to the total citrate available, then no quantitative determination of COF can be made. Ideally AX should be less than the citrate added (10 to 20 μ moles) and AY less than half this

amount of citrate. It was rarely possible to achieve this ideal state, the enzyme preparation possessing very variable activity from one preparation to another. Thus the citrate metabolised in the absence of CDP (AY) was found to vary over the range 3-15 μ moles/40 min. incubation period.

One observation of significance was that the pathway 2-metabolising activity of any one enzyme preparation (determined when excess CDP was added) was never independent of the same preparation's activity for metabolising citrate in the absence of CDP i.e., by pathway 1. Thus when AX was high, AY was reasonably high too. XY was never appreciable when AY was small. This would suggest that either the enzymes required for the respective pathways are closely associated within the yeast cells (e.g., situated on same cell particles) and suffer damage or activation simultaneously, or that both pathways have some enzyme reactions closely related to each other if not actually in common.

No simple saturation effects were found on adding excess CDP to enzyme preparations exhibiting appreciable activity for metabolising citrate by pathway 2, pointing to a dynamic rather than a static (e.g., simple coenzyme) function for CDP.

The addition of each of 3 independent samples of adenosine triphosphate (ATP) increased only slightly the oxidation of citrate both in the absence and presence of low concentrations of CDP in contrast to earlier finding (see p.8). The possibility must be considered that in Foulkes' experiments, the A.T.P. added also contained CDP. After the exploratory experiments the A.T.P. was omitted from the assay system. Whether CDP was present or not, magnesium ions appeared to have little effect on the stimulation of citrate metabolism. The magnesium was retained for further assays since it was

desired to prove that ammonium ions present in Foulkes' original experiments were not precipitated by the magnesium and phosphate ions also present. Magnesium ammonium phosphate is only precipitated at alkaline pH's (8+) but not at pH 7, i.e., under the conditions of incubation and assay.

- Table 1. a) Effect of addition of COF on citrate oxidation.
 b) Effect of dialysis and agitation on the activity of yeast particular enzyme preparations.
 c) Effect of addition of sodium ions.

10 μ moles citrate initially. Final vol. = 2.3 ml.

Addition of COF and sodium salts. One half the enzyme preparation was dialysed, the other half aged at 0° for period of dialysis.

Expt.		Addition	Shaking	Citrate metabolised
A + B	0.9% KCl	—	Continuous	0.0 μ moles.
	Dialysed enzyme	—	None	5.8 "
	" "	—	Continuous	8.4 "
	" "	COF	"	10.0 "
	non dialysed enzyme	—	None	4.4 "
	" " "	—	Continuous	8.6 "
	" " "	COF	"	9.3 "
C	Dialysed enzyme	—	"	6.0 "
	" "	NaHCO ₃	"	3.4 "
	" "	NaCl	"	2.2 "

Dialysis for periods greater than 4½ hours considerably reduced the activity of the enzyme preparation as was found by Foulkes (1951a, 1952).

Periods less than this failed to remove all the COF and citrate present in the cell-free preparations, which serve to provide the enzyme system for COF assay.

Addition of sodium ions in any form and replacement of the isotonic KCl (used in the extraction of the enzyme system) by isotonic NaCl, resulted in a very significant reduction of the total amount of citrate metabolised, (Table 1c).

Inhibition of the assay system.

Urea and cysteine (2 mg.) each inhibited the metabolism of citrate regardless of whether COF was added or not. These inhibitors, like the addition of sodium salts mentioned previously, affect both pathways 1 and 2, lending weight to the hypothesis that the two pathways are not unrelated.

Cysteine inhibition did not appear to be due to interaction with catalytic amounts of transition metals. Mammalian aconitase has been shown to require traces of ferrous ion as coenzyme (Dickmann + Cloutier, 1951. Morrison, 1954). Addition of the metal-chelating agents, o-phenanthroline (0.35 mg.) and 8-hydroxyquinoline (0.3 mg.) failed to effect either pathways 1 or 2 (Table 2), indicating that yeast aconitase and the other enzymes required for the functioning of the two pathways, if they are activated at all by transition metal ions, must bind the ions in very tight combination. Urea inhibition was simulated by a number of strong electrolytes, (large quantities of $(\text{NH}_4)_2\text{SO}_4$, KCl etc.), probably due to derangement of the osmotic balance and similar relationships between the active cell particles and the incubation medium, or inactivation by denaturation of the relevant enzymes.

Table 2. Effect of metal-complexing agents on Pathways 1 and 2.

Initial citrate = 10 μ moles. No magnesium added.

Additions	COF	Citrate metabolised
-	-	6.0 μ moles
-	+	9.5 "
Versene	-	6.3 "
"	+	9.2 "
Oxine	-	6.3 "
"	+	10.0 "

Versene = .4 mg. ethylene diaminetetracetic acid in K_2CO_3

Oxine = .3 mg. 8-hydroxyquinoline in 0.1 ml 10% ethanol.

Addition of the complexone, ethylenediamine tetracetic acid (versene, sequestrene) as the sodium or potassium salts, was without effect on both pathways 1 and 2. (Table 2). This reagent powerfully complexes all metal ions other than those of the alkaline metals (Gp. IA). COF activity is not associated therefore with simple catalysis or coenzymic activity due to metal ions.

Pathway 2 could be specifically inhibited by the addition of cyanide ions or semicarbazine hydrochloride-reagents which specifically react with keto compounds.

Activity of dried COF preparations.

It was reported (Foulkes 1953a) that attempts to obtain dried preparations of COF either by evaporation or concentration on freeze drying

(lyophilisation) led to loss of activity and that no detectable COF activity was to be found in the distillate or liquid air-cooled trap.

In contrast, the present experiments showed that all extracts of yeast submitted to freeze drying retained their activating effect on citrate oxidation, even in the dried state provided the pH was kept constant during concentration. In the absence of added buffer, the pH of all extracts examined fell significantly with drying, whilst the contents of the liquid air-cooled trap (i.e., distillate) became pungent in smell and alkaline to litmus. Ammonia was found in this distillate (smell, Nessler's reaction).

Believing that Foulkes' inability to obtain dried products might be associated with liberation of sulphuric acid in the active concentrates, due to dissociation of the ammonium sulphate present in all active samples, some COF preparations were desulphated before submitting to drying. Barium chloride was added to precipitate sulphate ions and excess barium removed with the addition of ammonium carbonate until barium ions were no longer detectable with rhodizonic acid. Freeze drying the desulphated material in the presence of phosphate buffer (0.1M, pH. 7.1) yielded a COF-active product. Control assays revealed that the mixture of inorganic salts resulting from the application of the desulphation procedure to solutions of ammonium sulphate also exhibited marked COF activity. This was traced to the presence of ammonium ions. (Table 3).

Table 3. COP activity of (a) dried yeast extracts
(b) inorganic salts

10 μ moles citrate initially

Additions	citrate metabolised	
—	6.0	μ moles
Concentrated yeast extract *	8.9	"
Dried desulphated yeast extract	8.7	"
Inorganic salts (NH_4^+ , Cl^- , CO_3^{--})	9.9	"
Na HCO_3	4.4	"
$\text{K}_2 \text{CO}_3$	6.2	"

* containing 0.44 M(NH_4)₂ SO₄

These additions do not express equivalent quantities.

Reality of the Ammonium ion effect.

The partial inhibition of citrate oxidation by sodium bicarbonate indicated above is of interest, suggesting that one possible catalytic role of the ammonium ion is to antagonise the inhibition due to presence of sodium ions - in this respect, ammonium ion simulating the known property of potassium ions. Subsequent findings revealed however that ammonia is actively removed from the assay system (see p. 33) regardless of whether the source be ammonium sulphate-free boiled yeast extracts or inorganic ammonium salts. All ammonium salts tested (carbonate, chloride, phosphate, sulphate, sulphamate) and aqueous ammonia exhibited activity, providing the pH was not disturbed by the addition of these salts. Both the yeast juice prepara-

tions (Foulkes' V280 and the large scale concentrate) contained ammonium sulphate (0.060 N and 0.88 N ammonium) respectively. The activity of a freshly prepared boiled yeast extract which contained no added ammonium sulphate was tested, found to be active in the assay system and shown to contain ammonium ions.

Table 4. Effect of (a) added ammonium salts
(b) ammonium sulphate-free yeast extract
on citrate oxidation.

10 μ moles citrate taken.

Additions		final citrate	citrate metabolised
		(μ moles)	(μ moles)
0.9%KCl	-	10	-
"	(NH ₄) ₂ CO ₃	10.5	-
"	Yeast extract (0.1ml.)	12.2	-
Enzyme	-	5.9	4.1
"	(NH ₄)H ₂ PO ₄	1.4	8.6
"	(NH ₄) ₂ CO ₃	2.1	7.9
"	Yeast extract (0.1ml.)	2.2	10.0

This experiment indicates that the ammonium ion effect is not due to interference in the method of citrate analysis.

Steam distillation of active boiled yeast extracts with borate buffer (0.3M, pH = 8.55) and concentration of the distillate in the presence of hydrochloric acid, gave solutions which reacted with Nessler's reagent (potassium mercuri-iodide) to give the characteristic brown precipitate

indicative of ammonium ions. The presence of other volatile bases in yeast (methylamine, etc.) being responsible for the activity of the extract was obviated by their inability to exhibit the COF effect upon the assay system. (Table 5)

Table 5. Effect of amino compounds on citrate oxidation.

Additions	Citrate metabolised
-	13.2 μ moles
MeNH ₂ .HCl	13.5 "
NH ₂ OH.HCl	13.3 "
NH ₄ Cl	20.0 "

(the final concentration of all these salts was .015 M.)

A large number of other amino compounds detected in the yeast concentrate by paper chromatography were similarly tested. Glycine, L-tyrosine, DL-aspartic acid, D-glucosamine, guanine and thymine were entirely without activity, whilst adenine, L-glutamic acid, L-glutamine, L-asparagine, DL-alanine, DPN, TPN and nicotinamide all exhibited slight activity, (see also Tables 17 + 18, pp. 42+43). Hydrazine hydrochloride likewise exhibited feeble COF activity.

The Ammonium ion content of whole yeast.

(1) Baker's yeast (D.C.L.) contained ammonium ion 0.02% by weight calculated on a dry weight of yeast basis.

Whole yeast washed three times on the centrifuge, to remove ammonium ion remaining from the growth medium, was suspended in distilled water and known quantities transferred by pipette to the Markham still. The rupture

of the cells and the liberation of ammonia was achieved simultaneously on steam distilling in the presence of excess borate buffer. The yield of ammonia in 3 experiments on separate samples of yeast was of the order of 60-65 $\mu\text{g/g}$. whole moist yeast, calculated for weight of yeast before washing. A series of experiments on dehydrating whole yeast to constant weight by

(a) oven heating at 105°

(b) liquefaction on warming in an oven followed by exhaustive concentration in a vacuum,

(c) disintegration by immersion in a solid CO_2 - ethanol mixture succeeded by exhaustive drying on the vacuum line,

- all gave a dry wt:whole yeast ration between 28.5 and 29.5%

A typical batch of yeast had an ammonia content 63 μg . per g. whole yeast and a moisture content of 71% giving a concentration of ammonia in freshly bought yeast of 217 $\mu\text{g/g}$ (12.8 $\mu\text{moles/g}$) dry weight. This figure probably represents a minimal value.

(ii) The ammonium ion content of a typical boiled yeast extract obtained from 31.75g. of washed yeast (equivalent to 8.05g. of dehydrated yeast) was 95 μmoles , corresponding to 11.9 $\mu\text{moles/g}$. (dry weight) of yeast.

Relation of COF activity to ammonium ion concentration.

All solutions of partially fractionated yeast juice and of ammonium salts exhibited COF activity over a particular concentration range only. In all cases it was found that the amounts of ammonium ion (added as salts or COF preparations) exhibiting activity were at least equivalent to the amounts of ^{extra} ~~extra~~ citrate metabolised. (Tables 6,7,8)

Table 6. Addition of various dilutions of a large-scale CCF preparation

Initially, 20 μ moles citrate. Incubated with shaking.

Dilutions of CCF	Ammonia Content	Citrate Metabolised	Increase in Citrate metabolism
	μ moles	μ moles	μ moles
-	-	12.7	0
x1	88	19.7	7.0
x10	8.8	19.7	7.0
x100	0.88	13.8	0.9
x1,000	0.09	12.7	0.0
x10 deamin- ated	< 0.1	12.4	-
Deamination blank	-	12.6	-

(1ml, 0.1M)

Deamination was carried out on 1ml. diluted CCF in the presence of KH_2PO_4 (1ml, 0.1M) at pH 5.2, with 0.2 ml 5% NaNO_2 and standing for 3 hours. (The nitrite concentration represents a 20-fold excess). 0.4 ml. of a 1% urea solution was added to neutralise the excess nitrite and stood for further 2 hours. On addition of Na_2HPO_4 (1.6ml, 0.1M) and solid K_2CO_3 to neutralise H^+ , the pH was restored to 7.1. A deamination blank carried out on distilled water and tested in the CCF assay system did not affect the oxidation of citrate. (Table 6)

Table 7. Effect of added ammonium sulphate.

Initially 10 μ moles Citrate, pH 7.02. No shaking on incubation.

(NH ₄) ₂ SO ₄ added (Final Molarity)	Ammonia equivalent (μ moles)	Final pH after Incubation	Citrate metabolised (μ moles)	Increase in citrate metabolised (μ moles)
-	-	6.85	6.3	0
.15M	300	6.98	6.5	0.2
.03M	60	6.90	9.8	3.5
.0075M	15	7.02	9.9	3.6
.0015M	3	7.02	8.5	2.2

High concentrations of ammonium salts, 0.15M and greater, evidently fail to exhibit COF activity. This result is of some significance in explaining Foulkes' failure to detect the activity of ammonium ions on the assay system, during the course of fractionating boiled yeast extracts with solutions of saturated ammonium sulphate.

Table 8. Effect of ammonia on citrate oxidation.

Initially 10 μ moles citrate, pH 6.48. No shaking on incubation.

Additions	Ammonium added (μ moles)	Final pH after incubation	Citrate metabolised (μ moles)	Increase in citrate metabolised (μ moles)
-	-	6.42	7.3	0
0.05M NH ₄ OH	10.0	7.04		
0.02 "	4.0	6.88	9.8	2.5
0.01 "	2.0	6.48	9.3	2.0
0.005 "	1.0	6.42	8.4	1.1
0.002 "	0.4	6.58	7.8	0.5

Note the close correspondence between increase in citrate metabolised and the amount of ammonium ion in the system.

A re-examination of the two partially fractionated COF preparations from yeast indicated that they both contained appreciable amounts of ammonium and sulphate ions, evidently derived from the use of saturated ammonium sulphate for the fractionation of boiled yeast juice (see Introduction, p. 12)

(i) Large scale preparation contained 0.88 N NH_4^+ .

(ii) Foulkes' most active preparation, V.280 contained 0.060 N. NH_4^+ .

Assaying these for COF activity against equivalent solutions of ammonium chloride indicated that the activity of these preparations was entirely accountable in terms of their ammonium ion content. (Table 9).

Table 9. Comparison of the effect of partially fractionated extracts and equivalent amounts of ammonium salts.

30 μ moles citrate initially. Incubated with shaking.

Additions (0.2ml)	Amount of ammonia (μ moles)	Citrate metabolised (μ moles)	Increase in citrate metabolised (μ moles)
-	-	14.4	0
Yeast extract * (1) x 10	17.6	23.3	8.9
0.084N NH_4Cl	16.8	23.3	8.9
Yeast extract V.280	12	21.7	7.3
0.063N NH_4Cl .	12.6	22.0	7.6

* Large scale preparation diluted ten-fold.

A similar correspondence was found between other dilutions and quantities of the COF preparations and their equivalent solutions of ammonium sulphate and ammonium chloride (see Table 10).

Table 10: a) further comparison COP activity of fractionated preparations from yeast

and equivalent amounts of ammonium ion.

b) direct correlation of uptake of ammonium ion and increased citrate oxidation.

30 μ mole citrate initially. Incubate with shaking. Volume = 2.8 ml.

Additions (0.2 ml.)	Ammonia equivalent (μ moles)	Citrate metabolised (μ moles)	Increased citrate metabolism (μ moles)	Residual ammonium ion (μ moles)	Uptake of ammonium ion (μ moles)
-	0.1	21.2	-	-	-
COP/10 [*]	17.6	30.0	8.8	9.3	8.3
COP/25 [*]	7.0	28.1	6.9	0.0	7.0
.084N NH ₄ Cl	16.8	29.9	8.7	8.8	8.0
.034N NH ₄ Cl	6.8	28.0	6.8	0.0	6.8

* 10- and 25-fold dilutions of large scale concentrate from yeast, containing 0.88N NH₄⁺.

Correlation of the uptake of ammonium ion with increase in citrate Metabolism

Ammonium ion analyses were performed on samples of COF and equivalent solutions of ammonium salts, the enzyme system used for COF assay and the products of enzyme-citrate-COF incubation. It was confirmed that ammonium ion is actively removed during incubation and in equivalent quantities to the extra citrate metabolised (Tables 10 and 11).

Combining the results given in these two tables, we find for six independent incubations of citrate and ammonium salts (or COF preparation):

	Increased citrate metabolism	Uptake of ammonium ion
(i)	8.8 μ moles	8.3 μ moles
(ii)	6.9 "	7.0 "
(iii)	8.7 "	8.0 "
(iv)	6.8 "	6.8 "
(v)	30.2 "	32.6 "
(vi)	13.0 "	12.8 "

i.e., nearly 1:1 relationship between extra citrate oxidised and the amounts of ammonium required to stimulate this extra oxidation.

Products of Pathway 2.

Both cyanide ions, (added as neutralised potassium cyanide) and semicarbazide hydrochloride at final concentrations =.01M., inhibited the ammonium-stimulated metabolism of citrate, i.e., pathway 2. (Tables 11 and 12). These inhibitors are known to react with keto compounds and effectively inhibit their metabolism. Foulkes (1951a) demonstrated that the metabolism of citrate by the yeast enzyme system used for COF assay in the presence of these inhibitors, results in an accumulation of α -ketoglutarate (as the cyanhydrin and semicarbazone respectively):

Table 11. a) Correlation uptake of ammonium ion with increased citrate metabolism.

b) Effect of semicarbazide on COF activity and Pathway 2.

Additions	Final Glutamate Opt. Density at Rf.22 (see p.36)	Initial NH ₃ conc. (μmoles)	Final NH ₃ conc. (μmoles)	Amount ammonia utilised (μmoles)	Initial citrate (μmoles)	Final citrate (μmoles)	Total citrate oxidised (μmoles)	Increased (due to NH ₄ ⁺) citrate oxidation (μmoles)
-	0.39	0	0	0	60	30.5	29.5	0
-	0.88	93	60.4	32.6	60	0.3	59.7	30.2
S-Cbz.HCl	0.54	93	80.2	12.8	60	17.5	42.5	13.0
S-Cbz.HCl	0.38	0	0	0	60	28.6	31.4	-

S-Cbz.HCl = Semicarbazide hydrochloride added to give final concentration = 0.01M.

Table 12. Cyanide inhibition of Pathway 2.

10 μ moles citrate, initially. Final $[CN^-]$ = .01M. or .001M.

Additions	pH ¹⁵⁰	Citrate metabolised (μ moles)	Increased citrate metabolism (μ moles)
-	7.10	3.0	-
$(NH_4)_2SO_4$	6.95	6.2	3.2
.01M. CN^-	7.2	2.9	0.0
$(NH_4)_2SO_4$ + .01M. CN^-	7.25	3.0	0.0
" + .001M. CN^-	7.15	5.9	2.9

$(NH_4)_2SO_4$ added equivalent to 70 μ moles ammonium ion.

Examination of the products of incubating citrate with ammonium ions or COF preparations by paper chromatography, revealed that COF-stimulated citrate oxidation was accompanied by significant increases in the amounts of several aminoacids and particularly of glutamic acid.

Chromatography in Phenol-ammonia.

Substances chromatographed	Ninhydrin-positive zones at R_f *
Incubation of citrate alone	No definite zones - general streaking
" " " + NH_4^+	.25, .39, .47
DL-Aspartic Acid	.185
L-Glutamic Acid	.25
Glycine	.47
DL-Alanine	.61

* R_f = $\frac{\text{distance travelled by substance}}{\text{distance travelled by solvent front}}$

Chromatography in butanol-acetic acid-water (4:1:5)

Substance chromatographed	Ninhydrin-positive zones at R_f
Incubation of citrate alone	.09, .14, .22, .29, .50, .68
" " " + NH_4^+	as above. V. strong at .22. Extra zone at .18
Aspartic Acid	.14/.17
Glycine	.18
Glutamic Acid	.21/.22
Alanine	.27

Elution of the zone at R_f .20 /.24 following chromatography in the butanol-acetic acid solvent system and development with ninhydrin, indicated that there was a qualitative correspondence between the extra citrate metabolised, the uptake of ammonium ion and amount of glutamic acid formed. Addition of semicarbazide (.01M) which depresses the uptake of ammonium ion and extra citrate metabolism, likewise depressed glutamic acid formation (Table 11).

No direct quantitative relationship between increased citrate metabolism, ammonium uptake and glutamate production could be established as the enzyme system readily metabolised glutamate. This was observed by the reduction in the optical density of the ninhydrin coloration at R_f .20/.24 following chromatography in the butanol-acetic acid system, when glutamate was added to the active assay system. An increase in the amounts of several other aminoacids and of glycine and aspartic acid in particular accompanied the metabolism of both citrate and glutamate in the absence of added ammonium ions (Table 13). The COF assay system was also shown to be capable of reductively aminating oxoglutarate to yield glutamate in the presence of added DPNH.

Table 13. Activity yeast preparation for glutamate formation and transamination, etc.

Incubation for 35 mins. Additions do not represent equivalent quantities.

Additions to enzyme system	Optical densities of zones (BuOH.HOAc.H ₂ O)	
	R _F .22 i.e. glutamate	R _F .18 i.e. glycine, aspartate
-	.31	.17
oxoglutarate, NH ₄ ⁺ , DPNH	.60	.23
citrate and NH ₄ ⁺	.53	.29
glutamate (800 μg)	.78	.27
glutamate (800 μg) + denatured enzyme prep.	1.05	.225

oxoglutarate added as sodium salt (14.5 μmoles).

Furthermore this glutamate-metabolising activity of the yeast enzyme preparation was not reproducible from one experiment to another, rendering it difficult to draw up a balance sheet in terms of extra citrate metabolised and ammonia uptake on one hand and total glutamate formation on the other. Over a series of experiments it was qualitatively established that glutamic production and, to a lesser extent, glycine formation and aspartic acid formation consistently paralleled ammonia uptake.

COF activity of whole yeast extracts.

Hitherto, it has been established that the COF activity of fractionated extracts from yeast is entirely attributable to the content of free ammonium ion. Unfractionated samples of boiled yeast juice however exhibited greater COF activity than was accountable in terms of their content of ammonium salts.

A typical preparation of boiled juice from 300 g. fresh washed yeast and concentrated to 20ml, was found to contain ammonium ions equivalent to 0.0145N. An equivalent solution of ammonium chloride exhibited only 26% the OGP activity of the unfractionated yeast extract. (Table 14).

Table 14. Comparison of the activities of an unfractionated boiled yeast extract and an equivalent amount of ammonium ions.

20 μ moles citrate initially. $\text{NH}_4\text{Cl} = 0.0145\text{N}$

Yeast extract equivalent to .0145N ammonium ion.

Additions	Content of Ammonium Ions (μ moles)	Citrate metabolised (μ moles)	Increased metabolism of citrate (μ moles)
-	-	12.6	-
0.2ml. Yeast extract *	2.9	23.8	11.2
0.4ml. " "	5.8	30.2	17.6
0.2ml NH_4Cl soln.	2.9	15.4	2.9
0.4ml. " "	5.8	17.5	5.0

* Containing 32.5 moles citrate/ml., taken into account in computing the total amount of citrate metabolised.

Treatment of yeast extract with silver nitrate.

Addition of silver salts to aqueous yeast extracts precipitates Coenzyme I (DPN) and other nucleotides therefrom (Kornberg + Pricer, 1953). Accordingly 1ml. of the yeast extract described above was treated with 1ml. 25% (w/v) silver nitrate when a heavy gummy precipitate was formed. This was separated by centrifugation, washed with 1ml. water and discarded. To the combined supernatant + washings was added 1ml. of an equivalent solution of chloride

ions (11.4%KCl) to precipitate all excess silver ions. The silver precipitation was accompanied by 83% reduction of the U.V. absorption of the extract, over the range 220-230m μ . 1ml. of the resultant nucleotide-free preparation of yeast extract was tested for COF activity against 1ml. of the original extract diluted 4-fold and also a water blank which was subjected to the same operations (Table 15).

Table 15. Effect of precipitation with Ag⁺ on activity of boiled yeast extracts.

Both Ag⁺.pptd. and diluted untreated yeast extracts contained 4.0 μ moles citrate and 3.6 μ moles ammonium ion /ml.

Additions (1ml.)	Citrate metabolised (μ moles)	Increase in citrate metabolised (μ moles)	Final [Glutamate] Opt. Density at R _F .22
-	6.0	-	.325
Diluted untreated extract	21.2	15.2	1.40
Ag ⁺ pptd. extract	9.5	3.5	1.35
" water blank	6.7	0.7	.280

Removal of the bulk of the U.V. absorbing compounds (nucleotides, etc.) was accompanied by reduction of the COF activity to 23% that formerly. The formation of glutamate was hardly affected, whilst the residual COF activity equivalent to the metabolism of 3.5 μ moles of citrate corresponded closely with the ammonium ion content of the nucleotide-free extract (3.6 μ moles).
Deamination of unfractionated yeast extract.

1 ml. of whole yeast extract was submitted to the deamination procedure described on p. 29 . After standing with nitrous acid , one half the res-

ultant solution was treated with urea as described, to remove excess nitrous acid and the other half left untreated. As controls, both a water blank and the equivalent solution of ammonium ions ($.0146N.NH_4Cl$) were subjected to similar treatment. These deaminated yeast extracts gave no titrateable ammonia on distillation with borate buffer (pH.8.55).

All these deaminated solutions were tested for COF activity (Table 16).

Table 16. Effect of deamination with nitrous acid on activity of boiled yeast extracts.

Untreated yeast extract diluted 4-fold, contained $8.1\mu\text{moles}$ citrate + $3.6\mu\text{moles } NH_4^+ / \text{ml.}$

Additions (1ml.)	Ammonia present (μmoles)	Citrate metabolised (μmoles)	Final Glutamate Opt.Density at $R_p.22$
-	0.0	6.8	.145
Untreated extract	3.6	18.4	.790
Deaminated " (i)	0.0	12.5	.545
" " (ii) *	0.0	15.0	.558
Water blank *	-	6.7	.172
Deaminated NH_4Cl . *	< 0.1	7.1	.150

* Excess nitrite removed by treatment with urea.

Failure to remove the excess nitrite (by urea treatment) evidently partly inhibits the metabolism of citrate in the absence of added ammonium ions.

Deamination, leading to removal of $3.6\mu\text{moles}$ ammonium ion in the yeast extract also results in loss of COF activity equivalent to the decreased metabolism of citrate by $3.4\mu\text{moles}$. The residual activity not due to ammonium ions = 71% activity of whole yeast extract. This is to be compared with re-

duction of activity to 23% when all nucleotides but not ammonium ions were removed by silver precipitation, i.e., at least 94% COF activity of this whole yeast extracts is due to its content of (a) ammonium ions and (b) nucleotides (DPN, TPN, etc.)

A repetition of this analysis of COF activity due to ammonia and nucleotide fractions accounted for 91% of total activity of another whole yeast extract (from 170 gm. yeast.)

COF activity of nucleotides, aspartate and other amino compounds.

The COF activity of the nucleotides, DPN and TPN was confirmed on observing an increase in the total citrate oxidised when these were added to the enzyme system (Table 17). Nicotinamide added with the intention of depressing the nucleosidase activity of the enzyme system, caused some stimulation in citrate metabolism. Thus the addition of 5 μ moles of nicotinamide stimulated citrate oxidation by 3 μ moles with an accompanying increase in the amount of glutamate. Evidently the nicotinamide exhibited some COF activity. Chromatographic examination of the nicotinamide sample failed to reveal the presence of ammonium salts.

The DPN + TPN tested were only partly purified samples (kindly provided by Dr. L. A. Stocken) of 40% and 15% purity respectively. The possibility must be considered that these were a source of ammonium ions either by simple decomposition or enzymic deamination of the nicotinamide and adenine. Not enough material was available to test these samples for their content, if any, of free ammonium ions.

A new factor from liver which stimulates the respiration of brain mitochondria (cf. Christie, Judah + Rees, 1953) and which bears some superficial resemblances to a nucleotide (precipitated by barium salts, degraded by acids,

etc.) was tested for COF activity. A barium precipitated sample provided by Drs. Rees and Judah was dissolved in water and excess Ba^{++} removed by treatment with K_2SO_4 . It was found to be almost devoid of activity (Table 17).

Table 17. COF activity of nucleotides and nicotinamide

20 μ moles citrate initially

Additions (0.1ml.)	Citrate metabolised (μ moles)	Increased citrate metabolism (μ moles)
-	5.5	-
0.1M. NH_4Cl .	12.0	6.5
.05M Nicotinamide	8.7	3.2
.002M DPN	8.0	2.5
ca .002M TPN	7.7	2.2
DPN/Nicotinamide	9.7	4.2
TPN/Nicotinamide	6.9	1.4
0.5% RJF.	5.8	0.3

RJF. = Rees-Judah factor from rat liver.

Foulkes (1955) reported that L-Aspartate exhibited COF activity, especially when added together with DPN. This could not be confirmed using the present assay system. L-Aspartate alone exhibited extremely feeble activity which was not affected either by the addition of DPN alone or DPN and nicotinamide. (Table 18).

In addition to nicotinamide itself, other amino group donors were found to exhibit a feeble COF activity in the assay system. These compounds included DL-Alanine, L-Glutamic Acid, L-Glutamine and L-Asparagine.

Table 18. COF activity of certain aminoacids.

Amino acids and amides were added in 0.2ml. 0.1M phosphate buffer (pH 7.1)

Additions	Citrate metabolised (μ moles)	Increased citrate metabolism (μ moles)
-	5.2	-
NH_4^+ (10 μ moles)	9.9	4.7
DL Alanine (1mg.)	6.8	1.6
L-Glutamic Acid (2mg.)	8.9	2.7
L-Glutamine (1mg.)	7.1	1.9
L-Aspartate (10 μ moles)	6.2	1.0
Aspartate /DEN	6.7	1.5
Aspartate /DEN/N'amide	6.2	1.0
N'amide	6.9	1.7

N'amide = Nicotinamide (5 μ moles).

DISCUSSION

The nature of the citrate oxidation factor(s).

The foregoing body of evidence indicates that the COF activity of unfractionated boiled yeast preparations is due to their content of the cohydrogenases, DPN and TPN and of ammonium ions. The activity of the fractionated preparations previously described (Foulkes, 1952, 1953a) is attributable to their content of ammonium salts.

The finding that ammonium ions exhibit the property of stimulating citrate breakdown in yeast extracts, is entirely in accord with Foulkes' observations on the nature of the citrate oxidation factor found in boiled yeast juice after precipitation of the bulk of the nucleotides with barium chloride and ethanol. The chief properties of the fractionated factor as reported by Foulkes (1953), may be summarised as follows:-

1. Stability over pH range 2-11. Normal acid or alkali at higher temperatures cause rapid and complete inactivation.
2. Phosphotungstic acid at pH 1.5 - 2 completely precipitates the factor.
3. It may be eluted from adsorbents (Fuller's earth or acid-washed Norite) with 0.1% ammonia.
4. Insoluble in ether and more soluble in propanol and butanol at an alkaline pH than under neutral or acid conditions.
5. It is taken up by the acidic resin Amberlite IR 100, from which it may be eluted at pH 3.5 with citrate buffers. Prior to elution under these conditions, the U.V. absorbing bases are first removed, indicating that COF is a stronger base than those of the latter type.
6. COF preparations retain their activity after deamination with nitrous acid

when the excess nitrous acid is destroyed by the addition of a 5-fold equivalent of ammonium sulphamate.

7. Ignition of the extract of yeast results in loss of activity.

These findings, indicative of the weakly basic nature of COF, do not preclude ammonia as the agent responsible for the COF activity of these nucleotide-free extracts of yeast.

Some further observations (Foulkes, 1952) are of interest:

a) Electrolytic desalting of COF preparations led to complete loss of activity under conditions which achieved a rapid desalting of dilute solutions of ammonium sulphate.

b) Active fractions separated from these preparations by chromatography on cellulose with phenol-water, still contained sulphate ions.

c) Boiled extracts of a number of vertebrate tissues (brain, liver, etc.) possessed COF activity.

There are however, some differences between the findings given by Foulkes and the results of the present investigation which deserve some comment.

These may be listed as:-

a) requirement of magnesium ions for activity of pathway 2 in the presence of COF (ammonium ions) and at low COF concentrations, of ATP.

b) reported lability of COF on heating with acid.

It has been found that the addition of ATP and magnesium ions often stabilises aged or damaged mitochondria and similar cell particles. The enzyme sequences required for the functioning of the tricarboxylic acid cycle (cyclophorase complex) and for the synthesis of glutamic acid have been shown to be associated with mitochondria in mammalian tissues (Still, Buell + Green, 1950. Green 1951,) and with cell granules in yeast, similar to mitochondria in character (Nossal, 1954c). The possibility that the added

ATP was a source of ammonium ions either by contamination or deamination to the inosine analogue (Dickens, 1951) cannot be discounted. The ATP preparation could also have served as a source of adenosine-5-phosphate, required as a coenzyme for the TPN coupled isocitric dehydrogenase of yeast (see p. 4.)

In a repetition of Foulkes' experiments (1952) on the loss of COF activity on heating with normal acid for 10 mins., it was found that whole yeast extracts suffered considerable reduction of activity presumably due to destruction of the nucleotides DPN and TPN, and that nucleotide-free preparations and solutions of ammonium salts appeared to lose activity only if the acid was neutralised with sodium hydroxide or sodium carbonate before addition to the assay system. The partial inhibition of citrate oxidation on addition of sodium ions has been repeatedly observed in the assay system (see p. 22).

Foulkes (1955) has now confirmed the COF activity of ammonium ions in Anheuser-Busch commercial baker's yeast. He also reports a residual COF activity in ammonium-free extracts of this yeast manifested in the presence of DPN, associated with L-aspartate which is present in quantities = 4.6 μ moles/gm. in this yeast. No such activity due to aspartate could be found using the assay system prepared from D.C.L. yeast, whether or not DPN and nicotinamide were present - the nicotinamide being added to depress DPN, TPN-ase activity. However, other amino donors were found to be feebly active in the assay system and include L-glutamic acid (sic), L-glutamine, L-Asparagine, D-alanine and nicotinamide.

The catalytic properties of both DPN and TPN in promoting citrate oxidation (in the absence of ammonium ions) are well established (Kornberg + Priar, 1951. Nossal, 1954a). Their combined activities account for at least 70% of the COF activity of whole boiled yeast extracts, as measured by the reduction

in activity following their removal by precipitation with silver ions and also on removal of ammonium ions with nitrous acid under conditions which fail to deaminate nicotinamide and its derivatives (Schlenk, Hellström + v. Euler, 1938).

The mode of action of COF.

Ammonium ions are known to be capable of activating enzyme systems, particularly those involving the transfer of phosphate groups (Muntz + Hurwitz, 1951. Seits, 1949). In this respect they often simulate potassium ions. It might be thought that ammonium ions owe this property to an ability (cf. potassium) to overcome the inactivating effect of sodium ions, which is manifest in many enzyme systems (see for e.g. Hartman + Kalnitsky, 1952). This is somewhat borne out by a comparison of the rates of fermentation, and overall respiration of a 'sodium yeast' (Conway + Moore, 1954) and an 'ammonium yeast' (Conway + Breen, 1945) - yeast cells in which there has been enrichment of sodium and ammonium ions respectively at the expense of their potassium content. The ammonium yeast respire more actively than either the normal or sodium-enriched yeasts. The inhibitory effect of sodium ions on the oxidation of citrate in the assay system has already been noted but the COF activity was clearly not due to a simple reversal of the sodium effect, as shown by the active uptake of ammonium ions accompanying the increased metabolism of citrate, i.e., ammonium ions are dynamically involved in the stimulation of citrate oxidation.

A similar dynamic effect of ammonium ions on yeast respiration has been observed by a number of workers. Of particular interest, is the finding of Smythe (1939) that the activity of extracts of yeast or animal tissues which stimulates yeast fermentation and attributed to a novel coenzyme, "activator Z" is due to the content of ammonium salts in these tissues. Massart and Heymans

(1951) found that this effect was associated with the synthesis de novo of nitrogenous compounds within the cells and that this ammonium-stimulated respiration was reduced by inhibitors which effectively prevent ammonia assimilation. Similarly, azide poisoning of the assimilation of ammonia, inhibited the response of a biotin-deficient strain of yeast to the addition of biotin and ammonium ions, the addition of biotin alone failing to restore fermentation, respiration or growth (Winzler et al. 1944).

The direct relationship between the assimilation of ammonia and increased rates of cellular respiration has been observed for both baker's yeast (Runnström et al. 1940) and the edible yeast, Torulopsis utilis (Yemm + Folkes, 1954). The present work serves to define one locus at least, for the coupling of the uptake of ammonium ions with increased respiration, i.e., increased breakdown of citrate. This coupling can be envisaged at three levels, which are not mutually exclusive but rather, three descriptions of the one relationship.

(i) Coupling by phosphorylation or other energy transfer processes, whereby energy derived from the oxidative catabolic processes of the cell is made available for synthetic reactions, particularly amino acid synthesis.

(ii) Coupling by oxido-reduction mechanisms. The reductive amination of ketoacids to yield aminoacids oxidises the reduced phosphopyridine nucleotide coenzymes, which are reduced again by the simultaneous oxidation of other metabolites, such as triosephosphate and isocitric acid.

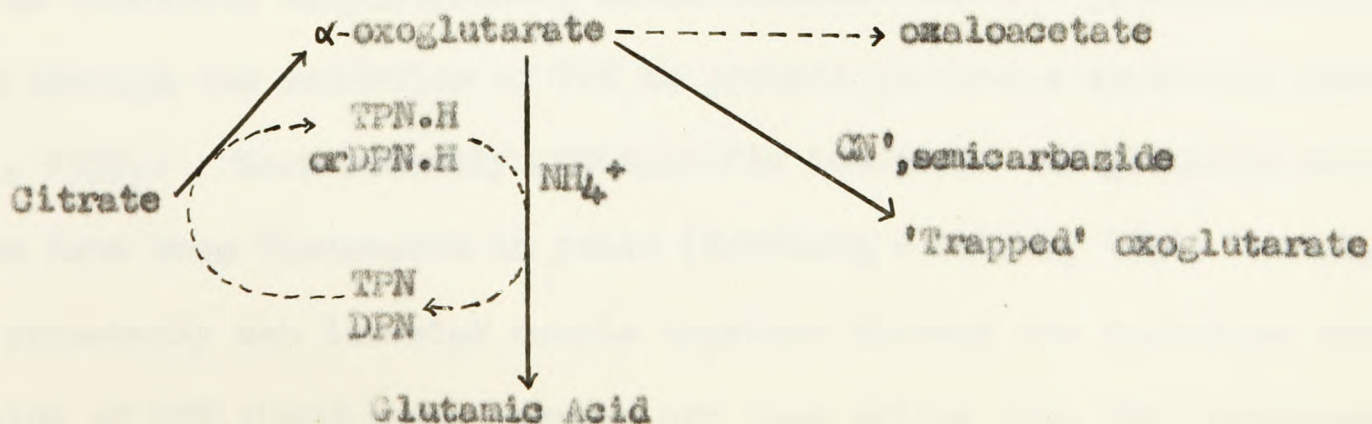
(iii) Coupling whereby the catabolic processes of the cell and of the individual stages of the tricarboxylic acid cycle in particular, furnish the carbon skeletons in the form of intermediates (α -oxoglutarate, oxaloacetate, pyruvate) for the synthesis of nitrogenous compounds.

Since it could be demonstrated that the supply of ammonium ions in nucleotide-free preparations of COF leads to the increased production of glutamate, and that the assay system was able to promote the formation of glutamate from oxoglutarate in the presence of ammonium ions, the COF activity of ammonium ions is evidently related to their effective removal of α -oxoglutarate from those sites in the yeast granule (pseudo-mitochondrion) where it is formed from citrate, together with the consequent oxidation of the reduced pyridine nucleotides. Both these effects, i.e., removal of the end product of citrate breakdown and the reactivation of the coenzymes required for the oxidative transformation of citrate to oxoglutarate, combine to promote the further oxidation of citrate.

The COF activity of L-aspartate on citrate oxidation by Anheuser-Busch yeast described by Foulkes (1955) and of other amino donors (alanine, glutamine, etc.), found in the present studies, may likewise be interpreted in terms of a similar removal of oxoglutarate from the sites of formation in the mitochondrion by furnishing free ammonia or by direct transamination reactions.

The nature of 'pathway 2'.

The second pathway for the oxidation of citrate in yeast proposed by Foulkes must be considered to be rather, a second pathway for the removal of α -oxoglutarate :-

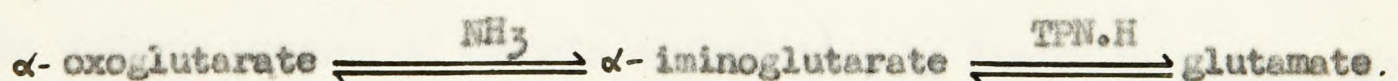


The properties of pathway 2 described by Foulkes (1953b) viz:-

- requires oxygen,
- one mole carbon dioxide liberated per mole citrate oxidised,
- end product is neither an α nor β keto acid,
- inhibited by cyanide,

are entirely explicable on the above scheme. An actual alternative route for citrate oxidation does not appear to be necessary.

The reductive amination of α -oxoglutarate is known to be a process of major importance in the assimilation of nitrogen by plants (Virtanen + Rautanen, 1952) and microorganisms (Wilson + Burris, 1953). The enzyme responsible for this amination, glutamic acid dehydrogenase is unique amongst the aminoacid oxidases on account of its widespread occurrence, high activity and specificity (Krebs, 1951. Cohen, 1954). It was first reported in yeast by Euler, Adler and Eriksen (1937) and shown to specifically require TPN (Co II) as the coenzyme. The reversible oxidation and synthesis of glutamic acid by this enzyme was found to be entirely analogous to the activity of glutamic acid dehydrogenases in a number of animal, plant and bacterial tissues (Adler et al 1938).



It was shown later that yeast, like plant and animal tissues, contains, a TPN-specific isocitric dehydrogenase, which couples with the glutamic dehydrogenase system through the mediation of TPN, to promote glutamate synthesis (von Euler et al., 1939). More recently DPN-specific isocitric and glutamic dehydrogenases have been discovered in yeast (Kornberg + Pricer, 1951. Nossal, 1954c), which presumably can likewise couple together through the reduction and re-oxidation of DPN, though these enzymes are less active than the corresponding TPN-activated systems.

In the assay system used in this investigation, the required nucleotide coenzymes were retained upon controlled dialysis. Exhaustive dialysis (6 hrs.) led to complete loss of activity, presumably due to loss of these coenzymes.

Nitrogen assimilation by yeast is chiefly restricted to ammonium salts and aminoacids. Commercial brewer's yeast and baker's yeast are grown in a molasses medium containing added ammonium salts as sole nitrogen source (White, 1954). Haine (1947) has demonstrated that the increase in the soluble nitrogen in Torulopsis utilis during the first half-hour of growth in the presence of ammonium salts, is mainly due to the formation of glutamic acid and glutamine. Aspartic acid was produced to a much lesser extent, excluding this aminoacid as a primary product of nitrogen assimilation. More recently the direct pathway from acetate (or pyruvate) to oxoglutarate and hence to glutamate has been demonstrated with C^{14} -labelled acetate and N^{15} -ammonium ions. Measurement of the relative isotope content of various aminoacids isolated from baker's yeast (Labbe et al., 1952. Wong et al., 1953) as well as from other microorganisms (Goldechmidt + Koffler, 1953) and animal tissues (Aqvist, 1951, Högstöm, 1953) indicate that the highest initial concentration of N^{15} is found in the glutamic acid fractions, i.e., the reductive amination of the oxoglutarate is the primary process of ammonia assimilation by diverse tissues. These observations, together with the present work on the catalytic property of ammonium ions on citrate oxidation, emphasise that the tricarboxylic acid cycle functions in yeast as a fundamental pathway for the provisions of intermediates for amino acid synthesis, whatever significance is to be attached to its role as a pathway for the terminal respiration of acetate and pyruvate.

Some of these findings concerning the nature of CCF and of pathway 2 have been published in the Biochemical Journal (Whitehouse et al. 1954).

SUMMARY

The properties of factors present in boiled yeast juice which stimulate the oxidative breakdown of citrate in baker's yeast, have been investigated. The activity has been shown to reside predominantly in the nucleotide fractions (DPN, TPN, etc.), and to a lesser extent in the ammonium salts present in yeast cells. The activity of the Citrate Oxidation Factor described by Foulkes and concentrated from yeast extracts, lies wholly in their ammonium ion content.

The activating effect of ammonium ions is accompanied by their removal and the simultaneous production of glutamic acid. Correlation of the uptake of ammonium ion with the stimulated oxidation of citrate indicates that the activity of ammonium salts lies in their ability to promote the reductive amination of α -oxoglutarate.

These findings are discussed in relationship to the functioning of the tricarboxylic acid cycle and alternative pathways for the oxidation of citrate, in yeast.

PART II

BIOCHEMICAL STUDIES OF INTESTINAL MUCINS

INTRODUCTION

In mammals, the intestinal mucosa is one of the most metabolically active tissues. Considerable attention has been devoted to studies of its permeability and its role in the biochemical utilisation of metabolites derived from the gut. Comparatively little is known, however, of its biosynthetic functions, particularly with regard to the synthesis of the mucins by the various glands of the mucosa. There is increasing evidence that different regions of the gastrointestinal tract, even within one species, produce chemically and biologically different mucins. Though gastric mucins of various animals have been extensively investigated (cf. Babkin, 1950. Kent & Whitehouse, 1955), far less is known of the chemistry and function of the intestinal mucins.

Chemistry and Biosynthesis of intestinal mucins.

Duodenal mucin is secreted by Brunner's glands, in response to nervous and hormonal stimuli and contact of the mucous membrane with hydrochloric acid and other irritants. Elsewhere in the intestine, mucin is elaborated by the so-called 'goblet' cells, the number of which is rather greater in the colon and rectum than in the small intestine (Florey, 1954). In addition to the Viscosity-raising components, which are only present in amounts of 0.5% w/v and less, these mucins have an appreciable content of inorganic ions - chloride, bicarbonate, sodium, etc. The presence of bicarbonate ions renders duodenal juice alkaline, though the actual alkalinity and concentration of bicarbonate ion seems to vary from one animal species to another. Undoubtedly one function of the mucous secretion is to partially neutralise the strongly acid stomach contents passed into the duodenum, and simultaneously afford a mechanical protection to the mucosa. In the duodenum, the

digestive action of pepsin is considerably diminished due to the increased pH but it is doubtful whether other components of the mucin directly protect the mucosa from peptic digestion. To ensure neutralisation and mechanical protection, there is a constant replacement from the Brunner's glands of the alkaline film of mucous. Little is known concerning the fate of mucous secreted into the alimentary canal but since only small amounts are excreted in the faeces, with the exception of such pathological conditions as mucous colitis, enteric inflammation, etc., the bulk of the mucin must be either digested or absorbed in the lower regions of the gut.

The mechanical properties of mucous secretions suggest that they have a protective and lubricant function. Thus pig pyloric and duodenal juices above pH 4.7 behave as viscous but true fluids, but in more acidic solutions they become gelatinous - a state which may increase their mechanical efficiency (Florey, 1955). The duodenal mucins tend to spontaneously lose their viscosity on standing at room or body temperatures (cf. Hartiala + Grossman, 1952) even when precautions are taken to preclude bacterial contamination and multiplication. This spontaneous decline in viscosity is not influenced by pH over the range 2.4 - 10.0 and so does not appear to result from enzymic action. However, the addition of proteolytic enzymes or the enzyme activators, cysteine and glutathione, is reported to accelerate the spontaneous depolymerisation of dog duodenal mucin (Hartiala + Grossman, 1952).

There is at present little accurate data concerning the structure of the organic components of mucins. One reason for our ignorance lies in the difficulty of preparing in any reasonable state of purity, the individual substances present in mucous secretions since they readily suffer chemical degradation (e.g., with alkali) and spontaneous depolymerisation. Much information has

been accumulated about some of the polysaccharide fractions of extracts of whole mucosae, but it is very doubtful whether these studies are relevant to a discussion of the chemistry of mucous secretions. Thus several monopolysaccharides have been identified in gastric mucosal extracts, including a carbohydrate with blood group A activity, two forms of chondroitin sulphate and heparin (Smith et al., 1952) - but the two latter polysaccharides are almost universally associated with structural components of tissues (see Kent + Whitehouse, 1955) and it is not unlikely that in the gastric mucosa, they are also associated with the structural elements and not with the secreted or true epithelial mucin.

A search of the literature reveals that the very few chemical studies made on the intestinal mucins are actually based on an examination of the material isolated from whole mucosae. Tryptic digestion of calf intestine has been found (Zittle, 1948) to liberate a polysaccharide with blood group A activity capable of cross reacting with type XIV pneumococcal antiserum. Similarly, Satoh (1949) reports the presence of A, B and O blood group substances in human intestines, again following drastic chemical treatment of the whole thoroughly washed intestines. It is probable that the very act of cleaning removes the bulk of the true epithelial secretion and possibly the mucosal cells as well. Werner (1953) in an extensive survey of epithelial and associated mucosubstances, described the isolation and composition of carbohydrate fractions from scrapings of pig duodenum, jejunum and ileum and from human ileal mucin obtained by means of a fistula. Extraneous protein was removed by successive digestion with trypsin, papain and pancreatic extracts and the residual polysaccharides fractionably precipitated with ethanol. The principal carbohydrate fraction so obtained contained gluco-

samine, galactosamine, galactose and fucose and was firmly bound to a polypeptide unit. In overall composition, this fraction closely resembles a blood group carbohydrate but no serological properties were described. The enzyme-treated mucosal preparations also contained a strongly acidic component with a composition akin to the chondroitin sulphates, i.e., aminosugar, hexuronic acid and acid-hydrolysable sulphate groups, in an approximately equimolecular ratio. This finding is of interest in connection with some autoradiographic studies of the intestinal mucosa made by Boström and other workers, following the uptake by the intestinal epithelium of parenterally administered inorganic sulphate labelled with ^{35}S . This work is discussed more fully later (p. 59). Therefore it appears that in material obtained by scraping the intestinal mucosa, there are at least two mucopolysaccharide components - one neutral and resembling the blood group carbohydrates, the other a sulphated polysaccharide. Werner found some slight variation in the composition of these mucopolysaccharides obtained from the different regions of the intestine, but it is difficult to know how much significance is to be attached to such a finding.

Our knowledge of the biosynthesis of the intestinal mucins is chiefly restricted to histological work on the goblet cells. Within these cells, there is considerable quantity of material which stains with mucicarmine and gives the periodate-Schiff reaction for polysaccharides. This material is considered to be the immediate precursor of extruded mucin and is often termed 'mucinogen'. Droplets stainable with neutral red can be observed in the region of the mitochondria in living goblet cells of rats and mice (Florey, 1955). These are considered to be in turn precursors of mucinogen, being transformed into the latter (i.e., intracellular mucin-

staining material) in the area of the Golgi body. Nothing is known of the chemical changes, if any, which mucinogen suffers on extrusion.

Metabolism of sulphated aminopolysaccharides. Studies with ³⁵SO₄

Among the animal mucopolysaccharides, those esterified with sulphuric acid and containing aminosugar constituents, (designated ^{Sulphated} aminopolysaccharides), constitute an important group with a wide distribution in different organs and tissues. Thus chondroitin sulphate in one or more of its known forms (Meyer + Rapport, 1951) is one of the main constituents (the ground substance) of cartilage and connective tissue. Heparin, a highly sulphated aminopolysaccharide present in the mast cells, liver, etc., with very marked anticoagulant and lipaemia-clearing properties, is likewise associated with tissue elements of the body (blood vessels, etc.) A third type of sulphated aminopolysaccharide, distinguishable from both chondroitin and heparin by not containing a uronic acid as a major component, has been recently described and found to be present in bovine cornea (Meyer et al., 1953) and also in rabbit and human subcutaneous connective tissue (Consden + Bird, 1954). These acidic polysaccharides associated with mesenchymal tissue are readily detected by their property of binding basic dyestuffs with accompanying metachromasia. The general chemistry and biological properties of these aminopolysaccharides have been fully reviewed elsewhere (Kent + Whitehouse, 1955). These substances evidently play an important role in different biological processes but very little is known as yet regarding their metabolism.

The metabolism and 'turnover' rate of the N-acetyl groups in the chondroitin sulphate(s) of cartilage and skin tissue has been studied following the administration of ¹⁴C-labelled acetate (Boström + Hansson, 1952b).

Schiller et al. 1955). Rather more attention has been devoted to the metabolism of the ester sulphate groups in these aminopolysaccharides.

The incorporation of inorganic sulphate into these sulphated polysaccharides has been followed both in vivo and in vitro by chemical and autoradiographic methods using ^{35}S -labelled sodium sulphate as an isotopic tracer. The isotope used in these studies, ^{35}S , is a β emitter possessing radiation properties very similar to those of ^{14}C , with a half life of 87 days. The bulk of the isotopic sulphate administered to animals is rapidly excreted in the urine and faeces, approximately 67% being eliminated by these routes within 24 hours but in contrast to the general and rapid fall in the amount of the isotope present in the liver and blood, the concentration of ^{35}S was found to significantly increase in both bone and articular cartilage (Dziewiatkowski, 1949a, Dziewiatkowski, Benesch + Benesch, 1949). Subsequently, detailed studies were made on the sites of ^{35}S -sulphate fixation in embryonic tissues (Layton, 1950, Friberg + Ringertz, 1954) and in the whole adult animal (Boström, 1953). Those tissues known to contain chondroitin sulphates and heparin, e.g., aorta, tracheal cartilage, mast cells of skin, cornea, etc., all exhibited a marked activity in binding the isotopic sulphate and retaining it for several days after administration.

It has been generally concluded that isotopic sulphate retained by cartilage, bone and mesenchymal tissues is present in the form of ester sulphate groups, as in chondroitin sulphate (and heparin). This conclusion has been amply confirmed by the isolation of these substances (^{35}S -labelled) from these tissues and demonstration that the radioactivity resided in the hydrolysable ester-sulphate groups (Boström, 1953. Vaughan, Kent + Steddon, 1955). Comparative measurements of the radioactivity of other sulphur-containing frac-

tions in animal tissues (inorganic sulphate, methionine, cysteine, taurine) 24-28 hours after administration of ^{35}S -labelled sulphate support the view that ^{35}S administered as sulphate is mainly retained in these tissues as ester sulphated polymers (mucosubstances).

Of particular interest in connection with the present investigation, are the findings that in addition to mesenchymal tissues, certain glands and epithelial sites, particularly the submaxillary glands and the epithelial layers of the gastrointestinal tract, likewise exhibit marked uptake of ^{35}S -labelled sulphate, as detected by autoradiography (Bélanger, 1953. Boström, 1953. Davies + Young, 1954. Florey + Jennings, 1955). This sulphate-fixation was particularly associated with the mucous-secreting glands and since the radioactivity was retained during the procedures of washing and histological 'fixing', it is reasonable to assume that it represents incorporation of sulphate into a sulphated polymeric component of the mucin produced at these sites.

In recent years, evidence has been forthcoming which indicates that the biosynthesis of aminopolysaccharides and related mucosubstances, hyaluronic acid, collagen, etc., is under the influence of hormones, e.g., ACTH and other factors (discussed in Kent + Whitehouse, 1955). For example, deficiency of vitamin C is reported to impair the incorporation of ^{35}S -sulphate into the chondroitin sulphate of guinea pig costal cartilage (Reddi + Norström, 1954). Cortisone, which is known to depress protein synthesis and growth of embryonic tissues, also exerts profound influence on the synthesis and turnover of mucosubstances. This has been particularly studied with reference to the metabolism of the aminopolysaccharides.

It has been demonstrated by histological means that cortisone administration in animals is accompanied by a considerable reduction in the total

amount of metachromatic ground substance, i.e., chondroitin sulphate and related compounds of regenerating connective tissue and healing bone (for references, see Jennings + Florey, 1954). Layton (1951) was able to show that cortisone-treated chick embryonic tissues and intact animals (rats) fixed considerably less $^{35}\text{SO}_4$ than the corresponding non-cortisonised controls, the embryonic tissues being found to take up progressively less sulphate as the concentration of cortisone in the medium was increased. Furthermore, in cortisone treated animals, decreased sulphate fixation was accompanied by a higher excretion of sulphate in the urine. Boström and Odeblad (1953) confirmed these results, finding that cortisone administration markedly depressed the incorporation of $^{35}\text{SO}_4$ into both skin and cartilage. Since these findings were also supported by the results of experiments with cartilage slices in vitro, the cortisone effect must be regarded as a lowering of the rate of exchange of ester sulphate groups in chondroitin and heparin with inorganic sulphate, rather than an inhibition of the complete synthesis de novo of the whole aminopolysaccharide macromolecule. This exchange reaction studied in vitro by Boström and Hansson (1952a, 1953) is strongly inhibited not only by cortisone but also by a number of enzyme inhibitors. What relationship this cortisone inhibition of sulphate exchange in mesenchymal tissues has with the action of cortisone as a palliative for the 'collagen diseases' (arthritis, etc.) is not understood at present.

By contrast, cortisone is found to have little effect on the generation of an epithelial mucin - that produced by the goblet cells of colon, as judged by histological criteria (Jennings and Florey, 1954).

The present work is concerned with studies on the incorporation of sulphur into rabbit duodenal mucin, using ^{35}S -labelled tracer compounds. Some preliminary findings on the composition of this and related mucins are also described.

EXPERIMENTAL METHODS

³⁵S-labelled sodium sulphate and ³⁵S-methionine were obtained from the Radiochemical Centre, Amersham.

N-Acetylglucosamine was prepared from glucosamine hydrochloride by White's (1940) method.

QUANTITATIVE ANALYTICAL PROCEDURES

Total carbohydrate (other than aminosugars) was measured by the anthrone colorimetric method (Dreywood, 1946) using the modification of Loewus (1952). The degree of heating attained on mixing the sulphuric acid with the other reagents was found to be critical (cf. Scott & Melvin, 1953). Attempts to regulate heat development by maintaining the tubes at a fixed temperature (boiling water bath) led to reduction in the intensities and change of colour from green to red of the final colorations. Satisfactory reproducible results were obtained on ensuring maximal heat production by thoroughly shaking the anthrone-carbohydrate mixture immediately after addition of the concentrated sulphuric acid. The final colorations were measured on a 'Spekker' photoelectric absorptiometer using Ilford 607 filters (max. transmission at 670 mμ) against a reagent blank.

Galactose, over the range 20-300 μg, gave a linear calibration curve. Using hard glass tubes, specially selected for uniformity, multiple determinations were reproducible to within 5%. The coloration from 100 μg. galactose was found to be equivalent to 60 μg. glucose and to 360 μg. glucuronic acid. Carbohydrate content was calculated as galactose.

Aminosugars in quantities of 0-700 μg. gave no coloration. Proteins (pepsin, serum albumin) did not appreciably interfere.

Uronic acid was determined on whole mucosubstances by the carbazole-

sulphuric acid method (Dische, 1947). The optical densities of the final colorations were measured after 2 hours, on the 'Spekker' using Ilford 604 filters (max. transmission at 520 m μ), and compared with glucurone standards.

Aminosugar content was determined after acid hydrolysis (see later) of the mucosubstances by the Elson-Morgan colorimetric method. Two procedures were employed in attempts to overcome interference due to interaction between aminoacids and reducing sugars (see Kent + Whitehouse, 1955) also present in the hydrolysates.

1. After hydrolysis with hydrochloric acid and partial neutralisation with sodium carbonate, the aminosugars (and aminoacids) were separated from other reducing sugars by absorption on Dowex 50 (acidic) ion exchange resin (Boas, 1953). The aminosugars were eluted with 2 N hydrochloric acid and determined by a modification of the Elson-Morgan procedure due to Boas (1953). Since glucosamine could be only incompletely recovered from the columns and to an irregular extent, this method for separating aminosugars from other reducing carbohydrates was later abandoned in favour of the second.

2. On hydrolysing the mucosubstances with 2 N hydrochloric acid for 16 hours, the bulk of the non-aminosugars is destroyed with concomitant humin formation (cf. Werner, 1953). 3-5mg. samples of dried mucins were therefore hydrolysed in sealed tubes for 16 hours at 105°, filtered from humin and the aminosugars determined by Boas's procedure (1953). Glucosamine standards were heated with hydrochloric acid under identical conditions. Calculation of aminosugar content was based on these hydrolysed glucosamine values, for which a linear relationship between final colour density and quantity was found. The recovery of glucosamine after hydrolysis was consistently of the order 70-75%.

Final colorations were measured on the Spekker using Ilford 604 filters,

after standing for 2 hours.

Reducing sugar was measured colorimetrically using the benzidine-acetic acid reagent (Jones + Pridham, 1954). Glucosamine gave no coloration whatsoever whilst N-acetylglucosamine gave colorations 40% those obtained from equivalent amounts of galactose.

Nitrogen was determined by the micro-Kjeldahl technique. Whole mucin (1ml.) or dried preparations (2.5mg.) were ashed for 2 hours with the sulphuric acid-selenium dioxide-potassium sulphate reagent. Aliquots of the diluted ashed material were distilled with alkali in a Markham still and the liberated ammonia estimated titrimetrically as described previously (p.17).

Phosphorus. Total phosphorus was determined colorimetrically after ashing, using the reagents and procedure described by Jones, Lee and Peacock (1951). Inorganic phosphate was determined directly without prior ashing. Final colorations were measured on the Spekker using Ilford 608 filter.

Chloride ion. Whole mucin was titrated against 0.01N silver nitrate using potassium chromate as indicator (Mohr 1856).

Sulphate. Inorganic and ester sulphate was determined by precipitation with benzidine after hydrolysis of whole mucins with 25% (v/v) formic acid for 16 hours at 105° in sealed tubes. Full experimental details of the method are given in Part III of this thesis. The analyses were performed on duplicate samples.

Acid hydrolysis.

a) For aminosugar estimations 3-5mg. samples of the freeze-dried mucin were heated with 2N hydrochloric acid at 105° in sealed tubes for 16 hours. Under these conditions there was extensive destruction of the non-aminosugar reducing sugars. The hydrolysate was filtered from humin and carefully

neutralised with sodium hydroxide.

b) For chromatographic identification of sugars present in the dried mucins, 5-10 mg. samples were heated with N sulphuric acid at 105° in sealed tubes for 6 hours and neutralised by stirring with solid barium carbonate. After filtration, the neutral salt-free hydrolysates were evaporated to dryness in vacuo and redissolved in the minimum quantity of water for application to paper chromatograms.

c) Some attempts were made to hydrolyse dried mucins with the polysulphonyl (acidic) resin, Dowex 50 (Dow Chemical Company, Midland, Michigan). The dry resin was ground in a mortar, loosely packed into a column and washed twice with 2N hydrochloric acid, removed from the column and repeatedly washed by decantation with distilled water until the washings were pH5, and finally dried at 37° . 5-10mg. samples of mucins, chitin, serum albumin and starch were dissolved or suspended in 2ml. distilled water and heated for 6 hours with 850mg. dried resin in sealed tubes at 105° . The hydrolysates were filtered from the resin and found to be appreciably acidic. They were therefore neutralised with barium carbonate, before being concentrated for application to chromatograms. Aminoacids were retained by the resins.

Water blanks heated with the resin became appreciably discoloured and gave a precipitate with barium chloride, indicative of the presence of free sulphuric acid since this precipitate failed to dissolve in concentrated hydrochloric acid.

The makers claim that these resins are stable to heating for 16 hours at 175° under neutral, acidic and alkaline conditions.

Qualitative analytical methods.

Paper chromatography was carried out using Whatman no. 1 paper, and the following solvent systems:-

n-Butanol-acetic acid-water (4:1:5)

Phenol, saturated with water and containing 8-hydroxyquinoline
(5mg./100ml.)

n-Propanol-borate buffer, pH 6-8 (1:3)

n-Butanol-pyridine-water (3:1:1)

Tertiary butanol - 2,6. lutidine-water (1:1:2)

Reducing sugars were detected on spraying with aniline hydrogen phthalate (Partridge, 1949) or anisidine hydrochloride (Hough, Jones + Wadman, 1950) in water-saturated n-butanol. Stannous chloride was added to the anisidine reagent as a stabiliser, excess being removed by filtration. (cf Jones + Pridham, 1954). Aminoacids and aminosugars were detected with 0.5% ethanolic ninhydrin. Sprayed chromatograms were heated at 105° until maximal colour development (ca. 10 mins.)

Glucose, galactose and mannose were separated by chromatography in the butanol-pyridine system. Glucosamine and galactosamine were distinguished after spraying chromatograms, run for 36 hours in butanol-acetic acid, with ninhydrin (sprayed twice and heated at 105°) and re-chromatographing the ninhydrin-treated aminosugar spots for a further 36 hours in the same solvent system together with lyxose and arabinose. Glucosamine was detected by the formation of some arabinose, galactosamine by lyxose formation after reaction with the ninhydrin (Blix, Svenserholm + Werner, 1950. Stoffyn + Jeanloz, 1954).

Chondroitin sulphate and heparin were detected by their metachromatic staining reaction with toluidine blue (Pasternak + Kent, 1952). Dried papers were stained with the dye (0.5% in 65% ethanol, acidified with glacial acetic acid) and excess of the dyestuff washed away by immersion in 95% ethanol.

Paper electrophoresis: The apparatus consisted of two glass dishes (9"x4"x2") containing buffer, which served as end compartments. The electrodes were 6 inch lengths of platinum wire lying along the bottom of these dishes and sealed into glass tubes containing mercury through which contact was made to a D.C. rectifier, capable of delivering up to 30 m.amps at 1,000 volts. The voltage was adjusted by means of a potentiometer. The paper was clamped between two glass plates (9"x15"), sealed at the edges with silicone grease to minimise evaporation, for electrophoresis at low voltages. At higher voltages (with consequently greater heat development) the paper was immersed in a centre compartment containing chlorobenzene. Where possible, mucin samples were submitted to electrophoresis between the glass plates to minimise denaturation with the mucosubstances by contact with the chlorobenzene. Whatman no. 1 paper, in 22" lengths, was used throughout, being dipped or sprayed with the buffer, dried, the materials spotted onto the paper and the paper sprayed again, lightly blotted and placed in the apparatus. The voltage was kept constant throughout a run, the current rising over the first hour. The buffers used were acetate, pH 3.5 (0.05M), borate pH 8.35 and 8.6 (0.3M) and barbitone pH 8.6(0.06M). Both the borate and barbitone buffers contained sodium chloride (0.3M) to raise the conductivity.

Monosaccharides, aminoacids and metachromatic aminopolysaccharides were detected by the reagents described above. Proteins were stained with bromphenol blue (Pasternak + Kent, 1952) or iodine vapour (Greenway, Kent + Whitehouse, 1953).

Diphenylamine colour reaction for deoxypentoses (Dische, 1930) and sialic acid (Werner + Odin, 1952) was carried out on whole mucin samples. Diphenylamine was crystallised from ligroin and dissolved in glacial acetic

acid-sulphuric acid (9:1 v/v) to give a 1% solution. This was heated with an equal volume of mucin or sugar solution in a briskly boiling water bath. Deoxypentoses and sialic acid give blue and purple colorations respectively after 10 mins. heating. Fructose alone of the common sugars, interferes in this test (Anderson + MacLagan, 1955).

Mucin samples.

Whole mucin samples were obtained from adult rabbits. The rabbit was used as a source of duodenal juice since the Brunner's glands extend almost 40 cm. (from the pylorus to the entry of the pancreatic duct) and secrete almost continuously. For the collection of mucin samples, two methods were used:

1. Survival preparations :

Fistulae, 10-15 cm. long, were prepared by an operation similar to that described by Florey and Harding (1934). No juice was collected until at least 10 days after operation. The rate of secretion through the fistulae was fairly regular, of the order 1 - 3 ml./hour. Collections were made at half-hour intervals or greater. After a few weeks, the animals tended to deteriorate exhibiting symptoms of 'dehydration'. The volume of juice diminished over this period, becoming more viscous in consistency.

2. Acute experiments with perfusion of the duodenum :

Florey and Harding (1934) showed that Brunner's glands were stimulated by passage through the duodenum of hydrochloric acid of about the concentration found in gastric juice (ca. $N/15$). This technique was used for some experiments, the animals being anaesthetised with urethane, to check the possible effects of bacterial action on the components of juice obtained

These animal experiments, the injection of isotopic tracer compounds and the collection of the mucin samples were undertaken by Professor H.W. Florey, Dr. M. A. Jennings and Mr. J. Kent, of the Sir William Dunn School of Pathology, Oxford.

from isolated (non-sterile) fistulae. $N/15$ hydrochloric acid was perfused through 10-15 cm. lengths of the duodenum at a rate of approximately 6ml./hr. The glands secreted continuously for periods up to 12 hours. The outflow bore all the character of diluted normal mucin (viscous, alkaline pH, etc.)

All mucin samples were stored either at -15° or under toluene at $+2^{\circ}\text{C}$.

Dried mucin preparations were obtained by freeze drying after exhaustive dialysis to remove inorganic components, etc. The yield of exhaustively dried material rarely exceeded 1.5 mg./ml original mucin. These preparations were only sparingly soluble in water.

Dried pig pyloric and duodenal mucins were isolated by Dr. N.G. Heatley, (Sir William Dunn School of Pathology, Oxford) by filtration of whole mucin on sintered glass filters and freeze-drying the washed residue. These samples were almost insoluble in water. The yield of dried mucin was generally 1-2 mg./ml. original pyloric or duodenal juice - obtained by means of fistulae.

^{of}
Measurement radioactivity.

Blood, mucin and urine samples were counted directly after plating 0.1ml. samples onto polythene cupped planchettes (2 sq. cm.) and drying (20 min.) under an infra-red lamp. A conventional Geiger-Muller apparatus (Dynatron Radio, Maidenhead) was used throughout, with a thin end window E.H.M.2 Geiger-Muller tube (G.E.C.) operating at 1500 v. Samples were counted in duplicate for periods of 10 mins./sample.

Radioactive substances on paper chromatograms and electrograms were detected by cutting the paper into strips (1x2.5cm.) and counting each strip individually. The ordinary monitoring apparatus was insufficiently insensitive for adaptation to scanning these paper strips. Through the courtesy of Dr. B. C. Loughman (Department of Agriculture, Oxford) a limited number

of strips were examined overnight by an automatic scanning and recording machine. All counts were corrected for background (12-25 counts/min.) No correction was made for self absorption by the plated samples as these were almost of "infinitesimal thin-ness".

In the experiments with $^{35}\text{SO}_4$, specific activities were determined after hydrolysis of whole mucin fractions and estimation of the total sulphate present.

Dialysis procedure.

Dialysis was conducted in one inch cellophane membranes against distilled water (two changes) for 3 days at $+2^\circ\text{C}$. Penicillin G, as the sodium salt, and streptomycin phosphate were added to all mucin samples submitted to dialysis at initial concentration 1:1,000 (w/v) and replenished at this initial concentration whenever the sacs were opened for sampling etc. Toluene was also added to sacs containing undiluted mucin. The pH of the mucin fell from 9.5 to between 5 and 6 after 48 hr. dialysis.

Experiments with ^{35}S -labelled sulphate.

Four perfusion (acute) experiments were carried out (Rabbits M.S. 60, 62, 64, 67) of duration 7-10 hours. After the first hour (control period) 0.1mC. sodium ^{35}S -sulphate was injected into the ear. Mucin samples, i.e., issuing perfusate, were collected half hourly. Two of these rabbits received cortisone acetate subcutaneously at the rate of 20 (M.S. 62) or 25 (M.S. 64) mg. per kg. body weight daily for 3 days before, and on the day of experiment.

Two preliminary experiments were carried out on fistulated animals, which had been injected with 0.1 mC. $\text{Na}_2^{35}\text{SO}_4$, in which duodenal juice only was collected - their duration being 6 (M.S. 55) and 7 (M.S. 54) hours respectively. Four fuller experiments on two rabbits (M.S. 58, M.S. 65)

3 expts.) in which the same dose of $^{35}\text{SO}_4$ was administered, were continued until radioactivity had virtually disappeared from both juice and blood. For blood samples, 0.1ml. was withdrawn hourly from the ear. Urine samples were also collected, as passed. One of these rabbits (M.S. 65) was treated with cortisone for two experiments at the rate of 20 mg./kg. for 3 days before, and during the period of collection.

Two experiments were performed in which one fistulated rabbit (MS 76) was administered 20 mC. $\text{Na}_2^{35}\text{SO}_4$. Prior to the second experiment, cortisone was administered as previously. Mucin samples were collected at hourly intervals, including controls.

Experiments with ^{35}S -labelled methionine.

Two perfusion (acute) experiments were carried out for duration of 10 hours (rabbits M.S. 66 and 68) and after the first hour 0.1mC. ^{35}S -methionine injected. In one, the rabbit (M.S. 68) received cortisone, 25mg. per kg., for 3 days before and on the day of experiment.

The same amount (0.1mC) of ^{35}S -methionine was twice injected into a fistulated rabbit (M.S. 58) and samples collected of blood, urine and duodenal juice until the respective radioactivities had fallen to 5% initial activity.

Enzymic experiments.

Dialysed mucin was incubated at 37° with commercial pepsin preparations (B.D.H., Parke Davis) at pH 1.5 (hydrochloric acid), with crude glycerol extracts of pancreas (B.D.H. 'Liquor Pancreaticus') and a medicinal pancreatin preparation (Bengers) at pH 8.5 (barbitone, borate buffers), with trypsin (B.D.H.) at pH 4.7 (acetate buffer) and with papain (B.D.H.) at pH 5.0. The papain preparation was activated by pre-incubation with

cysteine (0.05M) at 37°. Undialysed mucin (pH 9.5) was also incubated with the pancreatic preparations. As controls, these enzymes were incubated with serum albumin and N-acetyl glucosamine. All incubations were carried out under a layer of toluene or in the presence of a mixture of penicillin G (Na. salt) and streptomycin phosphate (1:10,000). Proteolytic digestion was followed by chromatographing boiled or ethanol-denatured incubation mixtures in butanol-acetic acid-water (4:1:5) and spraying with ninhydrin to detect aminoacids.

Dialysed mucin was also incubated with a commercial preparation of testicular hyaluronidase (Bengers) at pH 5.8 and 7.1 (phosphate buffers) at 30° and 37°C.

All buffers used were 0.1M. with the exception of the barbitone which was 0.06 M.

Other measurements.

pH was determined using a pH-meter (Cambridge Instrument Co.)

Refractive index was measured with a Philpot interference refractometer, using monochromatic light from a mercury vapour lamp ($\lambda = 546.2 \text{ m}\mu$).

RESULTS

Mucin production (ex fistulae)

The rabbit was chosen as a source of duodenal mucin in view of the almost continuous excretion of mucin into the duodenum. The regularity and rate of mucin production were examined in the following experiment. Total solid content of hourly fractions was determined by measurement of the nitrogen content and the refractive index increment of mucin samples freed from cell debris and suspended matter by prolonged centrifugation at 200 g (Table 19). These indicated that the variation in solid content in a given volume was no greater than $\pm 15\%$ over a series of fractions taken at hourly intervals, though the actual volume obtained from the fistula at regular intervals varied rather more widely ($\pm 30\%$).

Table 19. Variation volume and solid content of mucin sample.

Duodenal juice collected from rabbit M.S. 54 at hourly intervals.

1 ml. samples ashed for nitrogen determinations.

Time	Volume	Observed Refractive Index	Refractive Increment (Δn)	mg.N/100 ml.mucin
1 hr	4.05ml.	.650	.105	45.7
2 "	3.20	.629	.084	36.0
3 "	3.40	.638	.093	44.8
4 "	2.95	.643	.098	44.8
5 "	3.70	.643	.098	43.4
6 "	2.10	.660	.113	47.7
7 "	3.0	.649	.102	49.8
8 "	2.70	.650	.103	46.6
Water blank		.546	-	-

Absolute refractive increment = $\Delta n \times 1.203 \times 10^{-2}$ for $\lambda = 546.2 \text{ m}\mu$.

Incorporation of $^{35}\text{SO}_4$ into duodenal mucin

(1) Fistulae experiments.

In preliminary experiments, rabbits with duodenal fistulae were injected intravenously with approximately 0.1mC. $\text{Na}_2^{35}\text{SO}_4$ dissolved in distilled water (pyrogen free). 0.1 ml. of the mucin, collected at half-hourly intervals after administration of $^{35}\text{SO}_4$, was dried on a planchette and counted directly. Table 20 indicates the variation in the radioactivity of mucin samples with time, for one such experiment.

Table 20. Activity of mucin samples following injection $\text{Na}_2^{35}\text{SO}_4$.

Mucin obtained by means of a fistula (rabbit M.S. 94)

Activities are uncorrected for variation in total SO_4 present.

Initial (injected) activity equivalent to 9.4×10^6 counts/min on the counter used.

Hours after injection	Vol. (ml.)	Counts/min per 0.1ml.	Total activity of fraction	% initial activity
$\frac{1}{2}$	1.4	81	980 counts/min	1×10^{-4}
1	2.8	175	4600 " "	5 "
$1\frac{1}{2}$	2.6	156	3750 " "	4 "
2	1.4	149	2,000 " "	2 "
$2\frac{1}{2}$	2.0	168	3400 " "	3 "
3	1.5	142	1950 " "	2 "
$3\frac{1}{2}$	1.9	147	2500 " "	2.5 "
4	1.5	129	1750 " "	2 "
$4\frac{1}{2}$	1.9	103	1750 " "	2 "
5	0.9	83	650 " "	0.7 "
$5\frac{1}{2}$	1.8	87	1350 " "	1.5 "
6	1.6	53	670 " "	0.7 "
24/25	2.9	25	370 " "	0.4 "

Corrected for background (11.6 counts/min.)

Though it is doubtful if the attempts to assess the total activity of each half-hourly fraction have any real significance, the results do indicate however, that the radioactivity incorporated into the mucins represents a very small percentage indeed of the original radioactivity injected into the animal as $\text{Na}_2^{35}\text{SO}_4$; the maximum radioactivity incorporated into any one fraction from this animal being 0.0005% and the activity issuing in the first 6 hours after injection totalling only 0.003%.

Preliminary studies of the most active fraction (obtained $\frac{1}{2}$ -1hr. after injection) indicated that the activity was mainly due to inorganic sulphate. When submitted to paper electrophoresis in acetate and borate buffers (pH 3.5 and 8.35 respectively,) the sole detectable activity was found to migrate with the same mobility as inorganic sulphate. Both heparin and chondroitin sulphate (from bovine trachea) moved more slowly than free sulphate. The addition of barium chloride, which precipitates both heparin and free sulphate, did not however precipitate all the activity from this most active fraction - the residual activity being some 20% of the initial activity. A later fraction (collected $2\frac{1}{2}$ hours after injection) similarly treated with excess barium chloride retained 40% of the initial activity. Since there was a copious precipitate of barium carbonate due to bicarbonate present in native mucin which would tend to absorb radioactivity from solution, this residual activity suggested that in the later mucin fractions an increasing proportion of the ^{35}S is present in some form, other than free inorganic sulphate, which is not precipitable with barium ions (i.e., not heparin). That this bound form of ^{35}S was an ester (i.e., hydrolysable) sulphate group was indicated by the finding that all the measurable activity of various mucin samples was precipitable with barium ions following hydrolysis with 2N hydrochloric acid for 8 hours at 105° .

These observations were repeated on mucin samples obtained from a different animal (M.S.58). Sodium ^{35}S -sulphate (0.1mC) was injected twice, the second injection being given 2 hours after the first. After each injection a marked rise in the mucin activity was found (fig. 4) the total activity of these maxima being mainly due to the presence of inorganic $^{35}\text{SO}_4$ *. These maxima were no artefacts due to irregular excretion of free and esterified sulphate in the mucin, since determination of the specific activities of these fractions, following analysis for total sulphate, confirmed the reality of these maxima (Table 21 and fig. 5). Whether or not the activities of the various mucin samples were corrected for variation in total sulphate present (by calculation of the specific activities), the maximum activity was found in mucin fractions collected $1\frac{1}{2}$ - 2 hours after injection of the $\text{Na}_2^{35}\text{SO}_4$ (figs. 4 and 5).

* Specific activities were determined as radioactivity due to ^{35}S in the total sulphate present arbitrary units of counts/min/ μg . inorganic and acid-hydrolysable sulphate.

Table 21. Specific activity mucin samples following 2 injections $\text{Na}_2^{35}\text{SO}_4$

Mucin obtained by means of a fistula (rabbit M.S. 58)

Specific activities evaluated following hydrolysis mucin samples and determination of total sulphate.

Second injection $\text{Na}_2^{35}\text{SO}_4$ given 2 hours after the first - each dose =

0.1mC. Total activity injected equivalent to $2 \times 8.5 \times 10^6$ counts/min.

Hours after 1st injection	Vol. ml.	Counts/min per 0.1ml. mucin	Total SO_4 per 1 ml. mucin	Sp. Act. Mucin Counts/min/ μg	Activity 0.1m. blood (counts/min)
control	1.1	24	122.5 μg		23
$\frac{1}{2}$	1.5	364	110 "	31	
1	0.7	586	66.5 "	85	922
$1\frac{1}{2}$	0.8	454	94 "	46	
2	0.8	363	83.5 "	41	345
2nd injection					
$2\frac{1}{2}$	1.0	548	103 "	51	
3	1.3	605	84 "	69	741
$3\frac{1}{2}$	1.2	778	102 "	74	
4	1.3	964	115 "	82	734
$4\frac{1}{2}$	1.0	762	99 "	73	
5	0.7	598		58	491
$5\frac{1}{2}$	1.2	528			
6	0.7	489			500
23	1.6	108			
24	1.8	97			108
48	2.4	49			69

* Corrected for background count.

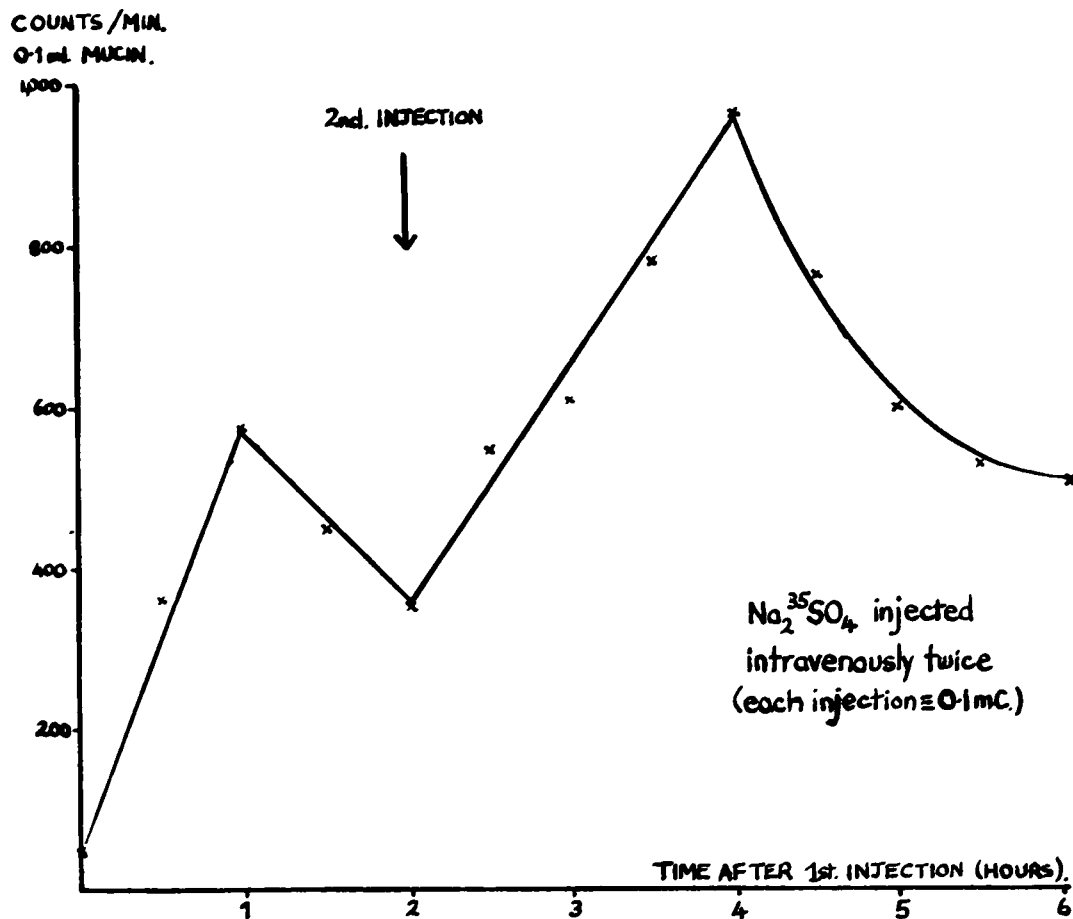


FIG. 4: INCORPORATION OF ³⁵S INTO DUODENAL MUCIN.

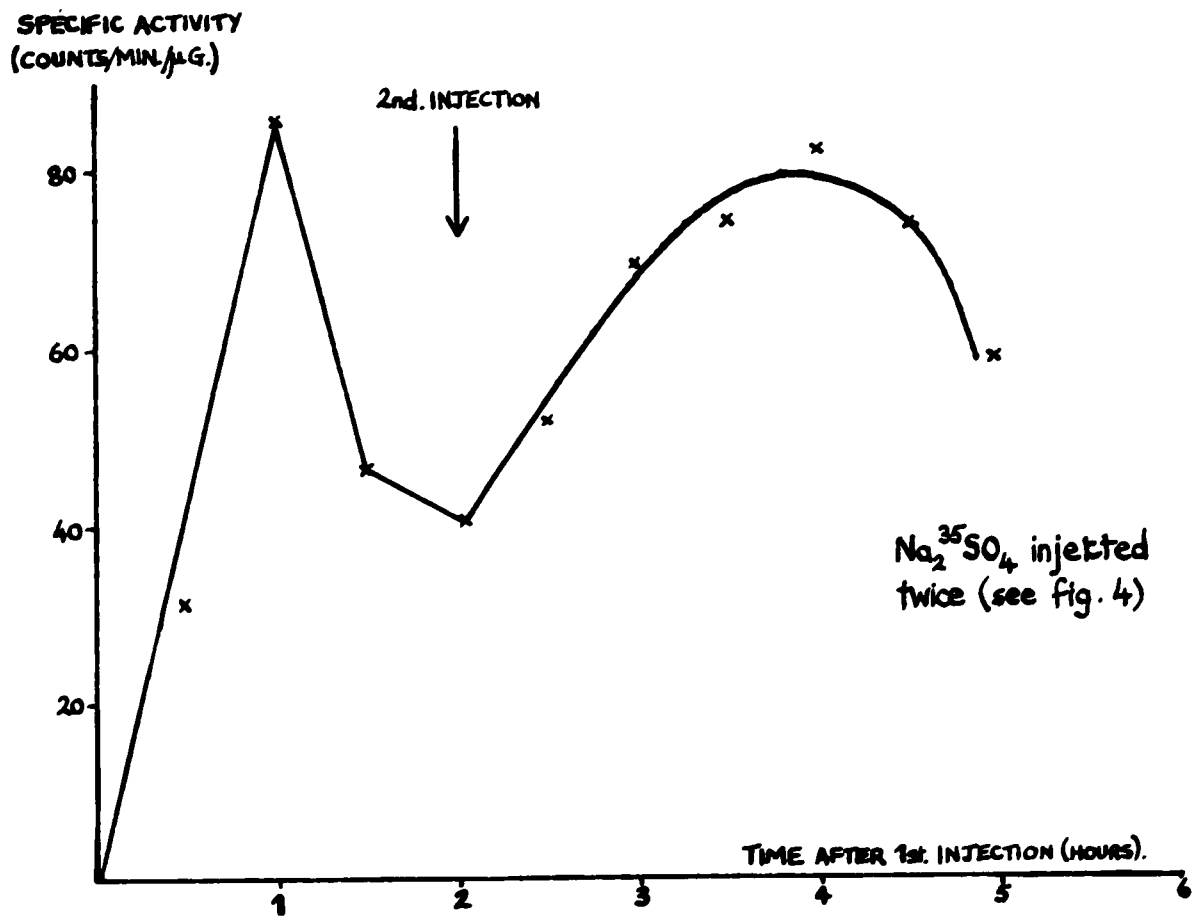


FIG. 5: SPECIFIC ACTIVITY OF (TOTAL) ³⁵SO₄ IN MUCIN.

The fraction of the initial radioactivity (injected as $\text{Na}_2^{35}\text{SO}_4$) emerging in the mucin samples collected over the 6 hours following the first injection was only 0.004% in this rabbit (M.S. 58) - cf. the figure 0.003% obtained previously (p. 75) from another animal (M.S. 54). The isolated lengths of duodenum in these fistula animals represent about one third the total length of the duodenum in an adult rabbit and since the activity emerging in all mucin fractions collected after 6 hours can be no greater than ten times that found in the first 6 hour fractions, the maximum incorporation of ^{35}S into rabbit duodenal juice following injection as $\text{Na}_2^{35}\text{SO}_4$ can be no greater than 0.1%.

By contrast 20% of the administered radioactivity was excreted in the urine within 6 hours and 65% within 24 hours, with an accompanying decline in the radioactivity of the blood (see Table 21).

Examination of these more active mucin fractions confirmed the presence of some of the ^{35}S in a non-dialysable, non barium-precipitable component of the mucin. The free inorganic sulphate ($^{35}\text{SO}_4$) could be further separated from this ^{35}S -bearing component by electrophoresis on paper (acetate buffer, pH 3.9, 20 volts/cm. for 1 hour). Under these conditions, free inorganic ^{35}S sulphate migrated towards the anode with a maximum at 21.5 cm., accompanied by some 'tracking' of the radioactivity along the electrogram when run in the presence of non-radioactive mucin. In the later mucin fractions (3hr., 5 hr., 24 hr.,) a radioactive electrophoretically-immobile component was detected, whilst in the 24 hour fraction, no inorganic $^{35}\text{SO}_4$ was present. The ratio of the immobile activity: inorganic $^{35}\text{SO}_4$ activity increased with increasing time of collection of mucin after injection of ^{35}S sulphate. These findings of the presence of two radioactive components on the paper electrograms and the varying ratio of their radioactivities with time of collection of the mucin

sample, were confirmed on taking autoradiographs of the electrophoretic strips. X-ray films were stood in contact with these strips (max. activity = 100 counts/min.) for 7 weeks before development.

(11) Acute experiments

The technique of stimulating mucin production by passage of $\text{H}/_{15}$ hydrochloric acid through isolated lengths of rabbit duodenum (Florey & Harding, 1934) was employed for some experiments, mainly to serve as a check on the possible effects of bacterial action on the components of the juice from isolated fistulae, by minimising bacterial growth and contamination during the actual mucin collection. The rabbits were anaesthetised with urethane throughout. The inflow cannula was attached to a burette containing $\text{H}/_{15}$ hydrochloric acid and 1 ml. of acid was run in every 10 minutes. Within a few minutes the outflow was viscous and almost or completely neutral. These characteristics were maintained to the end of the experiments indicating that the glands secreted continuously throughout. The volume of the outflow varied; with a short length of isolated duodenum it usually exceeded that of the inflow by 20-30% but with longer lengths it was usually less than the inflow, presumably because absorption exceeded secretion in the lower part of the isolated duodenum, where the acid had been neutralised.

Measurement of the chloride ion in various perfusate (mucin) samples, indicated that there was some absorption of chloride ion (initial $[\text{Cl}'] = .068\text{M}$) to regulate the chloride ion concentration at $.058\text{M} \pm .001$. The initial and final (post mortem) perfusates had an appreciably greater chloride ion content. The apparent reduction of chloride ion concentration with active perfusion and mucin secretion is not attributable to a dilution effect. (Table 22)

Table 22. Chloride ion content of mucin obtained in acute experiments.

The duodena of Rabbits M.S. 66 + M.S. 67 were perfused for 9 hours at rate of 6 ml./hr.

Chloride titrated directly against .01N. AgNO_3 .

MS 66

MS 67

Time after perfusion initial	Vol. (ml.)	Titre AgNO_3 per ml. mucin	Vol. (ml.)	Titre AgNO_3 per ml. mucin	pH at 20°
Control	2.9	9.80	2.9	7.31	3.2
1 hr.			3.1	6.00	
2½ "	3.2	5.83	3.0	5.66	9.6
5½ "	3.7	5.75	2.5	5.78	
7 "	3.3	5.84	3.2	5.62	8.9
Post mortem	2.7	7.71	1.9	8.01	8.3

The average chloride ion content of several mucin samples ex fistulae was 0.054N.

Injection of $^{35}\text{SO}_4$, resulted in a ~~smaller~~ rapid incorporation of ^{35}S into the mucin (Table 23). in these acute experiments, cf. the results of the experiments with fistulated animals (Table 21, p. 77).

Table 23. Incorporation of $^{35}\text{SO}_4$ into duodenal mucin in acute experiments.

0.1mC. $\text{Na}_2^{35}\text{SO}_4$ injected into rabbits M.S. 60 and 67

N/15 HCl perfused at rate of 6ml./hr.

MS. 60

MS. 67

Hours after inject ⁿ	Vol. (ml.)	c/min. 0.1ml. mucin	Total SO_4 /ml. mucin	Sp.Act.* c/min/ μg .	Vol. (ml.)	c/min. 0.1ml. mucin	Total SO_4 /ml. mucin	Sp.Act.* c/min/ μg
Control	2.8	26	6.1 μg	0	2.9	24		0
$\frac{1}{2}$	3.0	104	11.6 "	67	3.8	31		
1	2.9	181	11.9 "	130	3.5	170	27.6 μg	53
$1\frac{1}{2}$	3.5	293	10.3 "	258	3.3	197		
2	3.0	338	14.0 "	224	4.3	209	26.9	69
$2\frac{1}{2}$	3.0	299	17.3 "	158	3.7	187	26.5	62
3	3.3	291	20.0 "	132	3.5	214		
$3\frac{1}{2}$	3.5	159	28.0 "	48	3.9	172	21.0	70
4	2.6	111	19.6 "	43	4.0	176		
$4\frac{1}{2}$	3.8	112	21.3 "	40	3.9	100	14.0	54
5	3.0	104	13.3 "	59	4.0	117	12.8	72
$5\frac{1}{2}$	4.1	98	14.0 "	52	3.5	129		
6	2.7	73	16.0 "	29	3.25	136	20.7	54
$6\frac{1}{2}$	3.2	69	16.0 "	27	2.9	84	14.7	41
7	-	-	-	-	3.0	122		
$7\frac{1}{2}$	-	-	-	-	3.0	130	16.1	67
8	-	-	-	-	2.4	160		

* Corrected for background count.

Rather more activity was present in mucin samples obtained in these acute experiments than from fistulae experiments; 0.006% initial activity injected as $\text{Na}_2^{35}\text{SO}_4$ appearing in the mucin within 6 hours (rabbits M.S.60, 64 and 67), cf. figures of 0.003% (M.S.54) and 0.004% (M.S.58) for the corresponding period from fistula animals.

The specific activities of consecutive mucin samples were rather erratic but it is doubtful how much this is to be attributed to irregular incorporation of activity or irregular mucin production. Some of these mucin samples contained appreciable amount of inorganic phosphate (60 - 360 $\mu\text{g/ml.}$) which can seriously interfere in the micro-estimation of sulphate (see also p. 87 + 158) and hence the evaluation of the specific activities.

Electrophoresis, precipitation with barium chloride, and dialysis of some of these mucin samples confirmed the reality of the findings with mucin from fistulae, i.e., that there were at least two radioactive components present in the later mucin fractions - one being inorganic $^{35}\text{SO}_4$ and another, a macromolecule not appreciably precipitated with barium ions and which liberated inorganic $^{35}\text{SO}_4$ on acidic hydrolysis.

Influence of cortisone on $^{35}\text{SO}_4$ incorporation.

Preliminary experiments.

Cortisone was administered by subcutaneous injection of cortisone acetate to both fistula animals and to animals later submitted to the acute experiments, in amounts 20-25 mg/kg. body weight for at least 3 days prior to administering ^{35}S sulphate.

The acute experiments (rabbits M.S.62 and 64) indicated that cortisone administration was without significant effect on the variation of the specific activity of the mucin fractions with time (Table 24 and cf. Table 23). A second maximum for incorporation of radioactivity into the mucin was discern-

able at 4½-5 hours (fig. 6) being rather greater in these acute experiments with cortisonised animals than in corresponding experiments without prior cortisone treatment (Table 23 and fig. 6).

Table 24. Incorporation of $^{35}\text{SO}_4$ into duodenal mucin in acute experiments following administration of cortisone.

0.1mC $\text{Na}_2^{35}\text{SO}_4$ injected into rabbits M.S.62 and M.S.64.

Hours after inject. ⁿ	MS. 62.				MS. 64.			
	Vol. (ml.)	c/min /0.1ml mucin	Total SO_4 */ml. mucin (μg)	Sp.Act.* c/min/ μg	Vol. (ml.)	c/min /0.1ml mucin	Total SO_4 */ml. mucin (μg)	Sp.Act.* c/min/ μg
Control	2.1	20	52.5	0	3.3	21		0
½	3.4	104			4.0	31	8.0	13
1	2.5	439	38	110	4.2	157		
1½	2.2	640			4.5	204	15.1	121
2	2.3	755	27.4	268	3.7	267	10.5	234
2½	2.3	767	47.6	158	4.5	140	10.1	119
3	1.2	821			4.8	106		
3½	1.5	680			4.3	178	18.7	84
4	3.4	615	42.6	140	4.6	200		
4½	2.3	515			4.6	196	16.5	106
5	1.4	557	25.0	206	3.9	112	18.5	50
5½	1.0	597			3.6	94	13.8	53
6	2.5	661	39.0	164	3.5	112		
6½	1.6	568			3.5	117		
7	1.8	471	26.3	170	3.3	84		
7½	3.1	416			3.2	97		
8	2.5	378			3.8	88		

* Corrected for background count.

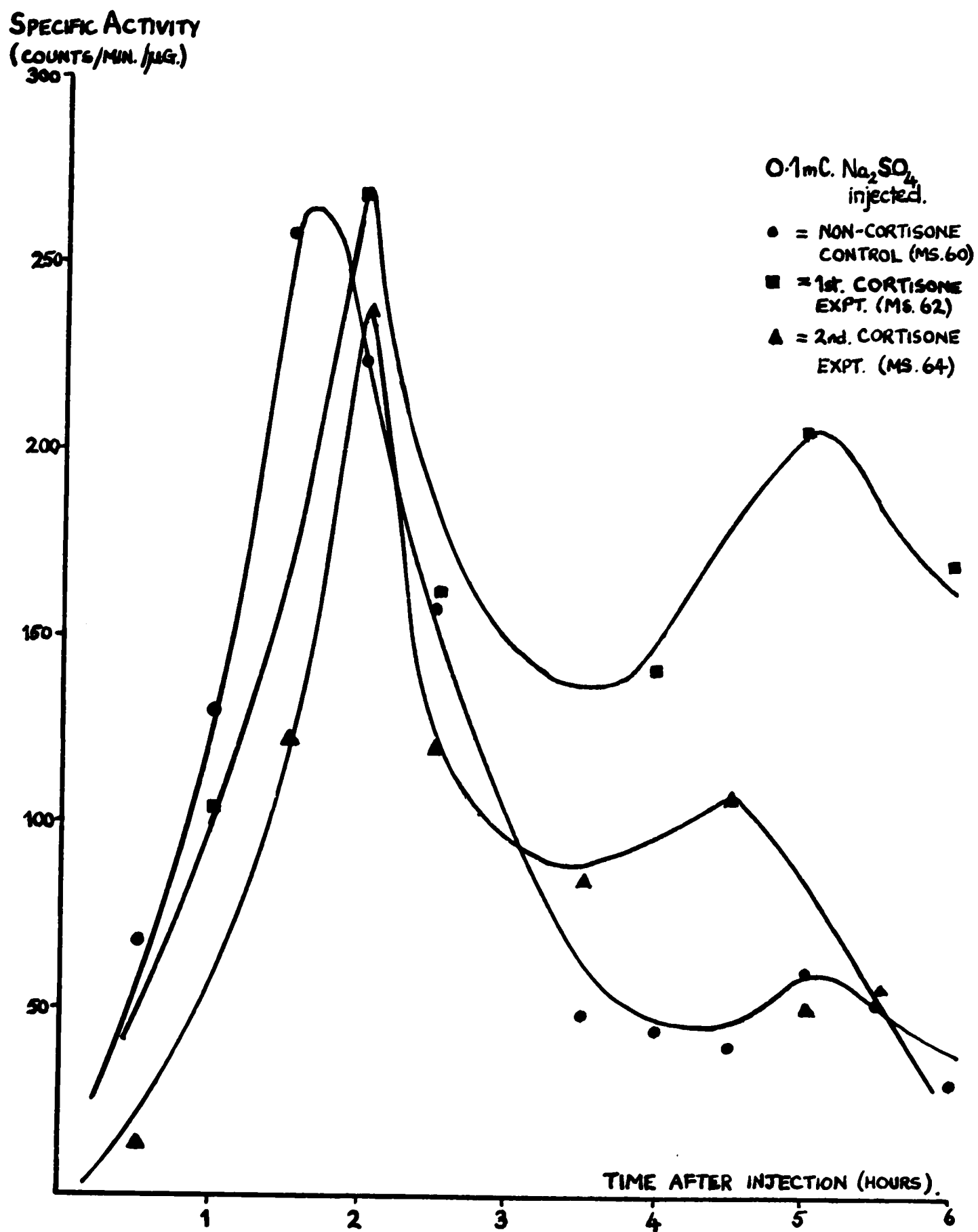


FIG. 6: INFLUENCE OF CORTISONE ON $^{35}\text{SO}_4$ INCORPORATION.
(ACUTE EXPERIMENTS)

One fistulated animal (M.S. 58)^{was} injected with 0.1mC. Na₂³⁵SO₄ and the activity incorporated into the mucin, determined. After three days, when the radioactivity of the urine had fallen to 100 counts/min./0.1ml. (maximum was 10,200 counts/min./0.1ml.) and the blood radioactivity to background count, the animal was submitted to cortisone treatment for 5 successive days. On both the fourth and fifth days, 0.1mC. Na₂³⁵SO₄ was injected. Table 25 records the activity of the mucin and blood and the specific activity of the mucin. These results are far more ambiguous than those obtained in the acute experiments concerning the influence, if any, of cortisone administration on the incorporation of ³⁵S into duodenal mucin.

In the experiments following cortisone treatment, the blood activity was found to decline more rapidly than in the control(non-cortisone) experiment (see column 6, 11 + 16 of Table 25) but whether this is to be specifically associated with the administration of cortisone or to a non specific effect associated with the passage of 5 days between the control and the first cortisone experiment, is difficult to determine. One effect of the time interval between experiments is indicated by the reduction in the volume of the mucin obtained at half-hour intervals (columns 2,7 + 12, Table 25.)

The very low specific activities ~~as~~ calculated for the mucin samples obtained in the cortisone experiments, (columns 10 + 15, cf. column 5, Table 25) call for some comment. Undoubtedly one factor contributing to this, namely the seemingly much higher content of total SO₄^{="}/ml. mucin in the "cortisone" samples, may be related to the reduction in the volume of mucin secreted.

It is, however, surprising that the activities /0.1ml. of the smaller volumes of mucin are not proportionately greater on account of the reduction in the actual volumes secreted, unless of course this is indicative of a reduction in the synthesis of the mucosubstances as well. Whether this is to be attri-

buted to the cortisone or increasing 'dehydration' of the animal is a moot point. The mucin obtained in these cortisone experiments was found to have a very high content of inorganic phosphate ($300-750 \mu\text{g/ml.}$) as compared with the phosphate content ($80-120 \mu\text{g/ml.}$) of the mucin obtained from the same animal in the control experiment. It is well known that the presence of phosphate ions constitutes a considerable source of interference in the determination of sulphate by precipitation with benzidine - the method essentially used to estimate total sulphate content of the mucin samples (see Part III). The coprecipitation of benzidine phosphate is largely prevented by carrying out the precipitation at pH 2 (Welcher, 1947) but the error due to phosphate is greatly increased when the precipitation mixture is allowed to stand at low temperatures, i.e., under conditions required for the micro-estimation of the sulphate ion. Using the method described in Part III, it was found that phosphate in amounts of $200 \mu\text{g.}$ gave no precipitate with the benzidine reagent and in quantities greater than $50 \mu\text{g/ml.}$ actually inhibited the precipitation and estimation of sulphate ions. Nevertheless the possibility must be considered that the high phosphate content of the mucin samples obtained in the cortisone experiments is actually seriously interfering in the determination of the total sulphate content of these mucins and that little weight is to be attached to the much lower specific activities calculated for the cortisone experiments, as compared with those obtained in the control experiment.

Table 25. Specific Activity of mucin and blood samples after administration $0.1\text{mC Na}_2^{35}\text{SO}_4$ to

cortisone-treated fistula animal (W.S.58)

c/m = counts/minute.

Specific activities corrected for background count.

Control Expt. (13.7.54)

1st Cortisone Expt. (19.7.54)

2nd Cortisone Expt. (20.7.54)

Hours after inject ⁿ	Vol. (ml.)	Mucin Act. c/m/ 0.1ml.	Total SO ₄ ³⁵ /ml. mucin	Sp.Act. Blood Act. c/m/ 0.05ml.	Vol. (ml.)	Mucin Act. c/m/ 0.1ml.	Total SO ₄ ³⁵ /ml. mucin	Sp.Act. Blood Act. c/m/ 0.05ml.	Vol. (ml.)	Mucin Act. c/m/ 0.1ml.	Total SO ₄ ³⁵ /ml. mucin	Sp.Act. Blood Act. c/m/ 0.05ml.
Control (½hr.)	1.0	21	μg	0	21	0.5	21	0	0.3	19	47.1μg	0
½	1.5	169			54	0.5		8.6	0.3	32		2
1	1.1	488	12.0	390	297	0.4	271	65	0.8	171	62.4	24
1½	0.9	421			250	0.3	250	60	0.35	221		33
2	1.0	405	17.5	220	184	0.5	250	60				206
2½	1.6	394			170	0.9		39	0.6	220	78.0	124
3	0.8	270	18.3	136	117	0.6	158	29				107
3½	1.4	228			137	0.5		25	0.6	196	60.0	50
4	1.1	182	28.1	57	98	0.6	110	28				
4½	1.1	138			92	0.5		22	1.2	111	41.7	43
5	1.4	105	20.6	41	73	0.5	81	18				

Hours after injection	Vol. (ml.)	Mucin Total Act. c/ml. 0.1ml.	Mucin Total SO ₄ /ml. mucin (μg.)	Sp. Act. c/ml. μg. 0.05ml.	Blood Act. c/ml. 0.05ml.	Vol. (ml.)	Mucin Total Act. c/ml. 0.1ml.	Mucin Total SO ₄ /ml. mucin (μg.)	Sp. Act. c/ml. μg. 0.05ml.	Blood Act. c/ml. 0.05ml.	Vol. (ml.)	Mucin Total Act. c/ml. 0.1ml.	Mucin Total SO ₄ /ml. mucin (μg.)	Sp. Act. c/ml. μg. 0.05ml.	Blood Act. c/ml. 0.05ml.
5½	0.8	90	18.4	38		0.75	87	42	15		1.0	99	56.0	14	29
6	1.1	80	17.0	35	51	0.6	81	44.7	14	31					
6½	0.8	83				0.5	66		11		1.0	52	87.2	4	27
7	1.1	73			44	0.7	44	45.5	7	31					
7½	1.0	60	21.7	18		0.9	40	45.5	5						
8	1.5	56			23	0.9	34	43.0	3	25					
8½	0.9	63				0.75	28				2.25	33	26.5	5	24
9	1.2	50				0.9	20				0.4	30	25.8	4	
23/24	1.7	33			25	0.75	19			19	1.1	24			23
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Table 25.
(cont.)

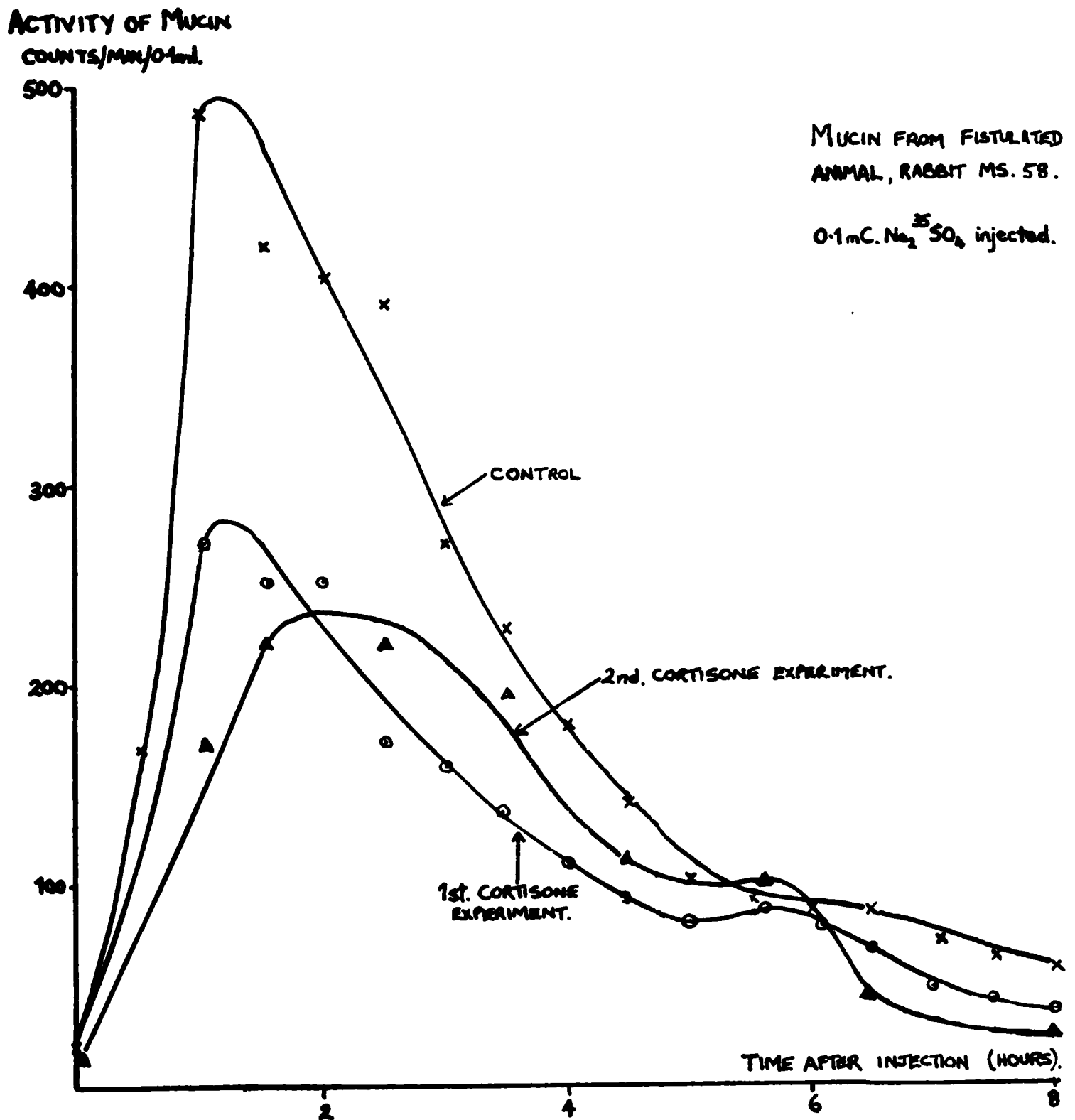


FIG. 7: COMPARISON OF THE RADIOACTIVITIES OF MUCIN FROM A FISTULA, BEFORE AND AFTER CORTISONE ADMINISTRATION.

A more valid comparison is perhaps to note the measured activities of 0.1ml. mucin samples (columns 3, 8 + 13. Table 25, and Fig. 7) in both the control and the two cortisone experiments. Even allowing for the considerable discrepancy in the proportion of mucin actually counted, due to the differences in volume produced in the 3 experiments, it is clear that far more activity appears in the mucin in the early fractions (mainly as inorganic sulphate), after injection of $^{35}\text{SO}_4$ in the non-cortisone experiment, than in the two experiments when an almost identical quantity of $\text{Na}_2^{35}\text{SO}_4$ was injected after cortisone administration. This is to be correlated with the more rapid decline in the blood activity in the two cortisone experiments, i.e., $^{35}\text{SO}_4$ was less readily available for incorporation into the mucin. No simple correlation could be found between the amount of activity excreted in the urine, the activity in the blood and the administration of cortisone, for if anything, the urinary $^{35}\text{SO}_4$ excretion was lower in the period following cortisone treatment. The greater rate of removal of $^{35}\text{SO}_4$ from the blood in the cortisone experiments was therefore not simply due to a greater excretion through the kidneys.

Experiments with the administration of a massive dose of $\text{Na}_2^{35}\text{SO}_4$.

In view of the ambiguous findings of these experiments on the fistulated animal, M.S.58, a further series of experiments was undertaken using another rabbit (M.S.76) and a comparison made of the incorporation of $^{35}\text{sulphate}$ into mucin both before and after cortisone administration. This animal was given a liberal supply of water and over a period of 2 months it exhibited none of the signs of dehydration, as found in the previous experiment. In order to be able to analyse the radioactivity of the mucin samples, highly active mucin fractions were obtained from the animal following injection of 20mC. $\text{Na}_2^{35}\text{SO}_4$. This represents a 200-fold increase in the amount of $^{35}\text{SO}_4$ in-

jected over that administered in the previous experiments.

Aliquots of the mucin samples collected at various time intervals were diluted before counting. These diluted solutions were then dialysed in cellophane for 5 days together with added (carrier) sodium sulphate. After 12 and 36 hours dialysis more carrier sulphate was added. The reduction in activity with dialysis was measured. The residual activity after precipitation of the inorganic sulphate (and bicarbonate) from the mucin with excess barium chloride in the presence of added carrier sulphate, was also determined. These results are presented in tables 26 and 27, see also figs. 8 and 9. (pp. 95 & 101).

Table 26. Activity of mucin fractions(ex fistula) after incorporation
of $^{35}\text{SO}_4$.

Rabbit H.S.76 injected 20 mc $\text{Na}_2^{35}\text{SO}_4$.

Mucin diluted to 22.5 ml., except where stated, and 20 ml. dialysed for 110hrs.

Activities in counts/min./0.1ml. (average of duplicate samples).

I.A. = Initial Activity.

Hours after inject ⁿ .	vol. (ml.)	Vol. taken (ml.)	I.A. dil. mucin	<u>Dialysed Mucin</u>		<u>Ba⁺⁺ pptd. Mucin</u>	
				Activity	% I.A.*	Activity	% I.A.*
Control	2.0	1	15	11	-	14	-
+ $^{35}\text{SO}_4$ ⁿ		1	1005	16	0.5	16	0.2
1	1.7	1	319	26	5.8	117	40.8
2	1.5	1	465	82	18.4	205	55.8
3	1.5	1	318	97	33.8	196	74.3
4	1.2	1	305	93	37.2	154	62.4
5	1.35	1	205	73	40.7	112	63.7
6	1.20	1	146	64	40.7	80	61.4
7	1.25	1	127	59	49.8	72	70.4
8	1.2	1	100	54	59.3	64	71.8
9+10	8.2	6	211	118	69.0	152	88.0
11+12	4.1	3	100	55	64.5	70	80.3
23-28	5.5	4 dil.	152	84	68.7	102	80.0
32-36	5.3	to 11.5 [†]	152	103	69.5	104	82.0
48	1.6	-	162 ^x				
72	2.5	-	89 ^x				
96	2.0	-	40 ^x				

* Corrected for volume changes and background count

x Measured as undiluted mucin

† Diluted to 11.5ml. and 10ml. taken for dialysis.

Table 27. Activity of mucin fractions from cortisone treated animal after incorporation of $^{35}\text{SO}_4$.

Same fistula animal M.S.76 as previous expt. (cf. Table 26) injected 20mc. $\text{Na}_2^{35}\text{SO}_4$.

Mucin diluted to 22.5ml, except where stated, and 20 ml. dialysed for 120 hrs.

Activities in counts/min/0.1ml. (average of duplicate samples)

Hours after injection	Vol. (ml.)	Vol. taken (ml.)	Initial Activity dil.mucin	Dialysed mucin Act.	% initial activity *	Ba ⁺⁺ pptd. dil.mucin Residual Act.	% initial activity *
Control	2.5	1	13	14	-	13	-
+ $^{35}\text{SO}_4$		1	1720	36	1.5	27	1.0
1	2.75	1	421	44	7.8	107	26.8
2	1.4	1	602	86	12.5	124	23.2
3	0.8	0.72	321	87	24.1	112	40.3
4	1.7	1	421	142	31.8	152	42.5
5	1.5	1	266	110	40.0	103	44.2
6	1.2	1	190	78	6.8	85	50.8
7	2.0	1	118	71	55.2	65	61.8
8	1.1	1	110	59	47.5	64	65.7
9	1.1	1	103	57	50.6	59	63.2
10	1.5	1	65	45	61.6	37	60.0
11	0.75	0.75	53	42	76.2	38	78.0
12	0.5	0.5 [†]	65	39	55.0	42	72.6
25-28	4.8	5 [†]	118	84	68.5	93	93.5
30-36	8.5	7.5 ^{††}	103	63	55.6	73	83.4
48	1.4	-	117 ^x				

* Corrected for volume changes and background count

† Diluted to 12ml. and 10ml. taken for dialysis.

†† Diluted to 20ml. and 18ml. taken for dialysis.

x Measured as undiluted mucin.

ACTIVITY DILUTED MUCIN.
(COUNTS/MIN./0.1ml.)

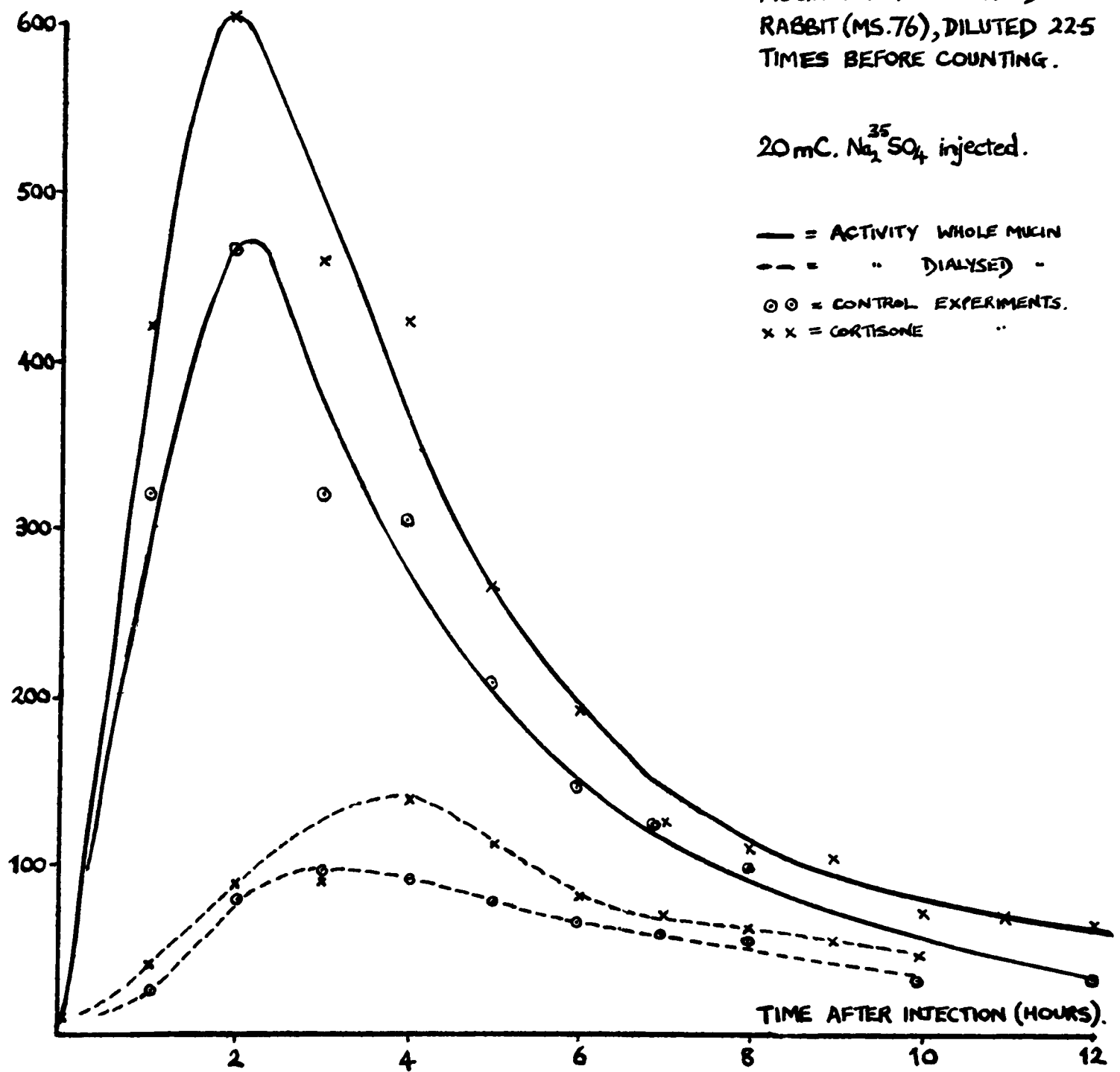


FIG.8: INFLUENCE OF CORTISONE ON SO_4^{35} INCORPORATION INTO MUCIN
AFTER ADMINISTRATION OF A MASSIVE DOSE OF $\text{Na}_2^{35}\text{SO}_4$.

These experiments, in which the activity of fistula mucin was measured at different time intervals (figs. 7 + 8) indicate that cortisone has no significant effect on the incorporation of ^{35}S into the mucin. This is in agreement with the results of the acute experiments, (fig. 6).

Radioactive components of mucin after $^{35}\text{SO}_4$ administration.

In some of the control (i.e., non cortisone) and the cortisone experiments in which $\text{Na}_2^{35}\text{SO}_4$ was administered to both fistulated animals as well as those submitted to the acute experiments, there was evidence of a second maximum in the activity/time curve. The first and large maximum regularly occurred at 1-2 hours after injection of the $\text{Na}_2^{35}\text{SO}_4$ and corresponded chiefly to the presence of dialysable ^{35}S . The second maximum found at a time of $4\frac{1}{2}$ -5 hours (figs. 6 + 7) corresponds fairly closely to the maximum (at 4 hours) in the activity of the non-dialysable ^{35}S contained in duodenal mucin (see fig. 8).

This non-dialysable radioactivity was associated with an electrophoretically immobile component of the mucin. Prolonged electrophoresis (30 volts/cm, 8 hrs.) on paper at pH 4.0 and 8.6 taking all precautions to prevent denaturation and precipitation of the macromolecular mucin components, still resulted in no migration of the radioactivity. Under these conditions inorganic $^{34}\text{SO}_4^{2-}$ (in the presence of inactive mucin), heparin and chondroitin sulphates (including ^{35}S -labelled chondroitin sulphate isolated from bone and cartilage by Mr. L.M. Steddon) were freely mobile. The non dialysable radioactivity was also found to be immobile in these solvent systems which have been employed for the chromatography of sulphated and other acidic mucopolysaccharides, viz:- n.propanol-phosphate buffer, 0.1M pH 6.8 (Kerby, 1953) and tert.butanol-lutidine (Consden + Bird, 1955). Heparin, chondroitin sulphate and inorganic $^{35}\text{SO}_4$ are all mobile in these solvent systems. Precipitation

of the dialysed mucin with excess acetone, and denaturation (and separation) of the bulk of the protein by shaking with an amylalcohol - chloroform (1:3:5) mixture, resulted in the simultaneous precipitation of the bulk of the carbohydrate present (as measured by the anthrone colour reaction) and of the radioactivity. The increasing ratio of this electrophoretically and chromatographically immobile radioactivity to the inorganic $^{35}\text{SO}_4$ present in the mucin, with time after injection, is indicated in Table 28 below.

Table 28. Electrophoresis and chromatography of some mucin fractions.

Mucin from fistulated animal (M.S.76) - non cortisone experiment.

Ascending chromatography in propanol-borate buffer.

$^{35}\text{SO}_4$ had R_F .85/.95, cf. heparin .9, chondroitin sulphate .3

Electrophoresis on paper sprayed with 0.1M borate buffer, pH 8.6 (5 $\frac{1}{2}$ hr.6volts/cm)

$^{35}\text{SO}_4$ migrated with max^B at 31.5cm, cf. heparin 11.5cm, chondroitin sulphate 10 cm.

Activities in counts/min. measured on dried papers.

Mucin fraction (hours)	<u>Chromatography</u>			<u>Electrophoresis</u>		
	Activity R_F .90	Activity R_F 0/0.5	(^{35}S) I.C.* $^{35}\text{SO}_4$	Activity 31.5cm.	Activity 0/1.0cm.	(^{35}S) I.C.* $^{35}\text{SO}_4$
2	200	26	.07	112	30	.18
4	220	60	.23	46	47	1.0
8	48	34	.60	18	26	2.3
32	26	42	2.2	14	51	2.0
Background		13			12	

Corrected for background counts.

* I.C. = immobile component.

Note. These figures do not represent total activity of inorganic sulphate and (^{35}S) macromolecular mucin components on the chromatograms and electrograms, since these activities given above are only those measured at the respective maxima. The ratios for (^{35}S) I.C./ $^{35}\text{SO}_4$ are not (quantitatively) comparable with each other nor the figures for the % residual activity after dialysis of the whole mucin (fig. 9).

Hydrolysis of the radioactive dialysed mucin samples with 1.75N hydrochloric acid in sealed tubes at 105° for 3 hours resulted in the liberation of over 90% of the radioactivity as $^{35}\text{SO}_4$ ". Table 29 records the activity of various dialysed mucin fractions and their activity after hydrolysis and precipitation of $^{35}\text{SO}_4$ " present by barium chloride, after carrier sodium sulphate had been added. The hydrochloric acid used to bring about hydrolysis was not neutralised before precipitation of the sulphate and since barium sulphate is slightly soluble in hydrochloric acid (Kolthoff + Sandell, 1950), it is probable that the small residual activities, after hydrolysis and treatment with barium, may represent some unprecipitated inorganic $^{35}\text{SO}_4$ " (cf. control experiment, table 39).

Table 29. Effect of acidic hydrolysis and barium precipitation of the hydrolysate on a) dialysed radioactive mucin b) residual activity of mucin after addition of barium chloride and carrier sulphate to whole mucin.

Hydrolysis with 1.75N HCl for 3 hours at 105°.

Counts made on 0.1ml. original (unhydrolysed) mucin samples = initial activity, and 0.2ml. barium precipitated hydrolysates (equivalent to 0.1ml. original samples) = final activity. Activities measured in counts/min.

Mucin samples from fistulated animal M.S.76 after injection 20mc. Na₂³⁵SO₄, diluted before dialysis and treatment with barium chloride and carrier sulphate (see Table 26).

Control = non-radioactive mucin and added ³⁵SO₄".

Fraction (time of Collection)	<u>Dialysed mucin</u>		<u>Ba⁺⁺ pptd. whole mucin</u>	
	Initial activity	Final Activity	Initial activity	Final activity
1st hour	117	23	26	12
2nd	204	25	81	17
3rd	196	29	97	18
4th	154	23	92	19
5th	112	24	72	16
6th	80	18	64	17
8th	64	17	54	17
10th	151	23	118	20
12th	70	21	55	19
24th	100	26	84	28
36th	105	26	103	25
control	2080	30	1665	25
Background		14		15

Though the amount of $^{35}\text{SO}_4$ bound in this macromolecular component(s) of mucin was greatest in the fractions collected 4 hours after intravenous administration of $\text{Na}_2^{35}\text{SO}_4$ (see fig.8, p. 95), the actual percentage of the total activity of the mucin present in the form of this non dialysable $^{35}\text{SO}_4$ -esterified mucosubstance(s) increased with time to a value of 70% after 12 hours (fig.9).

Determination of the residual activity of whole mucin after precipitation of the inorganic sulphate present with (excess) barium chloride and carrier sulphate, indicated that there was possibly a third ^{35}S -containing component in whole mucin in addition to the inorganic (or barium precipitable) sulphates and the $^{35}\text{SO}_4$ -esterified macromolecular component(s). This residual activity was in all cases greater than the non dialysable activity (fig. 9) and cortisone appeared to be without marked influence on the difference between these two activities. This consistent difference could not be attributed to incomplete precipitation of the inorganic $^{35}\text{SO}_4$ present in the mucin, since added $^{35}\text{SO}_4$ was almost completely (99%) removed. Furthermore, the conditions, due to the alkaline pH of whole mucin, etc., of counting after barium precipitation were such as to mask, if anything, this difference in activities because of β -particle absorption by the excess barium chloride (plated with the supernatant after barium precipitation). That this discrepancy was not due simply to corrections for the different volumes counted (due to addition of barium chloride and carrier sulphate) was shown by an examination of the concentrated dialysates. Concentration was effected in an air oven at 100° . There was an appreciable residual activity in these concentrates after precipitation of the inorganic $^{35}\text{SO}_4$ with barium chloride.

The term 'dialysate' used hereafter refers to the solution of substances which have diffused out of the dialysis sac, i.e., the liquid outside the sacs recovered after dialysis (diffusion) overnight (15 hrs.)

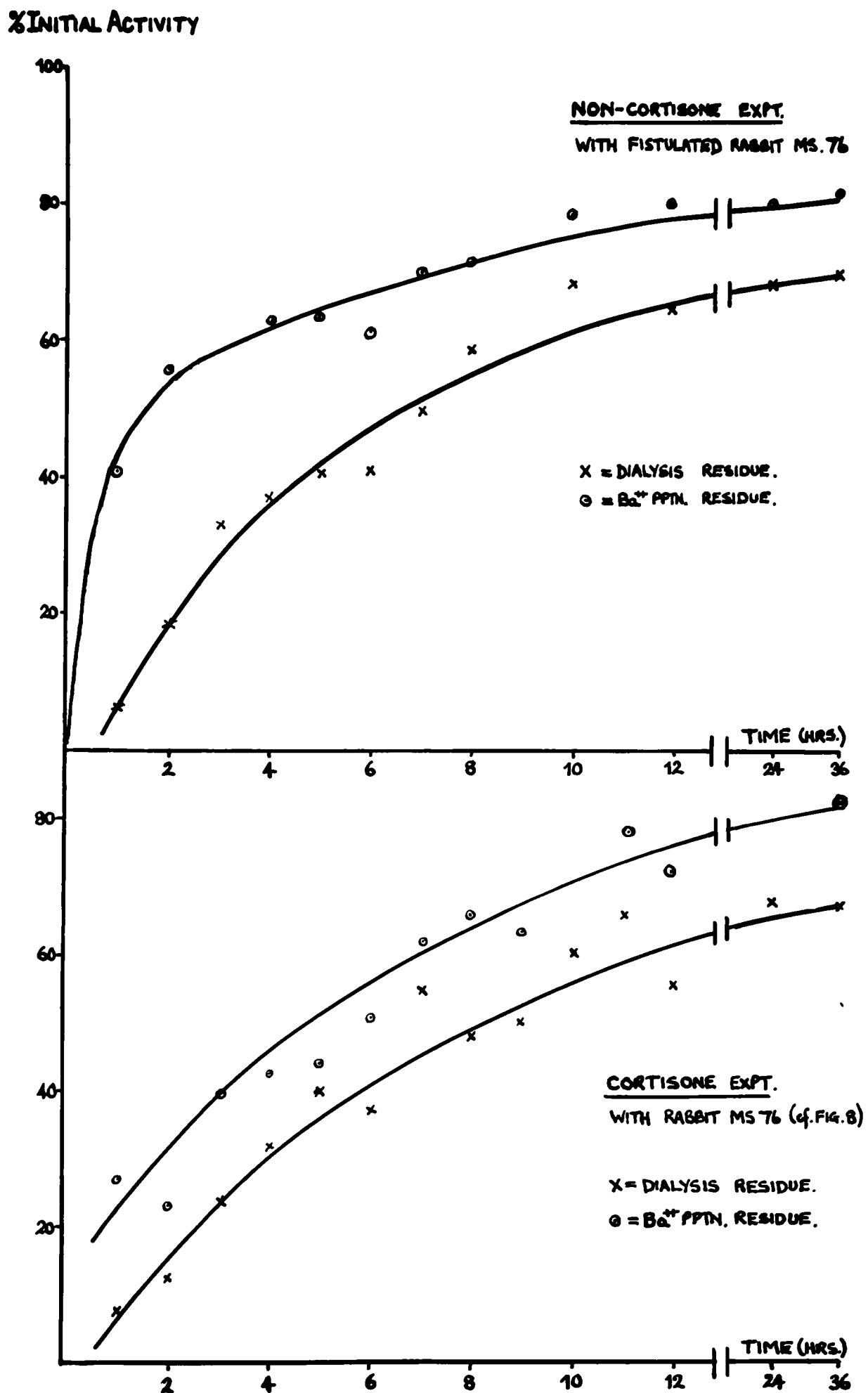


FIG. 9: RESIDUAL ACTIVITY OF MUCIN AFTER DIALYSIS AND Ba⁺⁺ PRECIPITATION.
(CORTISONE AND CONTROL EXPERIMENTS)

A typical concentrate (3ml.) from 180ml. of the original dialysate (2nd hour mucin sample) counted at 147 counts/min./0.1 ml. Non-radioactive sulphate was present (0.4mg./ml.) which had been added originally to the dialysing mucin to displace any inorganic $^{35}\text{SO}_4$ held in the mucin by salt formation, e.g., with basic protein. After treatment of 1ml. of this concentrate with 0.5 ml 1% barium chloride dihydrate (eqt. to 1.95mg. SO_4 , i.e., a 5-fold excess), 0.15ml. of the dried supernatant gave a count of 50 counts/min. Allowing for the background count (12 counts/min), this non barium-precipitable activity represents 28% of the radioactivity of the dialysate. Repetition of the barium precipitation did not decrease this figure. This particular mucin sample lost 81% initial activity after 110 hour dialysis so that the figure of 28% for the non precipitable activity of the dialysate concentrate actually represents 34.5% of the initial activity of this particular mucin fraction. This corresponds well to the figure of 37.4% obtained on subtracting the % non dialysable activity (18.4) from the % non barium-precipitable activity (55.8) of the whole mucin (cf. 2nd hr. fraction, Table 26).

Similarly for a concentrate (2ml.) of 100 ml. dialysate from another mucin fraction (4th hour sample), the percentage non precipitable activity was found to be 17.3% corresponding to 27.5% of the initial activity of the whole mucin. The difference in the % non barium-precipitable and % non dialysable activities for this mucin fraction was found to be 25% (Table 26).

Hydrolysis of the residual mucin samples and the dialysate concentrates after removal of inorganic $^{35}\text{SO}_4$ by barium precipitation indicated that the bulk of this residual activity was liberated as $^{35}\text{SO}_4$ after hydrolysis with 1.75N hydrochloric acid for 3 hours. $^{35}\text{SO}_4$ was identified as the hydrolysis product by its removal from solution by precipitation with barium chloride in the presence of added sodium sulphate (see Table 29, p.99). Hydrolysis for

5 hours followed by barium precipitation did not entirely remove the activity (residuum = 5 counts above background) but this is possibly due to the small solubility of the $\text{Ba } ^{35}\text{SO}_4$ in normal hydrochloric acid, as discussed on p. 98.

Attempts to further characterise this third radioactive component by chromatography and electrophoresis were not very successful due to the difficulty in counting (^{35}S) activities on paper, which correspond to less than 100 counts/min. when dried on a planchette. The absorption of the β radiation by the dried paper renders it difficult to locate feebly radioactive material after paper electrophoresis or paper chromatography, without recourse to use of a very sensitive detector or counting each small area of the paper for long intervals. It was shown that this third component was not taurine but it could not be obvi ated that it was cysteic acid, after chromatography of the dialysate in the amino acid chromatographic solvent systems, butanol-acetic acid-water and phenol saturated with water. In both these systems the radioactivity of the concentrated dialysate was found in the zone $R_F=0.0/0.1$ whilst $^{35}\text{SO}_4$ itself is immobile ($R_F 0.0/0.05$).

The presence of free inorganic ^{35}S sulphate in radioactive mucin samples was demonstrated by electrophoretic and chromatographic separation from other radioactive components, and loss of activity on precipitation with barium ions. Ester sulphates are only likewise precipitated in the presence of alcohol and it is unlikely that any ester sulphate would have an identical mobility with inorganic sulphate ions in a number of chromatographic systems, and after paper electrophoresis under conditions differing in pH etc. No evidence was found to suggest that the figure for % reduction in activity with barium precipitation represented anything other than the % inorganic sulphate in the various mucin samples.

Incorporation of methionine into duodenal mucin.

Preliminary experiments indicated that following the intravenous injection of 0.1 mC. DL ^{35}S -methionine, radioactive mucin was obtained from a fistula and that there was maximal incorporation of the radioactivity 4-4½ hours after injection of the labelled methionine. Table 30 records the variation in activity of the mucin (and blood) with time in one such experiment (see also fig. 10).

Table 30. Radioactivity mucin after injection DL- ^{35}S methionine.

Mucin obtained from fistulated rabbit M.S. 58.

0.1mC. methionine injected, equivalent to 10.5×10^6 counts/min.

Background = 19.31 counts/min. over period of experiment.

Hours after injection	Vol. mucin (ml)	Activity mucin c/m/0.1ml	Activity blood c/m/0.1ml	Hours after injection	Vol. mucin (ml.)	Activity mucin c/m/0.1ml.	Activity blood c/m/0.1ml.
-----------------------------	-----------------------	--------------------------------	--------------------------------	-----------------------------	------------------------	---------------------------------	---------------------------------

Control	1.5	27	164*	10	2.0	86	
1	1.6	41		11	2.5	83	
2	2.3	99	420	12	2.4	81	
3	2.0	120		24	2.0	45	298
4	2.0	119	487	30	2.0	43	288
5	2.3	102		48	3.3	39	307
6	1.9	73	444	72	2.9	39	360
7	2.3	95		96	2.5	37	226
8	3.0	93	424	145	2.0	43	299
9	2.0	94		200		33	264

* Residual activity from previous expt. 12 days before.

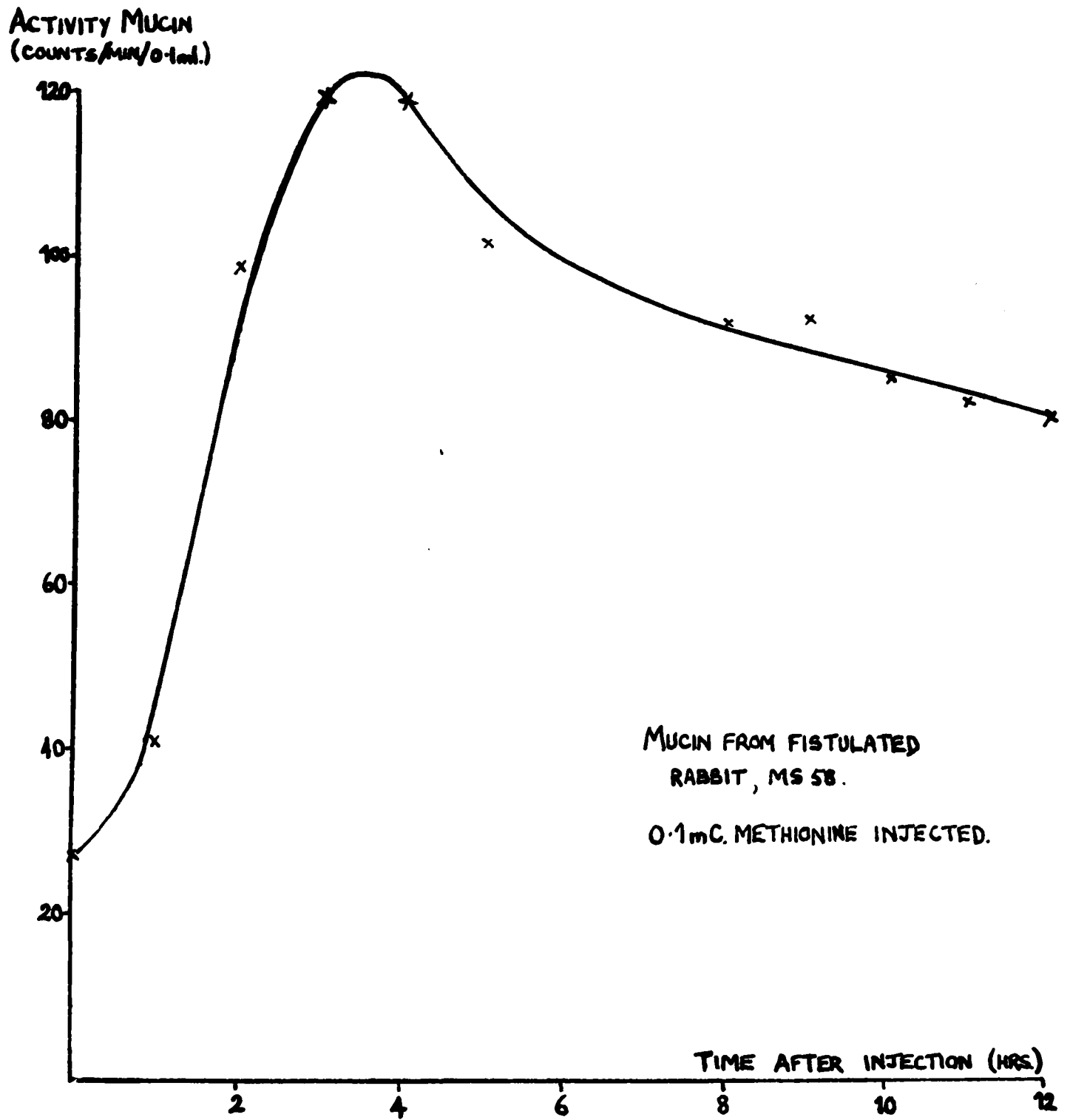


FIG.10: ACTIVITY OF MUCIN AFTER ADMINISTRATION OF ³⁵S-METHIONINE.

The blood and mucin activities were detectable over a much longer period of time (over 200 hours) than in the experiments in which $^{35}\text{SO}_4$ was injected into an animal. The high blood activity after the first few hours probably represents considerable incorporation of the labelled methionine into the serum proteins and the active circulation of ^{35}S compounds derived from the metabolism of methionine. In contrast to the blood and mucin activities, the activity of the urine fell much more sharply from a maximum of 2,000 counts/min./0.1ml. within four hours after administration of ^{35}S -methionine to a value of 65 counts/min./0.1ml. after 8 days. Examination of the urine activity by paper chromatography indicated that the bulk of the activity was due to two or three compounds other than methionine including inorganic sulphate, $^{35}\text{SO}_4$ appearing within 6 hours.

The maximum incorporation of ^{35}S into any one hourly mucin fraction appeared to be only 0.0002% of the activity initially injected as ^{35}S -methionine whilst the percentage of total activity contained within the combined fractions collected over the first 6 hours was only 0.0008%; cf. the figures of 0.0005% and 0.003 % respectively obtained in experiments in which 0.1mC. $\text{Na}_2^{35}\text{SO}_4$ was injected into the animals. Paper chromatography of the most active mucin fraction, obtained 4 hours after administration of the labelled methionine, in the butanol-acetic acid system revealed no detectable activity at R_F 0.35/.45 corresponding to free methionine. The activity appeared to reside wholly in an immobile spot R_F 0.0/0.06, i.e., whole protein or a polypeptide conjugate. An iodine positive-staining zone (protein) was found at R_F 0.0/0.06.

Hydrolysis of whole mucin followed by chromatography in butanol-acetic acid revealed the presence of at least 12 ninhydrin-reacting spots, including methionine, R_F 0.39 and cysteine, R_F 0.28. Hydrolysis of radioactive mucin gave a feebly radioactive zone at R_F 0.35.

Table 31. Incorporation of ^{35}S into mucin in acute experiments after administration DL ^{35}S -methionine and the influence of cortisone thereon.

Rabbit M.S.66 and cortisone-treated rabbit M.S.68 injected 0.1mc ^{35}S -methionine.

Injected activity equivalent to 10.1 ± 10^6 counts/min. on apparatus used.

Hours after injection	Cortisone expt. (M.S.66)		Cortisone expt. (M.S.68)	
	Vol. (ml.)	Activity mucin c/m/0.1ml.	Vol. (ml.)	Activity mucin c/m/0.1ml.
Control ½ hr.	2.9	15	4.0	24
½	3.1	33	2.9	24
1	3.5	53	3.1	42
1½	2.1	58	2.8	59
2	2.8	51	3.2	62
2½	3.2	52	3.0	59
3	3.5	49	2.8	60
3½	3.7	48	3.5	54
4	3.8	48	3.1	60
4½	3.5	43	2.6	45
5	3.8	37	2.9	40
5½	4.3	40	2.5	43
6	3.9	45	2.8	46
6½	3.2	46	3.0	41
7	3.3	44	2.2	51
7½	2.5	42	4.1	45
8	3.2	43	2.7	42

ACTIVITY OF MUCIN.
(COUNTS/MIN./0.1ml.)

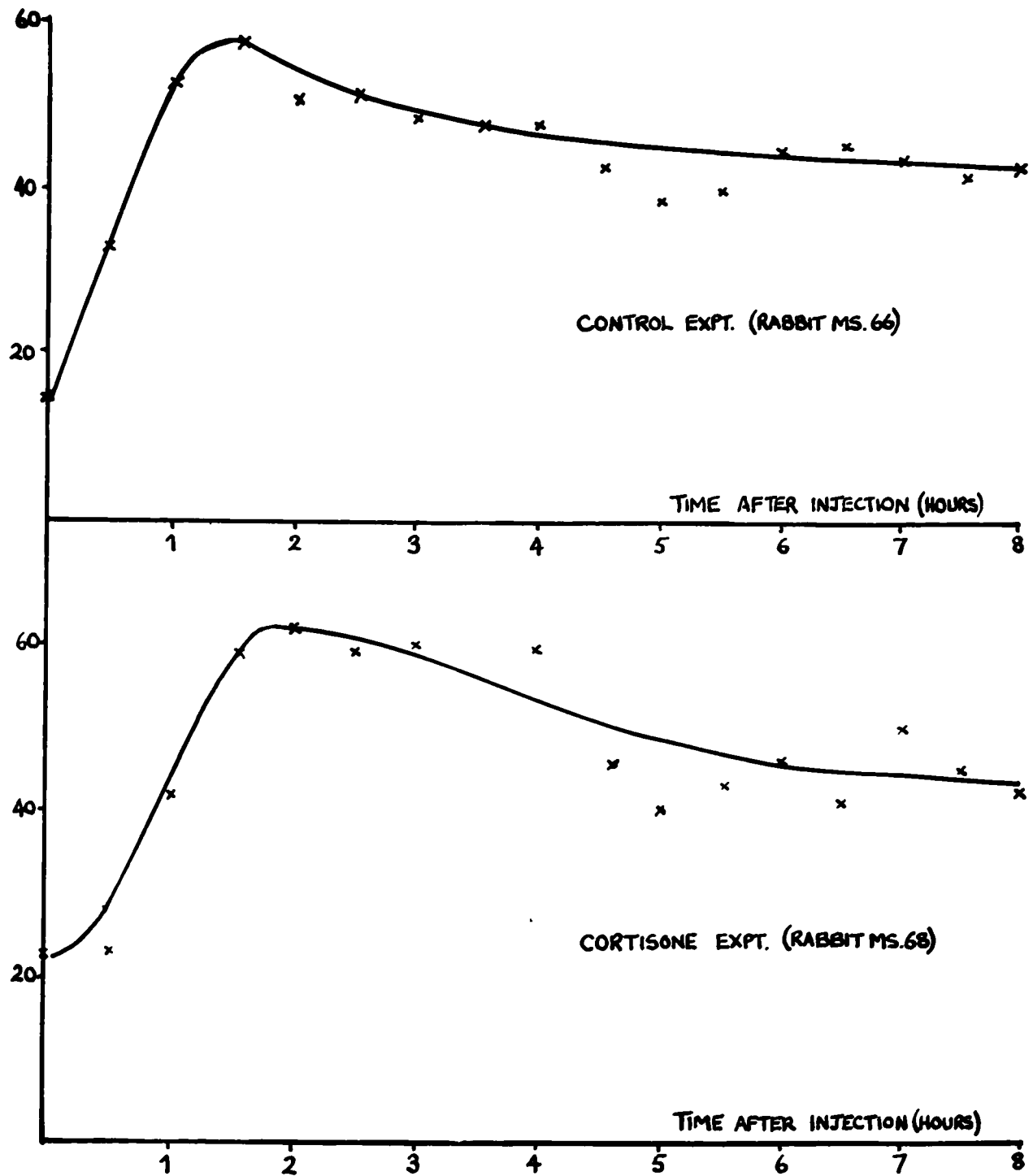


FIG. 11: INFLUENCE OF CORTISONE ON ³⁵S-METHIONINE INCORPORATION.
(ACUTE EXPERIMENTS)

In acute experiments, following the injection of ^{35}S -labelled methionine, a very low incorporation of activity was found with a maximum at $1\frac{1}{2}$ -2 hours. This (maximum) activity was not precipitated by barium ions nor appreciably diminished by dialysis for 3 days. As in the corresponding acute experiments with $^{35}\text{SO}_4$, rather more activity was found in the mucin elicited in acute experiments than that obtained from fistulae. Thus, in a typical acute experiment, the total activity present in the combined fractions obtained over the first 6 hours after injection of the ^{35}S -methionine was 0.0013% initial activity, cf. the figure of 0.0008% obtained from a fistula experiment after injection of the same amount of radioactive methionine.

Pretreatment of the animal with cortisone before submitting it to an acute experiment, appeared to be without effect on the rate and the level of ^{35}S -radioactivity incorporated into the mucin. (Table 31 and fig. 11).

Properties of rabbit intestinal mucin.

The mucin obtained from fistulae was opalescent, viscous and markedly alkaline, pH 9.5. In addition to its content of bicarbonate ions, chloride ion (ca. 0.05M), inorganic phosphate and inorganic sulphate were also present. On standing at room temperature for several days, the mucin lost its viscosity and opalescence and theropy filaments of mucous tended to pass into solution. Accordingly all samples were kept at $+2^\circ$ or -15°C .

On dialysis, there was a notable change in the physical and chemical properties of the mucin. After dialysis for 4 days, the pH fell to that of distilled water (ca. 5) and chloride, bicarbonate and sulphate ions were found to be absent. The dialysed mucin was far less viscous but contained stringy filaments of mucous which were not readily brought into solution by warming or shaking. The mucosubstances present in the mucin were more

readily precipitated by addition of ethanol, acetone, etc., from the whole mucin rather than from the dialysed samples. This was not due to change of pH alone since dialysed mucin made alkaline by the addition of barbitone or borate Buffers or sodium bicarbonate did not readily precipitate organic material with acetone or ethanol.

Addition of ammonium sulphate up to 30% failed to precipitate any protein or mucosubstances. Attempts to fractionally precipitate the mucosubstances from dialysed (i.e., salt free) mucin were without success. With ethanol, a turbidity was produced but further addition of ethanol did not facilitate precipitation. Glacial acetic acid rendered the dialysed mucin gelatinous and ethanolic precipitation of the gel gave a ropy product and removed the bulk of the organic matter from solution. Further addition of glacial acetic acid alone to the gel merely served to re-solubilise the partially precipitated material. Acidification with other acids (HCl, trichloroacetic) likewise rendered the clear mucin turbid but did not clearly precipitate the mucosubstances even on standing for several days.

Neither the whole mucin nor the dialysed material were specifically degraded by the proteolytic enzymes, pepsin, papain, chymotrypsin and trypsin. These enzymes were ~~not~~ without action on N-acetylglucosamine but readily degraded serum albumin. Under the conditions of incubation with pepsin at pH 1.5 there was some liberation of aminoacids but this was due to the action of the acid alone. Prolonged incubation at 37° in the presence of toluene and an antibiotic mixture (penicillin and streptomycin) led to marked solubilisation of the semi-insoluble mucosubstances, but this was a non specific effect being found in all the mucin controls. Attempts to remove preferentially ~~protein~~ ^{protein} by denaturation at an interface, on shaking with a chloroform amyl alcohol mixture (3.5:1), also removed the bulk of the carbohydrate and the

radioactive $^{35}\text{SO}_4$ incorporated into the non-dialysable components. The protein component(s) of the mucin was not readily stained with bromphenol blue making difficult all attempts to follow the separation of protein from other components by paper electrophoresis and chromatography. Testicular hyaluronidase was without depolymerase action on either the whole or dialysed mucin, as judged by retention of the opalescence and non-production of reducing sugars.

Freeze drying (lyophilisation) of the dialysed mucin yielded a somewhat deliquescent discoloured gum. Exhaustive drying of this gum gave a white fluffy solid material, which had lost almost all lyophilic properties in yields of only 1.0-1.5mg/ml. dialysed mucin. This material was only slightly soluble in water, even after prolonged shaking, in glacial acetic and (accompanying by some gel formation), phenol, pyridine and formic acid. It was insoluble in dioxan and β ethoxy^eethanol. On shaking, the material was soluble in 2% sodium carbonate and wet formamide. In all those solvents in which the material was only wetted or slightly soluble, it was observed that the white opaque particles gave gelatinous strands or transparent clumps of these filaments. Together with other observations, this would suggest that in the natural state, the mucosubstances are present in the mucin as sparingly soluble or even insoluble hydrated macromolecular structures or aggregates. In the cases of solution in formamide or aqueous sodium carbonate, addition of alcohol or acetone re-precipitated the material.

Though the proteolytic enzymes were freely soluble in formamide and 'formamide buffers' could be prepared by addition of varying proportions of sodium formate and 100% formic acid, no proteolytic digestion of either solubilised mucin or serum albumin could be obtained in dry or moist formamide solution.

Dr. N.G. Heatley (private communication) has found that neither the whole mucin nor the dried dialysed material possesses blood group activity.

Chemical composition of the isolated mucosubstances.

Some preliminary analyses were carried out on the (unfractionated) freeze dried material from rabbit intestinal mucin, as well as on dried preparations of the principal viscosity-raising components of pig duodenal and pyloric mucins. These latter, like the rabbit material, possessed no blood group activity (Dr. M.G. Macfarlane, private communication.)

After hydrolysis with N sulphuric acid and chromatography in butanol-acetic acid-water (4:1:5) and butanol-pyridine-water (3:1:1), the following monosaccharide components were identified.

Rabbit duodenal mucin:	Glucosamine, mannose, fucose and galactose
Pig duodenal mucin:	Glucosamine, galactosamine, fucose and faint spots associated with mannose and glucuronic acid.
Pig pyloric mucin:	Galactosamine, glucosamine, fucose, galactose, glucuronic acid and mannose(?)

No evidence of either a uronic acid or galactosamine could be found in hydrolysates of the rabbit duodenal mucin. Galactosamine and glucosamine were identified chromatographically by the production of lyxose and arabinose respectively after oxidative deamination as heating with ninhydrin (Blix, Svennerholm + Werner, 1950. Stoffyn + Jeanloz, 1954). Mannose, galactose and the 2-aminosugars were distinguished after chromatography in the butanol-pyridine system and paper electrophoresis in borate buffer, pH 8.6 (Conaden + Stanier, 1952). The presence of the uronic acid in the hydrolysates of the pig mucins is in agreement with the higher uronic acid content found in these mucin samples (see below) than in the rabbit mucin. Xylose was sometimes

also found on the chromatograms of these pig mucins, confirming the presence of glucuronic acid - the xylose arising by decarboxylation of the uronic acid during acidic hydrolysis.

Several aminoacids were also found to be present in all these mucin hydrolysates.

The presence of ester sulphate groups in these mucosubstances was indicated in the case of the rabbit mucin by the radioactive work with $^{35}\text{SO}_4$ described previously and the presence of inorganic sulphate in the hydrolysates.

The presence of 'sialic acid' (Kent + Whitehouse, 1955) in these mucin samples was detected by the direct colour reaction with the Ehrlich reagent, p. dimethylaminobenzaldehyde in ethanol:hydrochloric acid (1:1). This is also given by certain proteins, e.g., serum albumen, which contains the pyrrole aminoacids, proline and hydroxyproline, and may or may not contain sialic acid as well. It is difficult to distinguish this direct reaction for sialic acid from the typical pyrrole reaction with the Ehrlich reagent. Confirmation of the presence of sialic acid was found for the dialysed rabbit mucin with the production of the characteristic purple coloration on heating with diphenylamine in glacial acetic acid (Anderson + MacLagan, 1955. cf. Werner + Odin, 1952).

Some quantitative analyses on these mucin samples are recorded below:-

PPM = pig (J2) pyloric mucin

PDM = pig (J8) duodenal mucin, samples D5 and D9.

RDM = rabbit duodenal mucin, samples (i) and (ii).

	PPM	PDM(D5)	PDM(D9)	RDM(i)	RDM(ii)
2-aminosugars	16.7%	13.6%	12.5%	13.2%	12.7%
Non-aminosugar	(24.4)	15.0	21.1	10.3	12.7
(calculated as galactose)	(26.2)	16.3	20.5	11.3	13.5
Uronic acid	1.55		1.76		0.8
Sulphate	0.34	0.5	0.2	0.85	
Phosphate	-	0.75	0.6		
Nitrogen				12.5	

These figures serve to show that the overall composition of the mucin collected from ~~the~~ ^{the} one and same animal at different periods of time, may vary to a limited extent with the time of collection.

Use of ion exchange resins for acidic hydrolysis.

Attention was given to the use of these resins for hydrolysing the mucosubstances prior to identification of the monosaccharide components by paper chromatography and paper electrophoresis (Consden + Stanier, 1952). Theoretically, the use of these resins has the advantages that the hydrolysate does not require to be neutralised, the resin being conveniently removed by filtration, and also that aminoacids and aminosugars, which hinder the estimation and detection of the non-aminosugars in the hydrolysate, are removed by the resin.

Heating washed Dowex 50 (a sulphonated polystyrene) with distilled water resulted in the liberation of sulphuric acid and soluble brown products. Soluble mucosubstances were hydrolysed on heating at 105° for 5 hours. Thus the presence of fucose and mannose in rabbit duodenal mucin could be confirmed after chromatographic examination of the hydrolysate. Chondroitin sulphate was hydrolysed under these conditions to oligosaccharides, as judged by the aldose colour reaction given by spots of very low R_F values (<0.05), and to the monosaccharides, galactosamine, glucuronic, fucose(?) and a pentose; the latter being almost certainly xylose arising by decarboxylation of the uronic acid. In this respect, the use of ion exchange resins offers little advantage over the use of acid solutions for hydrolysis, since there is accompanying decomposition of uronic acids by either procedure. Insoluble material, e.g., chitin was hardly hydrolysed by the resin. Protein preparations, e.g., serum albumen, crystalline pepsin, were hydrolysed but required elution of the resin with hydrochloric acid

before the aminoacids could be detected. Both these proteins appeared to contain mannose and possibly galactose, which were detected after chromatography of the concentrated neutralised filtered hydrolysate.

The necessity for neutralising the resin hydrolysates before concentration and application to a chromatogram, gravely detracts from the (theoretical) advantages that the use of these resins has over normal acid hydrolytic procedures.

DISCUSSION

A mucosubstance (or mucocomplex) is defined as a biological product formed by associations between the major groups of biopolymers (Kent + Whitehouse, 1955). The modes of combination of these polymers range from stable chemical linkages, e.g., as in lipopolysaccharides, to absorption or ionic complexes of varying stability, e.g., chondroitin sulphate-chondroprotein complexes. Certain general distinctions may be drawn between the mammalian mucosubstances chiefly associated with epithelial sites or secreted into anatomical cavities and those of mesenchymal origin. The former serve as protective agents and biological lubricants whilst the latter play important roles in maintaining the structure of tissues, e.g., as ground substances. In addition to these functional differences (reflected in physical properties) between the two broad categories of epithelial and mesenchymal mucosubstances, marked differences exist in the chemical conditions of these two classes of mucocomplexes. Mucosubstances of mesenchymal origin are generally strongly acidic (metachromatic) containing uronic acid constituents and/or ester sulphate groups, e.g., heparin, keratosulphate, whilst those from epithelial sites (cyst fluids, gastrointestinal mucins) are much less acidic, highly hydrated and contain significant amounts of fucose, mannose and galactose.

Properties and composition of rabbit duodenal mucin.

By means of fistulae, it is possible to obtain a true epithelial mucin from the duodenum, uncontaminated by tissue components or by mucosubstances derived from other sections of the alimentary tract. Duodenal mucin is an opalescent viscous fluid containing appreciable amounts of inorganic constituents, notably bicarbonate and chloride ions, in addition to the viscosity-

raising agents (mucosubstances). The latter may be separated by dialysis and isolated by drying from the frozen state, in yields of 1-1.5mg./ml. native mucin.

With the removal of the inorganic constituents of the mucin, the contained mucosubstances tended to precipitate from solution as filaments. It is indeed doubtful whether these viscosity-raising agents can actually be considered to be in a state of true 'solution', even in the native mucin, since these macromolecular components may be separated by ultra-filtration on sintered glass filters (Heatley, 1954). Furthermore it is known that many mammalian mucins exhibit microscopic network structures. It is therefore suggested that these mucosubstances are instances of highly hydrated gels having essentially low solubilities. On drying from the frozen state, the mucosubstances were irreversibly denatured with loss of lyophilic properties (cf. Florey, 1955).

Attempts to bring these components into true solution by enzymic digestion, heating, shaking, etc., being unsuccessful, it was not found possible to apply classical fractionation procedures (electrophoresis, fractional precipitation, etc.), to separate the protein and mucopolysaccharide components. The resistance of the dialysed mucin to the action of the proteolytic enzymes, pepsin, papain, trypsin and chymotrypsin is not surprising in view of the function of the mucin to afford mechanical protection to the mucosa and lubricate the passage of foodstuffs in the presence of these digestive enzymes. Werner (1953) has described the successful application of proteolytic digestion procedures (Gardell, 1952) to separate mucopolysaccharide components from gastric and intestinal 'mucins' but since these 'mucins' were chiefly obtained by scraping the mucosae, it is more than probable that the

protein digested was extraneous material derived from the mucosal tissues (villi, etc.) rather than components of the mucin itself. Similarly, the present failure to separate a sulphated polysaccharide from the pyloric and duodenal mucins by electrophoresis is not surprising since such electrophoretically mobile substances, e.g., 'mucosin sulphate', have only been reported in those 'mucin' preparations (gastric and intestinal) obtained from mucosal scrapings (Werner, 1953) or whole minced mucosae (Zittle et al., 1948; Smith et al., 1952).

The incorporation of $^{35}\text{SO}_4$ into a non-dialysable component of the mucin and the low but significant sulphate content of the dried mucosubstances (0.85%) is in agreement with a number of autoradiographic studies (see Introduction) indicating that the mucous-secreting cells of the gastrointestinal tract exhibit an appreciable fixation of $^{35}\text{SO}_4$. This ester sulphate clearly does not correspond to a highly sulphated aminopolysaccharide, e.g., heparin or chondroitin sulphate, being present in small proportion ^{only,} since all attempts to separate the radioactivity from the bulk of the dialysed mucin, (by paper chromatography and electrophoresis under conditions in which the sulphated amino-polysaccharides were freely mobile) were without success. The use of procedures whereby chondroitin and heparin are separated from conjugated protein, likewise failed to dissociate with the $^{35}\text{SO}_4$ activity (or a fraction exhibiting the metachromatic properties of an acidic mucopolysaccharide) from the bulk of the dialysed mucin. That the $^{35}\text{SO}_4$ was ester-linked and not bound by ionic linkages was indicated by the non removal of the (residual) activity of the dialysed mucin after further dialysis in the presence of added excess inorganic sulphate. This procedure should displace the bulk of any $^{35}\text{SO}_4$ bound non specifically. Evidence that such binding could occur

was found with the considerable spreading or 'tracking' of the radioactivity regularly encountered after chromatography or electrophoresis of $^{35}\text{SO}_4$ in the presence of non-radioactive dialysed mucin.

Apart from the autoradiographic studies mentioned above, there has been no direct evidence that sulphated macromolecular components are present in epithelial mucins. The present observations that both inorganic sulphate and a dialysable sulphate ester are present in duodenal mucin in addition to the sulphated mucosubstance(s) indicate that $^{35}\text{SO}_4$ uptake by mucous-secreting glands is per se no indication that the mucins secreted contain sulphated macromolecular components. The finding of ester sulphate groups in the pig and rabbit duodenal mucosubstances suggests that partially sulphated mucosubstances may be more widely distributed than has hitherto been suspected. Previously, these acidic substances were considered to be associated only with mesenchymal sites in the animal organism (Kent + Whitehouse, 1955).

The composition of the aminopolysaccharide constituents of the duodenal mucosubstances appears to correspond to the serologically inactive 'fucmannan' described by Werner (1953) and isolated from both pig intestinal mucosal scrapings and the mucin obtained from a human ileal fistula. Werner did not however detect mannose amongst the monosaccharides liberated after acid hydrolysis. The finding of sialic acid in these mucosubstances is of interest. This is a new type of hydroxyamino compound characterised by its ability to give a 'direct' colour reaction with Ehrlich's reagent (in contrast to the 2-aminosugars) and other colour reactions with diphenylamine, orcinol etc, the constitution of which has still to be established (for full references, see Kent + Whitehouse, 1955). It is present in serum and urinary mucoproteins and many epithelial secretions, e.g., submaxillary and respiratory mucins, but has not yet been found in mesenchymal mucosubstances. It is not yet

known whether it is associated with the protein or the mucopolysaccharide components of these epithelial mucosubstances. Thus in their content of sialic acid as well as of galactose, fucose and mannose, these duodenal mucin components appear to reflect a general constitutional character which is associated with true epithelial secretions. The exceedingly low uronic acid content and the presence of these 3 neutral hexoses can no longer be regarded as typical of the epithelial mucosubstances alone (e.g., blood group carbohydrates of cyst fluids) since fucose, mannose and galactose have recently been found to be components of certain mesenchymal animal polysaccharides, which occur together with the highly sulphated aminopolysaccharides in reticular fibres (Glegg et al. 1953, Windrum et al. 1955) and the organic matrix of bone (Glegg + Rüdinger, 1955).

The variation in composition between pig and rabbit duodenal and pig pyloric mucosubstances (p.113), notably the much greater content of galactosamine in the pig mucins, is consistent with other differences found between the mucous secretions of different species. Thus in the rabbit and the goat, duodenal mucinogen (i.e., mucous gland contents) stains readily with mucicarmine but this is not so with most other laboratory animals (Jennings + Florey, 1955.)

The attempted use during these analytical studies of an acidic ion exchange resin (Dowex 50) to bring about the hydrolysis of mucosubstances is worth comment. Despite the makers claims that these polystyrene cross linked resins are indeed stable to heating up to 175° for several hours, considerable decomposition of the resin accompanied hydrolysis of the mucosubstances. The necessity of finally neutralising the liberated sulphuric acid largely detracted from the advantages to be gained by use of the resin. Dixon (1955) in a

recent paper has examined in some detail the use of various resins to effect the hydrolysis of ovomucoid. The long and tedious preliminary extraction of water soluble resin components described by Dixon in order to obtain satisfactory results with Dowex 50 and other resins, clearly confirms the present findings.

Biosynthesis of mucin components.

The regularity and continuity as well as the rate of overall biosynthesis was indicated by the continuous secretion of mucin into the duodenal fistula. These fistulae yielded 2-3 ml. mucin/hour which represents the synthesis of 3-4 mg. of the viscosity-raising components per hour by the cells of this isolated length of the duodenum. The appearance of ester bound $^{35}\text{SO}_4$ in the non-dialysable mucin fraction within 1 hour of administration of $\text{Na}_2^{35}\text{SO}_4$ (fig. 8, p.95) indicates the rapidity of the 'turnover' of the sulphate at least and possibly of the mucinogen, within the Brunner's glands. The high activity of the mucin within the first hour, due to the presence of free inorganic $^{35}\text{SO}_4$, a dialysable $^{35}\text{sulphate}$ ester, cannot be simply related to mucin production by the glands which synthesise mucinogen. These two latter radioactive components are probably excretion products but it is not known whether the glands which secrete the viscous mucin components, or other mucosal cells provide the actual pathway of excretion.

The finding of free inorganic sulphate in the mucin is perhaps rather surprising in view of the low level of sulphate in the animal body. However, the excretion of inorganic $^{35}\text{SO}_4$ was regularly found in all experiments with $\text{Na}_2^{35}\text{SO}_4$. Everett and Simmons (1952) in a study of the distribution and excretion of $^{35}\text{SO}_4$ in albino rats also found evidence that ^{35}S passes directly from the blood or tissues into the upper regions of the small intestine, but they did not distinguish between the free or combined

sulphate when measuring the radioactivity of the intestinal contents. No evidence was found that ^{any of} the radioactivity of the mucin within 12 hours after administration of ³⁵S-methionine, was due to the presence of $^{35}\text{SO}_4^{2-}$. Therefore it appears that any sulphate formed by metabolism of the methionine, is excreted through the kidneys and does not serve as a significant source of the sulphate incorporated into duodenal mucin.

The dialysable non barium precipitable sulphate fraction found as a third radioactive component of the mucin, following the administration of $\text{Na}_2^{35}\text{SO}_4$ to the animals, has not yet been identified. It is clear that it represents a simple ester sulphate, with inorganic $^{35}\text{SO}_4^{2-}$ being liberated on hydrolysis with 2N acid for 4 hours. Whether this represents an activated form of sulphate, such as might be required for sulphation of polysaccharides and sulphate conjugation of phenols, etc., cannot be decided. It is improbable however, that such an active-sulphate would be so readily excreted into the duodenum by the mucous-secreting cells, unless it were washed out along with the other cell contents during the actual process of extrusion of the mucin with accompanying cell rupture.

The intestinal tract is known to be the main extrahepatic site in the body for the sulphate conjugation of phenols (Williams, 1947). The utilization of $^{35}\text{SO}_4^{2-}$ administered parenterally, for the synthesis of other real sulphates has been amply demonstrated (Dwiewiatkowski, 1949b; Laidlaw + Young, 1952). It is therefore more than probable that this third radioactive component actually represents a phenol-sulphate fraction.

The total radioactivity of these $^{35}\text{SO}_4^{2-}$ fractions in duodenal mucin obtained over the first 6 hours after injection of $\text{Na}_2^{35}\text{SO}_4^{2-}$ amounted to no more than 0.1% of the total $^{35}\text{SO}_4^{2-}$ activity administered. It is clear that incorporation into mucin represents a very minor pathway for excretion

of sulphate from the body, for over the same 6 hour period 20% or more of the administered $^{35}\text{SO}_4$ was excreted in the urine. However a rough parallelism was found between the activity of the blood and the activity incorporated into the mucin (cf. Table 21, p.77).

The remarkable correspondence between the variation with time of the non-dialysable radioactivity of the duodenal mucin following the administration of both $\text{Na}_2^{35}\text{SO}_4$ and ^{35}S -methionine (figs. 8 and 10, pp. 95 and 105) with the maximum incorporation of activity at 4 hours in both cases would suggest that the ester (polysaccharide?) sulphate is associated with the methionine-containing mucoprotein in the mucin. The figure of 4 hours probably does not represent the actual time required for the synthesis of the mucosubstance de novo since there is evidence that ^{35}S -methionine exchanges with methionine already present in formed proteins which are rapidly labelled with the isotope (Gaitonde, Richter, 1955) and that sulphation may be one of the last processes in the overall biosynthesis of a sulphated polysaccharide. Meyer and his coworkers (1953) have isolated from bovine cornea a hitherto undescribed aminopolysaccharide, chondroitin, which in all respects, save degree of sulphation, possesses the constitution of a chondroitin sulphate. Since chondroitin sulphates are also present in bovine cornea, it is suggested that chondroitin is the precursor of these chondroitin sulphates, i.e., the carbohydrate skeleton is first formed and polymerised and only then, sulphated. Whether or not this is the case in the incorporation of $^{35}\text{SO}_4$ into the macromolecular component(s) of the mucin, it is clear that the rate of extrusion of the mucin is fairly rapid since non-dialysable ^{35}S is incorporated into the mucin within the first hour.

It is probable that the incorporation of ^{35}S as ester-sulphate requires the activation of the inorganic sulphate. The presence in liver of such

an activating enzyme for the enzymic conjugation of sulphate with phenols has been demonstrated (de Melo, Wizerkaniuk + Schreilman, 1955). Furthermore, the possibility that uridine coenzymes are involved the biosynthesis of mucopolysaccharides is suggested by the discovery of N-acetylamino sugar- and glucuronic acid-analogues (Storey and Button, 1955) of the uridine-diphosphate-glucose (galactose), originally described by Caputto et al. (1950), which functions as a coenzyme for the enzymic transformation of glucose to galactose. However the present system afforded no evidence concerning such intracellular processes.

Influence of cortisone.

The marked influence of corticoids and particularly of cortisone (Kendall's 'compound E', 17-hydroxy 11-dehydrocorticosterone) on condition of infection and inflammation is undoubtedly due in part to alterations in the nature of the ground substances of connective tissue and related mesenchymal mucosubstances. It is claimed that cortisone inhibits the production of (mesenchymal) mucopolysaccharides by altering the characteristics of mast cells (Asboe-Hansen, 1954). With cortisone administration there is an apparent diminution in the number of these cells with the accompanying loss of their granulation character, i.e., the property utilised for their detection (see below). It is also reported that the uptake of $^{35}\text{SO}_4$ by these cells as ascertained autoradiographically, is impaired by cortisone treatment (Asboe-Hansen, 1954) Boström, 1953) but it is very doubtful whether this finding reflects an actual depression in polysaccharide synthesis. Rather, it would seem to indicate a lowering of the rate of exchange of the $^{35}\text{SO}_4$ with the ester sulphate groups within these cells.

Asboe-Hansen (1954) has defined a mast cell as a "mesenchymal cell which in its cytoplasm contains granules and substances with mucopoly-

saccharide character". All other instances in which cortisone has been reported to impair the uptake of $^{35}\text{SO}_4$ " (see Introduction) have been associated with mesenchymal tissues e.g., costal cartilage slices (Boström + Mansson, 1953). The present investigation has however been concerned with the incorporation of ^{35}S into an epithelial mucosubstance and the site of this incorporation, the goblet cells and 'Brunner's glands', can by no means be classified as a mesenchymal tissue nor the description ('mast cells') given to these particular mucin-secreting cells.

In light of the considerable differences between mesenchymal and epithelial mucosubstances, some aspects of which have already been discussed, there is little justification for the facile assumption that cortisone would exert the same effects (i.e., on the exchange reactions between inorganic and esterified polymer-bound sulphate and possibly on the de novo synthesis of the mucosubstances) at epithelial sites, as has been demonstrated for mesenchymal tissues.

Thus the present findings that cortisone appears to exert no influence on the production of both the sulphated aminopolysaccharide and the protein constituents of duodenal mucin, as determined by the incorporation of $^{35}\text{SO}_4$ " and ^{35}S -methionine respectively, are no simple contradiction to the results reported by various authors working with mesenchymal mucosubstances. In support of these present findings, Jennings and Flory (1954) found that the administration of cortisone had no effect on the emptying of the colonic goblet cells in rats, cats and mice and there was no evidence to suggest that the mucin from the cortisone-treated animals differed histochemically or otherwise, from that obtained from non-cortisonised animals. Furthermore the rate of synthesis of the colonic mucin in the two groups of animals appeared to be identical. With the exception of the series of experiments

on a fistulated animal which was definitely suffering from progressive dehydration and wasting (M.S.65) and consequent reduction in the volume of mucin produced, there was no evidence in any of the present work that cortisone depressed the rate of production of duodenal mucin. Janowitz and co-workers (1953) have similarly found that the regeneration of mucin in the stomach after depletion by chemical irritation, was likewise unaffected by cortisone administration. The body of this evidence would seem to suggest that biosynthesis of the epithelial mucosubstances associated with the gastrointestinal tract is not under the direct influence of cortisone (or ACTH), and furthermore that in their differing response to the corticoid hormones, there is yet another distinction to be drawn between mesenchymal and the epithelial mucosubstances.

Preliminary communications concerning this work have already been given (Florey, 1955) and accepted for publication (Pasternak, Whitehouse + Kent, 1955).

SUMMARY

1. The incorporation of ^{35}S into rabbit duodenal mucin has been followed after intravenous injection of $\text{Na}_2^{35}\text{SO}_4$ and ^{35}S -methionine. ^{35}S -methionine was present in the mucin in the protein fraction in greatest amount 4 hours after administration.

The initial radioactivity of the mucin after injection of $\text{Na}^{35}\text{SO}_4$ was due to free inorganic $^{35}\text{SO}_4$, whilst after 4 hours there was maximum incorporation into a non dialysable fraction (sulphated mucosubstance). A third radioactive fraction, representing a dialysable ester sulphate which is not precipitated by barium ions, has been tentatively identified as a phenol sulphate (ethereal sulphate) in light of the known activity of the intestine, as the chief extrahepatic site for the detoxification of phenols by sulphate conjugation.

2. Cortisone, administered as the acetate, was without effect on the incorporation of ^{35}S -sulphate into this epithelial mucin, in contrast to its action on sulphate exchange and incorporation into mesenchymal mucopolysaccharides.

3. The composition of the carbohydrate component(s) of the mucin has been examined and compared with that of pig pyloric and duodenal mucins.

PART III

THE MICROESTIMATION OF FREE AND
ESTERIFIED SULPHATE

INTRODUCTION

As a first stage in the estimation of combined sulphur, the element is usually converted into ionic sulphate by vigorous oxidation. The inorganic sulphate is then estimated gravimetrically or volumetrically. A number of procedures are available for this oxidation, the reagent most commonly employed being hot concentrated nitric acid. Where however a compound contains esterified sulphate groups, oxidation is not longer essential and the sulphate may be liberated in the course of simple acidic hydrolysis.

The methods employed in the determination of ionic sulphate may be conveniently classified accordingly as they employ barium salts or benzidine (4,4' diamino-diphenyl), to bring about quantitative precipitation of the sulphate ions.

PROCEDURES INVOLVING THE USE OF BARIUM SALTS:

In the simple gravimetric estimation of sulphate, an excess of barium chloride is added and the washed and dried barium sulphate precipitate determined by weighing. The co-precipitation of carbonate and hydroxyl ions is prevented by conducting the precipitation under mildly acidic conditions. Small quantities of Agar-Agar or picric acid may be added to facilitate formation of a granular precipitate. For routine analyses, these gravimetric procedures have

been largely superceded by the more rapid methods involving direct titration of the sulphate against barium ions, using such indicators as rhodizonic acid or tetrahydroxyquinone (THQ).

Rhodizonic acid, a colorimetric reagent for barium ions, can only be used in the back-titration of an excess of barium but tetrahydroxyquinone is used for the direct titration of a barium salt with sulphate ions. In neither of these titrations is the end point very sharp, the accuracy attained being no greater than 1-3% on several mg. quantities of sulphate. Fritz and Freeland (1954) have recently described the use of Alizarin Red S (sodium alizarin - 3-sulphonate) functioning as an adsorption indicator, to give sharp end points in these titrations. These authors claim that by this means, 0.2 - 0.8 millimole samples of sulphate i.e. 20-80 mg. can be estimated with an accuracy of the order of 0.5%, which is equivalent to 5 μ g/mg. sulphate. A similar degree of accuracy is attained in titrating any excess barium ions, added to precipitate mg. quantities of sulphate, against ethylene diaminetetra-acetic acid (EDTA). EDTA is a powerful complexing agent (Complexone) for the alkaline earth metals and may be titrated directly with these using Solochrome-Black (Eriochrome Black-T) as an indicator. As an alternative procedure, the barium sulphate precipitate is dissolved in an ammoniacal solution of EDTA and the excess complexone then back-titrated with barium

or magnesium ions. (Belcher, Gibbons and West 1954. Sijderius 1954).

Various conductometric methods have also been given for following the direct titration of sulphate ions, (see for example Spillner & Voigt 1954. Paulson 1953) but whilst these allow rapid determinations, even using microburettes operated by a micrometer screw, not less than 300 μ g. of sulphate may be estimated with an error of + 1%. For the determination of microgram precipitates of barium sulphate, a number of nephelometric procedures have been described but these have not found general use due to difficulties of controlling conditions of temperature, contamination etc.

PROCEDURES EMPLOYING BENZIDINE AS A PRECIPITANT:

Benzidine is used extensively as a reagent in analytical chemistry (Welcher 1947) on account of (1) its great susceptibility to oxidation to give a brilliant blue meriquinonoid compound

(2) its ability to form complexes with metal salts

(3) its basic properties, particularly salt formation with strong mineral acids.

sulphate, selenate and tungstate ions are all quantitatively precipitated by the reagent under carefully defined conditions.

Chloride, phosphate and chromate ions are found to interfere in the quantitative determination of sulphate ions

(see for e.g. Cuthbertson & Tompsett, 1931), requiring any phosphate ions in particular, to be removed by prior treatment. Low concentrations of chloride ion (0.1M) can however be tolerated. This interfering effect of several ions is attributed to their property of effectively increasing the solubility of the precipitated benzidine sulphate. Fisher and Sprague (1951) have shown by electron microscopy studies that the incomplete precipitation of the benzidine sulphate in the presence of foreign ions is due, in part at least, to the prevention of proper crystal formation and growth. This is particularly apparent with chloride ions in concentrations greater than 0.2M.

Various investigators have found that the determination of sulphate using benzidine hydrochloride as the precipitant yields accurate results only if the precipitation is made from solutions with the pH carefully adjusted to the region 2.5-3.0. For example, Owen (1936) recommends precipitation from aqueous solutions at $\text{pH } 2.75 \pm 0.3$.

The precipitate of benzidine sulphate so obtained, is filtered, washed and estimated either by titration or colorimetrically (reviewed by Grant, 1939). Titration methods were favoured by the earlier workers who were determining mg. quantities of sulphate. Benzidine itself is neutral to phenolphthalein allowing the precipitated sulphate to be titrated directly (as sulphuric acid) against sodium hydroxide. Alternatively the benzidine may be

titrated with potassium permanganate, barium chloride and rhodizonic acid or potentiometrically, against potassium nitrite (diazo reaction) in the presence of hydrochloric acid (Keller & Munch, 1954). None of these titration methods is really suitable for the determination of microgram quantities of sulphate ion. One other method, which attempts to afford sufficient sensitivity without recourse to colorimetric procedures, deserves mention. Hoagland (1940) has described the estimation of quantities of the order 60-80 μg . sulphate with an error of 2% on determining the amount of carbon in the precipitate by combustion and measurement of the volume of carbon dioxide so produced.

Biochemists have developed a number of colorimetric procedures for the routine determination of both mg. and μg . quantities of sulphate as present in urine, blood and various biological extracts. By including acetic acid in the precipitation medium, the occlusion of excess benzidine on the precipitated benzidine sulphate is largely prevented. This occlusion can be a very considerable source of error, particularly when mineral acids are present, and render these sensitive colorimetric methods almost valueless for the estimation of μg . quantities of the sulphate ion.

Procedures for the colorimetric determination of the precipitated benzidine include diazotisation and coupling in alkaline solution with phenols (Kahn & Lieboff, 1928), thymol (Cuthbertson & Tompsett, 1931) or an aromatic amine

(Klein, 1944) oxidation with ferric chloride and hydrogen peroxide (Hubbard, 1930) and the use of the Folin amine/ aminoacid reagent, sodium naphthoquinone-4-sulphonate (Letonoff & Reinhold, 1936). The overall sensitivity of all these colorimetric methods rests however on the exactness with which the precipitate can be separated from the precipitation mixture and all excess benzidine removed by washing.

Klein (1944) reported that 10-150 μg . quantities of sulphate could be estimated following the quantitative separation of the precipitated benzidine sulphate by centrifugation in specially drawn out centrifuge tubes, whilst Dodgson and Spencer (1953) found that the centrifugation of these microprecipitates was greatly facilitated by the addition of powdered glass. Even with extremely careful manipulation, the latter authors could only directly estimate quantities of sulphate greater than 10 μg . smaller amounts being determined after addition of a known quantity (10-20 μg .) of sulphate. They also stated that amounts of sulphate less than 10 μg . were not quantitatively precipitated by benzidine, under conditions employing trichloroacetic acid at pH 2.0.

Thus it appears that of the methods available, none was really suitable or sufficiently sensitive for the estimation of quantities of sulphate (both free and bound in sulphated mucosubstances) of the order 2-20 μg . which are present

in small volumes of biological secretions e.g. mucins of the gastro-intestinal tract. Since these analyses were required for the evaluation of the specific activities of various radioactive mucin fractions, the volumes of which were only 0.3-1.0 ml., it became necessary to devise a method of sulphate analysis of sufficient sensitivity, yet capable of being used for a large number of routine determinations. This part of the thesis describes the development of such ^a method, capable of estimating as little as 2 μ g. sulphate to within an (overall) accuracy of 10%.

A nephelometric method was considered unsuitable on account of the relatively large amounts of organic matter present in mucin hydrolysates, which might be co-precipitated with either the barium or benzidine reagents and otherwise interfere. Attention was therefore directed towards a colorimetric procedure following benzidine precipitation, as being the most likely type to offer the requisite sensitivity.

EXPERIMENTAL METHODS AND RESULTS.

A.R. Reagents were used, except where otherwise specified. Benzidine was twice recrystallised from ethanol after decolourising with charcoal and ethanol.

The ethanol used was the commercial azeotropic grade (97%). Distilled water was obtained from 'Manesty' still. On concentration, it failed to give any precipitate with the benzidine reagent.

Only Pyrex glassware was employed, being regularly cleaned in A.R. concentrated nitric acid and repeatedly rinsed with distilled water.

pH determinations were made at 15° with a Cambridge pH-meter.

Colorimetric estimation of benzidine.

cf. Dodgson and Spencer (1953)

Recrystallised benzidine (335 mg.) was dissolved in the required amount of hydrochloric acid (200 ml., 0.185N) to give a solution of benzidine hydrochloride (467 mg.) This was diluted 50-fold to give a stock solution $\equiv 46.7 \mu\text{g./l ml.}$ which was kept at + 20°C.

Various volumes of the stock solution were taken and diluted with water to 2 ml. before diazotisation. 1 ml. 0.1% sodium nitrate was added, the mixture shaken well, and after 3 minutes, 5 ml. of a 0.5% (w/v) solution of thymol in 2N sodium hydroxide, added.

The diazotisation could be carried out at room temperatures.

The brilliant red coloration of diphenyl-tetrazothymol developed instantaneously and was stable for several hours. The colour densities were measured against a reagent blank in 2.5 cm. cells using a direct-reading Hilger 'Spekker' photoelectric absorptiometer with Ilford 603 filters (maximum absorption at 485 $m\mu$).

A linear relationship was found for colour density, plotted against amount of benzidine present. (Table 32 and fig. 12)

Table 32: Colorimetric estimation of benzidine:

Solution benzidine hydrochloride = $46.7 \mu\text{g./ml.}$ equivalent
to $17.5 \mu\text{g. sulphate/ml.}$

Vol. taken	Equivalent to	'Spekker' readings	
0.1 ml.	$1.75 \mu\text{g. SO}_4$	.668	.072
0.2 "	3.5 "	.134	.134
0.3 "	5.25 "	.206	.204
0.6 "	10.5 "	.410	.412
1.0 "	17.5 "	.720	.720
1.5 "	26.25 "	1.050	1.052
2.0 "	35.0 "	.705	.710

OPTICAL DENSITY
(SPEKKER UNITS)

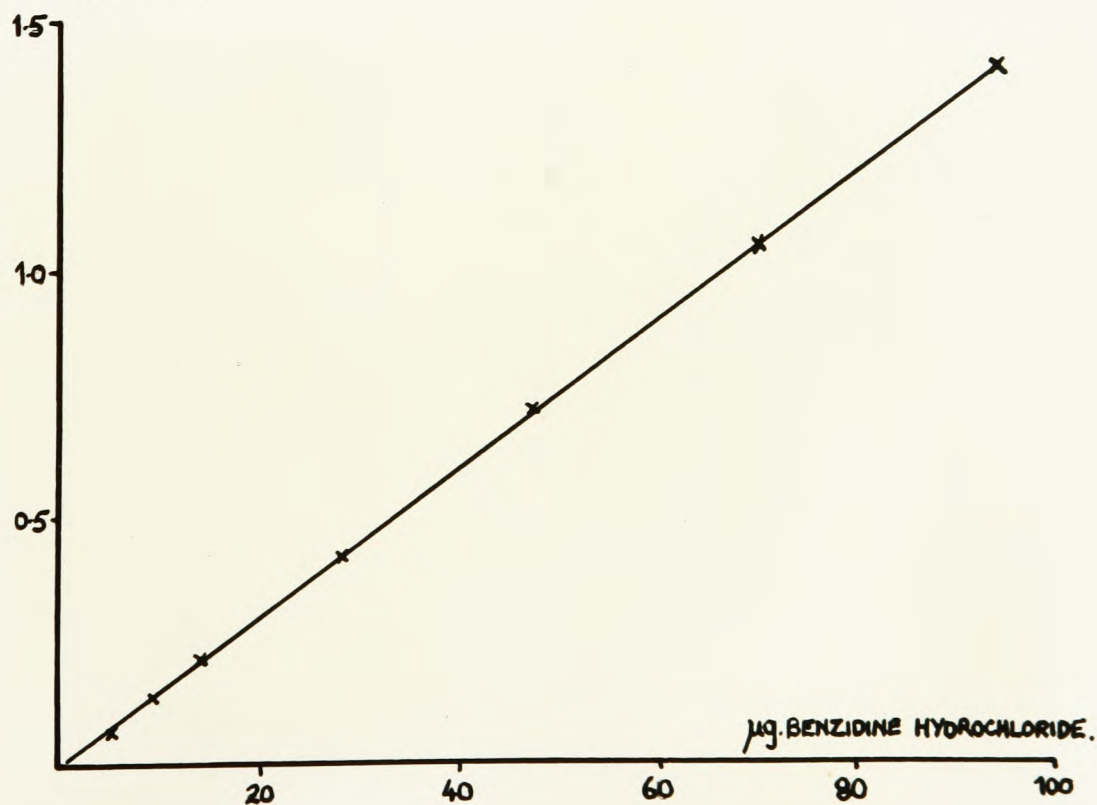


FIG.12: THE COLORIMETRIC ESTIMATION OF BENZIDINE.

Attempted Separation of benzidine sulphate by centrifugation.

Attempts were made to follow the colorimetric methods for the estimation of $10-75 \mu\text{g}$. given by Klein (1944) and Dodgson and Spencer (1953). These authors directed attention to the need for careful separation by centrifuging, of the microprecipitates from the precipitation medium, and after removal of excess benzidine by washing. Klein recommended the use of drawn-out (tapering) centrifuge tubes whilst Dodgson and Spencer advocated centrifugation in normal wide (15 ml.) tubes with the addition of small quantities (3.5 mg.) of powdered glass to assist the "packing down" of the precipitate.

Both these procedures were found to be distinctly unsatisfactory for the separation of $75 \mu\text{g}$. benzidine sulphate (from $25 \mu\text{g}$. sulphate) and smaller quantities. Excess benzidine could not be completely removed by washing due to formations of small air locks in the drawn-out tubes. Addition of powdered glass to facilitate centrifugation in normal tubes in our hands, did not prevent the breaking away of the precipitate from the walls of the tubes during removal of the washings by decantation. Loss of precipitate in this manner, or alternatively failure to remove the excess benzidine by washing and decantation, invalidated these (centrifugation) methods for extension to the determination of less than $10 \mu\text{g}$ quantities of sulphate ion.

Some attempts were made to coprecipitate the benzidine sulphate with starch and serum albumen, the latter serving as binders of the precipitate during the washing procedures. The benzidine sulphate was precipitated satisfactorily from aqueous solution together with starch (50 mg.) upon addition of acetone or alcohol. Unfortunately the starch precipitate absorbed considerable amounts of benzidine, as measured by obtaining diazotised benzidine coloration of optical density far greater than 1.05 Spekker units (see table 32) after washing and redissolving the precipitate from 25 μ g. sulphate ions.

These procedures, involving separation of precipitated benzidine sulphate by centrifugation, proving unsatisfactory, attention was therefore directed towards the use of filtration methods, for quantitative separation of the microprecipitates from the large excess of benzidine required for complete precipitation of the sulphate ions.

Preparation and use of immersion filters.

It was clearly undesirable in view of the minute precipitates obtained from 5-10 μ g. sulphate ions, to employ a method of filtration involving the transfer of these microprecipitates (together with the precipitation medium) on to a filter. The difficulties of ensuring a quantitative transfer of the precipitate from the vessel containing the precipitation mixture to the filter, suggested that the

filtration be actually carried out in this vessel. For this purpose, Professor Carl Emich of Graz had introduced in the late 1930s the use of immersion filters, also known as 'filter sticks'. These filters consist essentially of slender tubes, at the base of which is inserted a filter plate. On introduction into the precipitation mixture, the whole supernatant may be removed through the filter by gentle suction up the tube, whilst the required precipitate is retained on the filter plate or the walls of the vessel. When used in reverse, ie. pressure is applied down the tubes in place of suction, these filters serve as gas distribution tubes for aerating liquid media etc.

The manufacture of some filters was undertaken by Mr. H. Vincent (Department of Biochemistry, Oxford), allowing scope for experimentation to determine the porosity of the filters, which would retain the microprecipitates over a period of several washings, yet allow fairly rapid filtrations. Finally grade 3 sintered glass plates were chosen, being especially selected for evenness of porosity. These were cut and ground to 9 mm. diameter and sealed into hard glass tubes (7 X 200 mm.) drawn out to receive the plates. The plates were set back 1.5-2 mm. from the ends of these tubes.

Before use every tube was rigorously tested as follows:- A slow stream of air was gently drawn through the filters by

suction, after the actual tube of the immersion filter had been partially filled with industrial spirit. An even bubbling indicated no large site of leak and a uniform porosity. The overall porosity was roughly gauged by the rate of flow through the filter when the liquid was allowed to drain under gravity. Finally all the filters which had proved satisfactory in these arbitrary tests were individually marked and employed simultaneously in the determination of a standard amount of sulphate (usually 25 μ g.) under fixed conditions. In several such determinations it was generally noted that precipitates of the benzidine sulphate which gave weaker colorations than the average in the final colorimetric estimations of the precipitated benzidine, had been filtered through certain of these filters. These were discarded. Once the filter had been found to be reliable, it could be kept in constant use for 3 months or more. However, even the best filters gradually developed leaks with time and had to be discarded eventually. Those filters in common use were again tested at least once every three or four weeks.

A slow steady rate of filtration was maintained by adjusting the suction from a Bunsen (water) pump. The rate was never allowed to exceed 4 ml./min. The initial filtrations in which the precipitates were brought into contact with the filter were always at a much slower rate than the subsequent washings.

When not in use, the filters were kept immersed in dilute hydrochloric acid. They were repeatedly rinsed with distilled water and ethanol before use again.

Conditions for precipitation of the sulphate ions.

A survey of the literature (see Introduction) indicates that optimum conditions for precipitation of benzidine Sulphate include the control of the pH and the use of acetic acid to minimise occlusion of excess benzidine on the precipitate. In addition to the effect of pH, the concentration of benzidine was found to be critical for the reproducible and maximal precipitation of 2-10 μ g. sulphate ions.

(1) Effect of pH:
1936

Owen¹ found that conditions for the optimum precipitation of mg. quantities of sulphate included control of the pH at 2.75 ± 0.3 . The method given by Dodgson and Spencer (1953) for the estimation of 10-75 μ g. Sulphate involved the use of 20% trichloroacetic acid. The pH of the precipitation medium described by these authors was measured and regularly found to be within the range 2.0-2.2. Using this method, (but separating the precipitate by filtration and not by centrifugation), consistently lower optical densities were obtained in the final estimation of the precipitated benzidine than those obtained from simultaneous determinations made on the same amounts of sulphate ion precipitated at higher pH's, when the trichloroacetic acid was replaced by

10% and 25% formic acid.

The method finally developed, employed ~~an~~ glacial acetic acid and 25% formic acid in the presence of ethanol and acetone. The pH of this precipitation medium was 2.6-2.7 and did not appreciably change on standing at +2°C (ie. no appreciable esterification of the ethanol). Without the formic acid, the pH of the mixture was 2.8-3.0,

(ii) Effect of benzidine concentration:

Dodgson and Spencer (1953) precipitated microgram quantities of sulphate with 50mg. benzidine (5 ml. of a 1% solution in ethanol). Even in the presence of acetic acid to minimise occlusion, considerable contamination of the precipitate was suffered using this amount of benzidine, as evidenced by the appreciable optical densities, (0.10-0.40 'Spekker' units) finally obtained after carrying out the whole procedure for estimation of sulphate on sulphate-free distilled water samples. This was only partly improved by ~~an~~ further washing of the precipitate. It was found that the quantity of benzidine required for precipitation of 25 μ g. sulphate could be reduced 10 fold, ie. taking 1ml. of a 0.5% solution in ethanol. This corresponds to 5mg. benzidine per determination and represents a 100 fold excess for the precipitation of 25 μ g. sulphate, [184 μ g. benzidine being equivalent to 96 μ g. sulphate ion]. Further reduction of the amount of benzidine (to 1mg. and less) definitely improved the formation of a granular precipitate suitable for

filtration. With the reduced excess of benzidine and extension of the washing procedures, contamination of the precipitate was effectively prevented. Distilled-water blanks consistently gave final colour densities in the range 0.025-0.050 as measured on the Spekker photoelectric absorption meter.

(iii) Effect of standing:

After mixing the reagents, an immediate turbidity was apparent if sulphate was present in quantities of 5 μ g. or greater. After standing for 4-6 hours a (suspended) precipitate was obtained from sulphate in amounts of 20 μ g. For smaller quantities of sulphate, it was necessary to stand the precipitation mixture considerably longer for a coherent precipitate to form. On cooling to +2° and standing, a more granular precipitate was usually obtained. Accordingly, the very fine precipitate formed on mixing the reagents was allowed to coagulate by standing for at least 12 hours at +2°C. ^{To minimise} evaporation of the reagent mixture over this period, (to leave a 'crust' of relatively insoluble benzidine which could be a considerable source of interference), the precipitation was carried out in narrow test tubes which were then tightly corked and stood overnight in the refrigerator. The precipitation was regularly carried out in the presence of an ethanol-acetic acid mixture which served to raise the pH slightly, considerably depress the solubility of the

benzidine sulphate and minimise precipitation of the free benzidine.

Manipulation of the precipitate.

After standing at $+2^{\circ}$ overnight, the precipitate was usually sufficiently granular to allow filtration through porosity-3 filters. When the precipitate was still too fine, standing for a further period at $+2^{\circ}\text{C}$ always eventually resulted in the formation of a coherent precipitate.

After the filtration of the precipitate from the reagent-mixture, the precipitate and all the surface of the glassware which had been in contact with the benzidine reagent was washed with an acetone-ethanol (1:1) mixture in 10ml. portions and the washings removed by filtration. At least two such washings were necessary to remove all excess benzidine and give satisfactory blanks with distilled water. Therefore a threefold washing of every precipitate and filter was incorporated into the method as finally developed for the microestimation of sulphate ions. As a precaution against re-solution of the precipitate during these washings, the acetone-ethanol mixture was cooled in ice-water to keep the temperature less than 5° .

The thrice-washed precipitate was dissolved in dilute hydrochloric acid (0.6N). The volume of the acid taken (5ml.)

was such as to adequately cover all surfaces of the glassware which had been in contact with the original precipitation mixture and might carry traces of the precipitate. The acid was added down the centre of the filter to ensure extraction of all benzidine sulphate trapped in the body of the sintered glass filter plates. After standing for 45 minutes, the filters were drained by gentle application of pressure (from an air compressor) and removed. The solution of benzidine in the hydrochloric acid was then dissolved and estimated colorimetrically as described on p. 134 .

Hydrolysis of sulphated mucosubstances.

The use of hydrochloric acid for hydrolysis was rigorously avoided as it is well known that chloride ions constitute a source of interference in the determination of sulphate by benzidine precipitation (see Introduction). Concentrated solutions of trichloroacetic acid brought about the hydrolysis of mucosubstances but this was accompanied by partial separation of the hydrolysate into two immiscible layers on cooling.

In attempts to use N nitric acid (freed from nitrous acid by pretreatment with urea) and 10% perchloric acid, it was found that carbonisation of both carbohydrates and proteins occurred and that cysteine and methionine suffered oxidation. Both these aminoacids are present in the mucoprotein constituents of gastric and intestinal mucins (see p. 106) and are therefore always a potential source of

interference in the estimation of the sulphate contents of whole mucins. The oxidation of these sulphur-containing aminoacids could be followed chromatographically. The products of oxidation of cysteine gave a heavy precipitate with the benzidine reagent.

25% (v/v) formic acid was eventually found to be not only a suitable hydrolytic agent but also did not interfere with the subsequent determination of the sulphate ions in the hydrolysate. Accordingly the hydrolysate containing 25% formic acid could be incorporated directly into the precipitation mixture to minimise losses due to transfer, neutralisation etc.,.

Measured volumes of the mucin to be analysed (0.3-0.8ml.) and an equal volume of 50% formic acid were sealed in hard glass tubes, which had been subjected to rigorous washing to remove traces of sulphate and heated for 16 hours at 105°C. Under these conditions, starch and dextran sulphate were completely hydrolysed to glucose, and serum albumen to the constituent aminoacids, as detected by paper chromatography. The hydrolysis of polysaccharides, and whole mucins was generally accompanied by some browning and formation of insoluble humin. The latter was removed on filtering the hydrolysate through small sintered glass filters (porosity-2). The filters were washed with small volumes of 25% formic acid. Aliquots of the combined filtrates and washings were taken, made up to 1.0 ml. in

volume with 25% formic acid and submitted to the procedure for precipitation of all the (free) sulphate present. All the determinations of sulphate content were performed in duplicate.

Applications of the method.

This method for sulphate determination, the development of which has already been described and which is summarised in the appendix, p. 160, was rigorously tested by the following means:

(a) Applications to solutions of K_2SO_4 in 25% formic acid containing from 2 to 30 μg . sulphate and comparison with the colour developments from known solutions of benzidine hydrochloride.

(b) Hydrolysis and subsequent determination of the sulphate content of sulphated mucopolysaccharides, the sulphur content of which was known from independent determinations.

(c) Submitting (radioactive) $Na_2^{35}SO_4$ and chondroitin (^{35}S -labelled)-sulphate to the conditions of hydrolysis and sulphate precipitation and measuring the residual radioactivity and the activity of the final diazotised solutions.

(a) A.R. Potassium sulphate was exhaustively dried at $60^{\circ}C$. under reduced pressure before weighing. 182.5 mg.

K_2SO_4 in 100 ml. water gave a stock solution $\equiv 1000 \mu g.$ sulphate/ml. which was diluted initially with water and then the final dilutions made with 50% formic acid. The final solutions submitted to analysis contained 2-30 $\mu g.$ sulphate in 25% formic acid.

A linear calibration curve was obtained when the final colour density was plotted against total sulphate present. Some figures for a typical run are given below (table 33) and plotted in fig. 13.

Table 33: Calibration of the method for 0-20 $\mu g.$ sulphate.

Amount Sulphate	'Spekker' readings.		
0.0* $\mu g.$	.035	.040	.025
2.5 "	.156	.140	
5.0 "	.260	.215	.232
10.0 "	.460	.430	.442
12.5 "	.535	.545	
15.0 "	.624	.652	
20.0 "	.830	.850	
Benzidine standard equivalent to 17.5 $\mu g.$ SO_4	.715	.705	.695

* ie. 25% formic acid blank

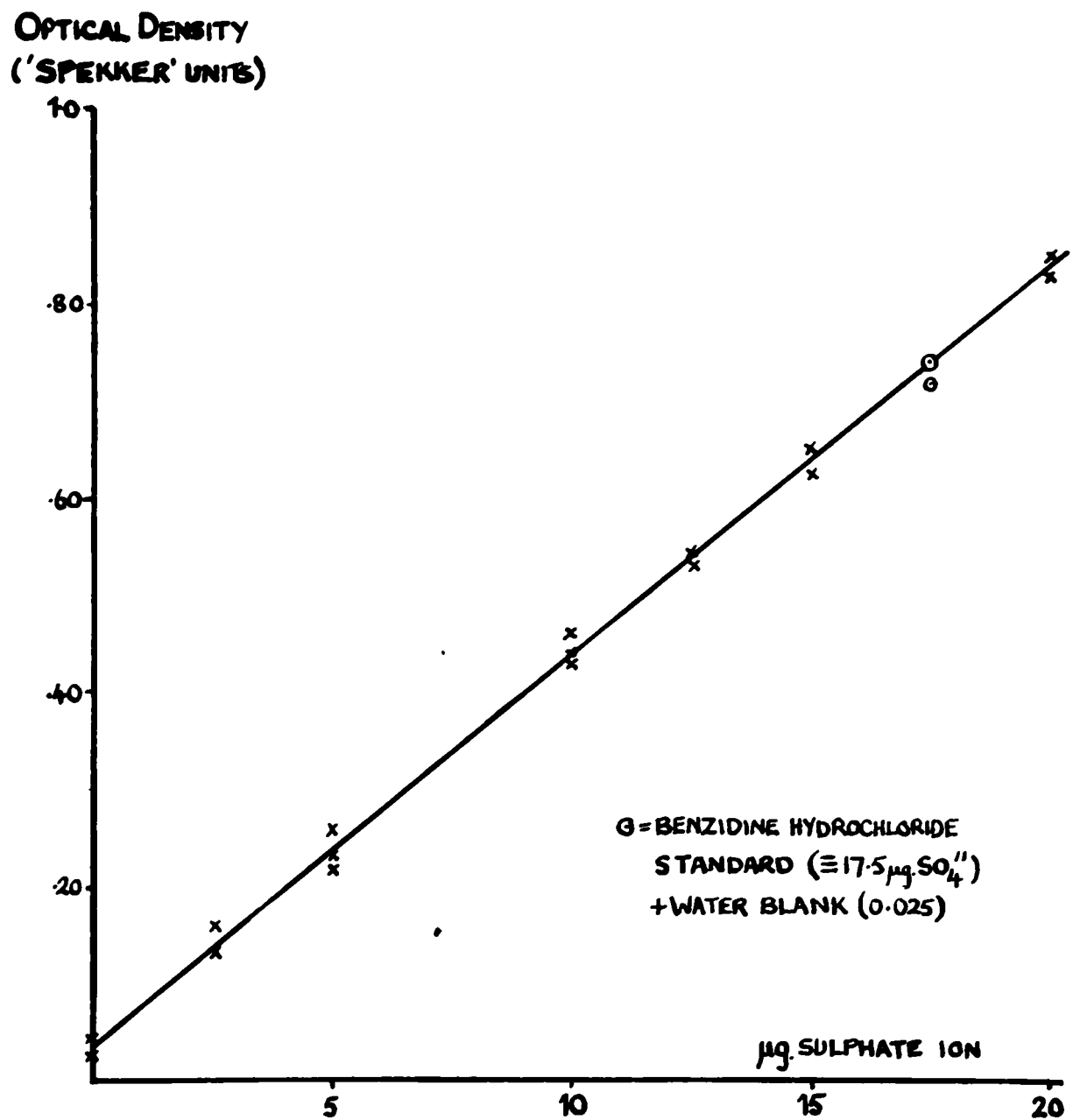


FIG.13: CALIBRATION CURVE FOR 0-20 µg. SULPHATE.

(b) The following sulphated mucopolysaccharides were hydrolysed and after suitable dilution, the hydrolysates analysed for sulphate content:-

(i) Dextran Sulphate (DS), (kindly presented by Dr. C.R. Ricketts), S content known = 17.5%

SO_4 " " calc. = 52.5%

(ii) Chondroitin sulphate (CSA)-chondroprotein complex, (extracted from bull nasal cartilage by Mr. C.A. Pasternak), S content known to be 2.6% (Protein freed CSA has an S content = 6.2%) This complex yielded several aminoacids on hydrolysis of which cysteine was certainly one, though the identification is somewhat ambiguous due to the presence of oxidised products, (cysteine sulphoxide etc.) on chromatograms of the hydrolysate. The SO_4 content of the C.S.A. complex was calculated assuming 15% non-sulphate sulphur (ie. S-containing amino acids).

The poorest agreement of the calculated values was 85% theoretical.

Table 34: Comparison of the calculated and measured sulphate content of hydrolysates of sulphated polysaccharides

(This table is complete on the following page).

Table 34: Comparison of the calculated and measured sulphate content of hydrolysates of sulphated polysaccharides.

Samples analysed	Calculated SO ₄ ^{''} equivalent	'Spekker' readings	SO ₄ ^{''} found
25% formic acid blank	-- μg.	•035 •035	--
0.2ml. DS hydrolysate (•134mg./ml)	14.0 "	•512 •476	14.3 μg. 13.1 "
0.15ml. CSA hydrolysate (1.14mg./ml)	11.3 "	•352 •392	9.6 " 10.6 "
0.25ml. CSA hydrolysate	18.8 "	•582 610	16.5 " 17.2 "
10 μg. SO ₄ ^{''}		•354, •368, •390	Av = •370 = 9.6 μg
Benzidine Standard equivalent to 17.5 μg. SO ₄ ^{''}	}	•615 •605	" = .610

N.B. These Spekker readings were lower than those given by identical amounts of SO₄^{''} and benzidine in the previous table, due to greater age of the diazo and coupling reagents used here.

(c) A radioactive chondroitin sulphate preparation was kindly provided by Mr. L.M. Steddon, isolated from rat tracheal cartilage following the administration of a massive dose of Na₂³⁵SO₄. (15mC./kg. body weight). The sulphate content of this sample was 2.22%, specific activity = 66.5 counts/minute/μg.

Hydrolysates of this ^{35}S - chondroitin sulphate and highly radioactive solutions of $\text{Na}_2^{35}\text{SO}_4$ to which 10 . free sulphate was added as carrier, were submitted in duplicate and triplicate respectively to the analytical procedure. The combined washings and filtrate were collected, concentrated and counted in a liquid counting chamber (vol = 10ml.) together with identical volumes of the final developed colourations.

Table 35: Activities of washings and final developed benzidine colorations when the method was applied to samples of $\text{Na}_2^{35}\text{SO}_4$ and ^{35}S -sulphate.

Initial material	10ml. samples counted.	Counts/min.
$^{35}\text{SO}_4$ labelled chondroitin.	Washings	19, 23
	Colourations	72, 90
Free $^{35}\text{SO}_4$	Washings	40, 25, 65.
	Colourations	686, 696, 665.
Water blank		18, 20, 18.

The greatest value for activity washings was 7%
activity colourations

Reproducibility and Accuracy.

The tests of the method outlined previously would suggest an accuracy and general ~~reproducibility~~ ^{reproducibility} of the order of 10% or perhaps better. A survey of the results of over 150 (duplicate) determinations confirms that reproducibility to within 10% was about the best - that could be achieved when the method was applied to the routine determination of 3-25 μg . sulphate.

An illustration of the overall reproducibility is given in the following determinations on (i) a mucin sample and (ii) a known sulphate solution.

Table 36: Reproducibility of results of sulphate analysis.

(i) Two differing volumes of the mucin sample were taken and hydrolysed independently, and duplicate analysis performed on the hydrolysates.

Vol. hydrolysed.	'Spekker' Readings.		SO ₄ " found.
0.45 ml.	.407		39.6 $\mu\text{g.}/\text{ml.}$
	.425		41.3 mucin
0.5 ml.	.470		41.7
	.446		39.4
Water blank	.055	.055	--
Benzidine $\equiv$ 17.5 $\mu\text{g.}$	SO ₄ " .700	.687	--

The reproducibility here is to within 6%.

(ii) 12 simultaneous determinations were performed on a solution containing $25 \mu\text{g} \cdot \text{SO}_4''/\text{ml}$ -1 ml. samples being taken for analysis.

Samples.	Spekker readings					
25 $\mu\text{g} \cdot \text{SO}_4''$ (12 determinations)	•960	•971	•975	•943	[1•16]	•922
Benzidine standards	[•864]	•980	1•060	1•005	1•048	•990
$\approx 17\cdot5 \mu\text{g} \cdot \text{SO}_4''$	•72	•71				
$\approx 35 \mu\text{g} \cdot \text{SO}_4''$	1•33	1•34				

Theoretical reading for $25 \mu\text{g}$. would be $1\cdot000 \pm \cdot025$
(cf. table 32 and fig. 13)

The readings above (disregarding those in square brackets) average as $\cdot984 \pm \cdot065$, the overall scatter being 13% of the average value.

Interference with the method.

"hole protein and polysaccharide tended to be coprecipitated and constituted a serious source of interference, giving high blank values for solutions free of sulphate due to absorption of benzidine. Hydrolysed protein in quantities of 1 mg. and greater did not interfere at all. Hydrolyses of polysaccharides in amounts greater than 300-350 μg . inhibited the quantitative

precipitation of 10-15 μg . sulphate. (Table 37). In the total absence of sulphate, these protein and polysaccharide hydrolysates gave no precipitate (contrast the polymers) under the conditions for sulphate precipitation.

Chloride ions in concentration up to 0.05 M interfered negligibly. Chloride ion was regularly found in mucin samples ~~submitted~~ submitted to analysis but never in concentrations greater than 0.06 M.

Table 37: Effect of polysaccharides, proteins and chloride ions on sulphate estimation.

Solutions containing 12.5 μg . SO_4^{2-} in 25% formic acid and submitted to analysis.

Additions (in formic acid solution) 'Spekker' readings.

SO_4^{2-} alone	*535	*545
" + 1mg. protein hydrolysate	*530	*555
" + 1mg. starch	*315	*305
" + 500 μg starch	*340	*360
" + 350 " "	*440	*382
" + 250 " "	*495	*525
" + 0.05 M NaCl	*536	*510
Water blank	*045	*040
0.1M NaCl	*076	*075

A more serious source of interference was provided by the presence of even microgram quantities of phosphate. Interference was not suffered from quantities of 25 μg . and less but greater amounts seriously inhibited the quantitative precipitation of sulphate. (Table 38).

Where possible the phosphate content was checked of all hydrolysates, submitted for sulphate determination, using the phosphomolybdate colorimetric method of Jones, Lee

+ Peacock (1951)

Table 38: Interference due to phosphate in the micro-estimation of sulphate.

SO_4 and additions.

'Spekker' readings.

10 μg . SO_4 .

•390 •415

" " + 10 μg . PO_4 .

•415 •395

" " + 50 μg . PO_4 .

•320 •310

15" . SO_4

•620 •635

" " " + 22.5 μg . PO_4

•595 •665

22.5 μg . PO_4

•055 •035

Water blank

•035 •040

DISCUSSION.

A summary of the method, the development and testing of which has already been described, is given in the appendix, (p. 160). The method has been used regularly over a period of several months and apart from the occasional trouble of a filter commencing to leak, proved entirely satisfactory for the determination of 3-20 μ g. quantities of sulphate ion present in hydrolysed mucin samples.

The methods previously given which most nearly approximated to this one in general procedure and sensitivity (Klein, 1944. Dodgson and Spencer, 1953), were limited both by the use of centrifugation to separate the precipitated benzidine sulphate, and the apparently non-quantitative precipitation of sulphate in quantities less than 15 μ g. This latter defect was undoubtedly due to the conditions of greater acidity arising with the use of hydrochloric acid and trichloroacetic acid respectively.

Dodgson and Spencer's claim that sulphate present in amounts of 10 μ g. and less, is not quantitatively precipitated by benzidine, must undoubtedly be related to their sub-optimum conditions of precipitation at pH 2.0-2.2. At pH 2.5-2.7, the quantitative precipitation and estimation of as little as 2 μ g. of sulphate was indeed found possible.

The present method, besides increasing the sensitivity 10-fold to allow the determination of microgram quantities of sulphate ion, is also less exacting than the above methods in the care and attention required during the washing procedures to remove all excess benzidine. It therefore lends itself particularly to the routine determination of the sulphate contents of a large number of analytical samples--
for which the method was indeed developed.

Nevertheless the method is time-consuming and suffers the disadvantages and limitations of both a gravimetric and a colorimetric procedure. No greater accuracy than to within 10% can be claimed for determinations on quantities of the order 2-15 μ g. sulphate.

The principal source of interference is the simultaneous presence of phosphate, in amounts greater than 25 μ g. per 10-15 μ g. sample of sulphate. A number of investigators who have used the benzidine-precipitation procedure for determining rather greater quantities of sulphate (see Welcher, 1947 for references) claim that the phosphate tends to be co-precipitated but that this may be minimised by increasing the acidity of the precipitation medium to pH 2.0. Under the present conditions, it appears that phosphate (50-500 μ g.) tends to prevent the precipitation of sulphate ions. Certainly any attempts to increase the acidity effectively reduced the efficiency of precipitation of micro quantities of sulphate and rendered the procedure non-quantitative. The gross phosphate interference may be eliminated following the removal of the bulk of the phosphate ions by precipitation with uranyl acetate (Yoshimatsu, 1926) or magnesium and ammonium ions (Fiske, 1921).

An account of this method for the micro-estimation of ester sulphate and free sulphate ions, has been accepted for publication in 'The Analyst'.

SUMMARY.

Following the extension and considerable modification of established colorimetric methods for the determination of sulphate ions, it has been found possible to estimate as little as 2 μ g. sulphate. The principal departures from former methods are the careful control of the acidity during the precipitation of sulphate ions by benzidine and the introduction of immersion filters to effect the quantitative separation and allow the thorough washing, of the micro-precipitates. Ester-bound sulphate is liberated by hydrolysis with formic acid prior to its estimation by this procedure. The present method (given in the appendix, p. 160) allows the routine estimation of 2-15 μ g. quantities of ester- and inorganic sulphate to within an overall accuracy of 10%.

APPENDIX.

Procedure for the micro-estimation of 2-15 μ g. ester sulphate
and free sulphate ions.

(as communicated to 'The Analyst')

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All glassware was cleaned with strong nitric acid, rinsed repeatedly in glass distilled water. Immersion filters were prepared by sealing sintered glass plates (9mm. diameter, 3 porosity) into hard glass tubes, the plates being set back 1.5 - 2mm. from the end. The plates were specially selected for evenness of porosity. Overall dimensions of the filters were 200 x 7 mm. They were kept immersed in 0.5 N hydrochloric acid and thoroughly rinsed with ethanol before use.

Benzidine (twice recrystallised from ethanol) dissolved in 98% ethanol to give a 0.5% solution, was stored at 0° and renewed every three weeks. A solution of thymol (0.5%^{w/v}) in 2N sodium hydroxide was used. 0.2 - 0.8 ml. samples of mucins or solutions of sulphated polysaccharides, containing between 5 and 50 μ g. sulphate, were heated with an equal volume of 50% A.R. formic acid (13N) in sealed tubes at 105° for 16 hours. When cool, the hydrolysates were filtered through sintered glass (porosity-2) to remove

humin etc. the residue washed with 25% formic acid and the combined filtrate and washings adjusted to a known volume (usually 2 ml.) with 25% formic acid. Determinations of sulphate were performed in duplicate on each filtrate.

Portions of the hydrolysates or solutions of sulphate in 25% formic acid and containing between 2 and 20 μ g . sulphate were transferred to 15 x 160 mm. hard glass tubes and the volumes made up to 1 ml. with 25% formic acid. 1 ml. glacial acetic acid, 3 ml. ethanol-acetone (1:1) mixture and 1 ml. benzidine reagent were added and adequate mixing ensured. The tubes were tightly corked and kept for at least 12 hours at 0°. The pH of the mixture remained constant throughout. The fine (suspended) precipitates were separated from the supernatant mixture, using the filters described above, with gentle suction. The rate of filtration was carefully controlled and never allowed to exceed 4 ml./min. The precipitates and filters were carefully washed three times with ice-cold ethanol-acetone mixture in 10 ml. portions, with complete suction to dryness between each washing. To each of the filtered precipitates, 2 ml. distilled water was added and 3 ml. N hydrochloric acid was placed in the centre tube of each filter. After 45 minutes the filters were then completely drained by applying gentle pressure from a compressor and removed. The benzidine solutions were diazotised at room

temperature with the addition of 1 ml. 0.1% sodium nitrate. After standing for 3 minutes, 5 ml. of the alkaline thymol solution was added with shaking. The density of the resulting colour was measured, against a reagent blank, in 5 mm. cells on a Hilger 'Spekker' photoelectric-absorptiometer using Ilford 603 filters (maximum transmission at 485 m μ .) The colorations were stable for at least 4 hours. A water blank, included in each series of determinations, consistently gave a reading in the range 0.025 - 0.050, indicating the complete removal of the excess benzidine reagent by the washing procedures. The amount of sulphate was read from a calibration curve prepared by diazotising and coupling known amounts of benzidine hydrochloride in identical volumes.

(26.3 μ g . benzidine hydrochloride equivalent to 10 μ g . sulphate).

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ABSTRACT

I. The properties of factors present in boiled yeast juice which stimulate the oxidative breakdown of citrate in baker's yeast have been investigated. The activity has been shown to reside predominantly in the nucleotide fractions (i.e., pyridine cohydrogenases) and to a lesser extent in the ammonium salts present in whole yeast cells. The activity of the Citrate Oxidation Factor (COF) concentrated from yeast extracts and described by Foulkes (Biochem. J. 54, 323 (1953)) was attributable entirely to the ammonium ion content of COF preparations.

This activating effect of ammonium ions is accompanied by their removal from the assay system and the simultaneous production of glutamic acid. Correlation of the uptake of ammonium ion with the increased citrate metabolism indicates that the activity of the ammonium salts lies in their ability to promote the reductive amination of α -oxoglutarate. The mechanism(s) whereby this serves to stimulate the further breakdown of citrate to oxoglutarate involves the mediation of the pyridine cohydrogenases (DPN, TPN.)

These findings are discussed in relation to the known functioning of individual stages of the tricarboxylic acid cycle and to possible alternative pathways for the oxidation of citrate in yeast.

II. A study has been made of the incorporation of ^{35}S into rabbit duodenal mucin after intravenous administration of $\text{Na}_2^{35}\text{SO}_4$ and DL- ^{35}S -methionine. Within 1 hour, ^{35}S -sulphate and ^{35}S -methionine were present in the non-dialysable mucosubstances which constitute the viscosity-raising agents of the mucin. The maximum incorporation of both the sulphate and the

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methionine into these mucosubstances was found after a period of 4 hours. Following the administration of $\text{Na}_2^{35}\text{SO}_4$, appreciable amounts of inorganic $^{35}\text{SO}_4$ and a dialysable ester 35 sulphate were excreted into the mucin.

The influence of cortisone on mucin production was studied using these isotopically-labelled compounds. In contrast to the many reports in the literature concerning the inhibitory effect of cortisone on the biosynthesis of sulphated mucosubstances in mesenchymal tissues, cortisone acetate was found to have no appreciable influence on the biosynthesis of the (epithelial) mucosubstances in duodenal mucin. Some studies on the composition of the duodenal mucosubstances of both pig and rabbit are also reported.

In part III, the development and use of a micromethod for the estimation of 3-15 μg . inorganic and esterified sulphate is described. This method was developed in response to the necessity of determining the micro-quantities of total sulphate present in small volumes (<1ml.) of various radioactive mucin fractions, in order to evaluate their respective specific radioactivities. In principle, the sulphate is quantitatively precipitated by benzidine, filtered in situ and the benzidine contained in the microprecipitate determined colorimetrically by diazotisation and coupling with thymol.