THE SYNTHESIS OF GLUCURONIDES

IN

LIVER HOMOGENATES

Thesis presented for the Degree

of

DOCTOR OF PHILOSOPHY

by

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July, 1953.

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INTRODUCTION

From a chemical point of view, glucuronic acid may be regarded as a derivative of glucose in which the primary alcoholic group has been oxidised, producing a carboxylic acid. The chemical properties of glucuronic acid have as yet been relatively little studied, but like all substances bearing a potentially reducing aldehyde group, it will form glycosides, and these 'conjugated' compounds, without reducing properties, are known as glucuronides.

In living organisms, glucuronic acid is found almost entirely in the conjugated form, and, although very widely distributed, remarkably little is known about its physiological function. As the great importance of the compound can be inferred from such widespread occurrence we may, before considering the various theories regarding its biosynthesis, briefly indicate the natural distribution of glucuronic acid.

In normal blood and urine small quantities of glucuronic acid exist, conjugated with various metabolites, such as many of the steroid hormones and related compounds; the administration of certain 'glucuronogenic' substances increases these levels very markedly, the foreign compounds, in whole or part, being excreted as conjugates with glucuronic acid. There is a great number of these glucuronogenic substances, including most phenols /

phenols, and many aromatic hydrocarbons, acids, ethers, aldehydes, ketones, nitrogen and sulphur compounds are capable of being rendered glucuronogenic following bodily transformation into hydroxylated derivatives (Williams, 1947). Conjugation of these substances, many of which are toxic, generally results in their increased solubility within the body and consequent rapid excretion. This gave rise to a belief that glucuronic acid conjugation was mainly a mechanism existing for 'detoxication' of poisons or unwanted metabolites, and for ensuring the transport of otherwise insoluble factors like the steroid hormones.

However, that glucuronic acid had a larger part to play became increasingly clear when it was shown to be present in many mucopolysaccharides, including those of such great importance as chondroitin sulphate, the ground substance of hyaline cartilage; hyaluronic acid, known to be present in the vitreous humour, synovial fluid and skin, and reported to be widely distributed in the tissues; mucoitin sulphate of the gastric mucin, and heparin, the powerful anticoagulant. Besides this, glucuronic acid also occurs in the cell-walls and gums of plants, and in many bacterial polysaccharides of antigenic significance (Artz & Osman, 1950).

In all these compounds from so many sources the/

the precise relationship of glucuronic acid to the rest of the molecule, its function, and its method of incorporation are all imperfectly understood. As further evidence of the great but obscure role played by this substance is the presence in almost every tissue of the enzyme β-glucuronidase which hydrolyses the glucuronides to the free acid and aglycone; the true physiological function of this enzyme is also problematical.

Glucuronic acid being thus revealed as a remarkably little-understood compound in spite of its common occurrence, we may turn to a survey of what has so far been discovered of its formation within the animal body.

The early experiments were performed on intact animals and consequently were difficult to interpret. It was first thought that the carbon chain was derived simply from glucose, by an oxidation occurring at carbon atom 6; during normal metabolism the aglycone joined on at this stage (Schmiedeberg & Meyer, 1879). Later, it was suggested that a glucoside was the intermediate during the formation of conjugated glucuronic acid, so that carbon atom 1 was protected and the oxidation at carbon atom 6 rendered more feasible (Fischer & Piloty, 1891). Although bornyl glucoside, on injection, gives rise to bornylglucuronide in the urine (Hildebrandt,1909), it seems most likely from the evidence that hydrolysis first takes place and the freed aglycone is then /

then conjugated with glucuronic acid. Indeed, the glucoside phloridzin is itself excreted unchanged but for the attachment of glucuronic acid at the site of a hydroxyl group (Schuller, 1911). Also, injected phenyl-β-glucoside gave the same ethereal sulphate excretion as did an equivalent amount of the free phenol (Pryde & Williams, 1936), indicating the hydrolysis *in vivo* of the glucoside; perfused preparations (Hemingway, Pryde & Williams, 1934) which could synthesise phenylglucuronide from free phenol were unable to do so from phenyl-β-glucoside, presumably because they could not perform the necessary hydrolysis.

That pre-formed carbohydrates were, however, necessary for the formation of conjugated glucuronic acid was demonstrated by Schmid (1936), who showed that starved hibernating frogs, whose livers had a very low glycogen content, were unable to conjugate menthol; but similar animals in water containing glucose or glycogen could produce menthylglucuronide, behaving like well-fed specimens. Quick (1926), using a different subject, the starved depancreatised dog, had also come to the conclusion that glycogenesis was concerned with glucuronic acid formation; it seemed that glucose and glucuronic acid had the same precursor, for if a glucuronogenic drug was fed, glucuronic acid was apparently produced at the expense of possible urinary glucose. This glucuronic acid did not arise from specially catabolised /

catabolised protein, for there was no extra urinary nitrogen produced, but probably came from the usual protein breakdown in animals so treated; the pathway presumably lay through the glucogenic amino-acids. With normal dogs, Quick found that, provided the animals received sufficient carbohydrate, conjugated glucuronic acid was produced without any great increase in urinary nitrogen; with starved normal dogs, though, glucose fed with a glucuronogenic drug did not prevent extra protein breakdown to provide the glucuronic acid. Quick therefore concluded that glucose itself could not be used directly, and that the precursor of glucuronic acid was formed more readily from glycogenic amino-acids or glycogen than from glucose; such a precursor could very well be lactic acid.

This work on intact animals received support from Lipschitz & Bueding (1939), using tissue slices. They confirmed what had been suspected, that the liver is the main site of conjugation, and with slices from fasted guinea-pig livers showed that borneol or menthol was conjugated readily when lactate, pyruvate or dihydroxyacetone was added to the medium, but not appreciably when glucuronic acid or hexoses were present instead. The process was strictly aerobic and was inhibited by cyanide, fluoride and iodoacetate. Because of this, they suggested the synthesis derived energy from oxidative reactions and involved phosphorylation, the carbon /

carbon chain of glucuronic acid being formed from three-carbon precursors.

Continuing along these lines, Storey (1950), using o-aminophenol and directly measuring the glucuronide formed, found considerable conjugation in both fed and fasted mouse and guinea-pig liver slices, whether the three-carbon compounds were present or not; their addition made no difference. He also found that glucuronic acid itself, and to a lesser extent, gluconate and saccharate, were marked inhibitors. From this he postulated, by analogy with Cori's results (Cori, Cori & Green, 1943), wherein glucose competitively inhibited glycogen formation from glucose-1-phosphate under the influence of phosphorylase, that glucuronic acid might be competing with such a compound as a glucuronic acid-1-phosphate.

Storey also confirmed that phosphorylations were involved, by showing the inhibitory action of 2:4-dinitrophenol. Sulphate ion was found to inhibit as well, competing with glucuronic acid for the conjugation with o-aminophenol. He found very considerable increases with bicarbonate ion and suggested that carbon dioxide fixation is concerned in glucuronide synthesis.

Subsequent work on the problem has been mainly concerned with the use of isotopes,performed, with one exception, upon intact animals; it therefore fails to give a very coherent picture.

The /

The first workers to be considered (Mosbach & King, 1950) injected glucose uniformly labelled with carbon-14, and examined the C_6 of the glucuronic acid conjugated with borneol fed to the animal. They found one-sixth of the activity of the molecule in C_6, and suggested a direct conversion from glucose; but the latter may have been split and the two halves recombined with no dilution of activity. Using labelled bicarbonate they only found a little activity in C_6, which was to be foreseen, for in known carbon dioxide fixation it is the C_3 and C_4 positions in the liver glycogen which exhibit activity. Since some 90 per cent of the isotope was expired over the time of the experiment, the small amount found is hardly surprising, as little could be expected to be available for incorporation.

Further work (Douglas & King, 1952) revealed that glucuronic acid can be readily metabolised _in vivo_, for, if uniformly labelled and injected into guinea-pigs, one quarter of it was recovered as carbon dioxide within three hours. If borneol was fed, some 3 - 7 per cent of the injected glucuronic acid was found to be conjugated; this might suggest that pre-formed glucuronic acid could be used in the process of conjugation, but since less than one-sixth of the activity was found in the C_6, other pathways than direct use of the six-carbon chain are probable.

Glucose /

Glucose labelled at C_1 was fed by other workers to rabbits (Eisenberg & Gurin, 1952), and the glucuronic acid from the urine degraded to give C_6; C_1 & C_6; C_3 & C_4; C_2 & C_5, and C_4, C_5 & C_6 fragments. Most of the activity was at C_1, and glucose or some other six-carbon compound was suggested as the immediate precursor. If this were so, then the amounts of isotope at positions 3 and 4 should be equal, as should those at 2 and 5. Although the authors were seemingly able to prove these assumptions, an entirely different isotopic distribution can be calculated directly from their results, and it would be quite possible, from their evidence, that the glucose molecule had been broken in two and then recombined, during its utilisation.

When lactate, pyruvate, glucose and glucuronic acid were fed in the labelled form to rats, the isotopic carbon was found incorporated to the same extent in the urinary glucuronic acid from all these sources (Packham & Butler, 1952). However, if glucuronate was injected, it was twenty times as effectively incorporated and did not give rise to respiratory carbon dioxide (as it did with the guinea-pigs of Douglas & King). This would certainly suggest that pre-formed glucuronic acid is rapidly utilised in conjugation.

As a result of his experiments with injected glycerol labelled at the C_1 position, Doerschuk (1952) concluded that the biosynthesis utilised three-/

three-carbon intermediates made *in vivo* from glycerol and which did not pass through the body glucose pool with its accompanying dilution.

From all these intact animal isotopic experiments it seems probable that glucose may be converted to glucuronic acid fairly directly, but no conclusive evidence has been presented. Pre-formed acid can be used, possibly by way of phosphorylation; but this leaves the normal precursor still in doubt, though it may be a three-carbon compound.

These conclusions are borne out by the sole example of work *in vitro* with isotopic carbon (Bidder, 1952). It was found that both glucose and lactate, labelled at C_1 and C_3 respectively, could be utilised by fasted guinea-pig liver slices; the lactate, however, gave rise to a glucuronic acid having more of the isotope in C_4 to C_6 than in C_1 to C_3, suggesting the two three-carbon units to come from different sources.

Further whole animal experiments would seem to be unprofitable; preparations of the tissue itself and the responsible enzyme systems need to be investigated. The only characterised enzyme system so far shown to involve glucuronic acid is β-glucuronidase, but this enzyme, according to Levvy and co-workers (Karunairatnam, Kerr & Levvy, 1949), is not concerned in the synthesis of glucuronides.

Previous to the publication of the first paper dealing with the work presented (Dutton & Storey, 1951 /

1951), in which glucuronide conjugation by liver homogenates was reported, no successful attempts with such tissue preparations had been described. Subsequently, other workers (Bernstein & McGilvery, 1951, 1952) have claimed some synthesis of m-aminophenylglucuronide in homogenates, from the free phenol and potassium glucuronate or three-carbon precursors of glucuronate; this reaction was apparently similar to the sulphate synthesis which they were studying, in being aerobic and energy-consuming. However, the amount conjugated was small and they were only able to provide indirect evidence of the formation of the m-aminophenylglucuronide. Another group (DeMeio & Tkacz, 1952), working on phenol conjugation by liver, found synthesis of the glucuronide to be 33-40 per cent of that of the sulphate in sliced preparations, but only 5-10 per cent in homogenates. They, too, reported that the reaction seemed to be aerobic, but were also unable to characterise their product to any extent.

The work presented below was begun with the intention of studying further the nature of glucuronide synthesis in the liver, using disintegrated cell preparations (the so-called 'homogenates'); the use of such preparations is the next logical step after slice experiments, for it enables more consistent conditions to be attained, and prepares for eventual enzyme isolation.

In place of the overall results which are all that /

that can be ascertained from experiments on whole animals, the homogenate technique offers a simple and convenient method of directly demonstrating definite reactions, such as the production of o-aminophenylglucuronide on adding o-aminophenol. Whereas with the intact animal, its perfused organ or even slices of that organ, there is no certainty that the added substance is really reaching the responsible enzymes, or in what form or concentration it might do so, in the homogenate technique a much closer control can be exercised. The problems of diffusibility of substrate and inhibitors through the cell membrane, or of unequal tissue thicknesses with their consequent metabolic differences, do not exist in homogenates, where, provided reasonably complete cell disruption has occurred, equal volumes of the tissue preparation will behave identically, being to the same degree exposed to their environment. If the homogenate be used in a sufficiently dilute form, autolysis will be checked and by the addition of various metabolites and co-factors the enzyme system of part or all of the desired reaction may be encouraged above the level of others; subsequently the studied system may be isolated from the crude homogenate and its behaviour controlled to an extent impossible by any other means.

Therefore, since liver slices had already been used by Storey to investigate the biosynthesis of glucuronides /

glucuronides, liver homogenates were employed in the present work, which continues the study of glucuronic acid conjugation in the liver.

It was found during the course of this work that appreciable glucuronide formation could be obtained in liver homogenates, but only when a boiled liver extract was added to the medium (Dutton & Storey, 1951). The bulk of this work, then, concerns the isolation and purification of the substance responsible, and the behaviour of the enzyme system utilising it (Dutton & Storey, 1953).

After the tabulation of the various standard methods used, there follows a report of the experimental results, showing how, despite the trial of many compounds of metabolic importance, no significant synthesis was obtained unless a boiled liver extract containing the unknown factor was added. Evidence that the products of synthesis were indeed glucuronides is given, and a few observations on the homogenate enzyme are appended. Work on the nature of this synthesis with the crude factor is then described, and the dissimilarity of the system to that obtaining with sliced tissue (Storey, 1950) pointed out. Lastly, the course of isolation and purification of the factor is illustrated, with its analysis and structural investigation. In the Discussion the relationship of this work to the results/

results of previous investigators will be dealt with, and its bearing on the wider aspects of glucuronic acid metabolism indicated.

EXPERIMENTAL METHODS

Homogenate.- The preparation of the homogenate was conducted according to the principles of Potter (1948). The animals used were chiefly mice of either sex, with occasionally rats, guinea-pigs and rabbits; they were killed by dislocation of the cervical vertebrae.

The liver was rapidly excised, cooled in crushed ice, freed of gall bladder and weighed. 1 g. was disrupted in 9 ml. alkaline isotonic KCl, to give a 10 per cent homogenate. This initial dilution is claimed to slow down glycolysis; the usual subsequent dilution was 0·5 ml. in 3 ml. reaction mixture, giving a final dilution of 1 : 60, with 50 mg. wet weight of liver in 3 ml. medium.

Alkaline isotonic KCl consisted of 1·15 per cent aqueous KCl solution with enough potassium bicarbonate added to give a pH of 8 when equilibrated with air; this is supposed to neutralise harmful acids liberated on cell disruption.

The homogeniser was of the Potter-Elvehjem all-glass type, with longer pestle to reduce wear and increase grinding surfact; the tissue was homogenised rapidly in 3 ml. solution for 30 seconds, then for another 30 seconds more slowly in the full 9 ml., the tube being continually cooled in ice-water.

Substrate.- The substrate was usually re-sublimed o-aminophenol, taking advantage of the excellent /

excellent rapid method of determining o-aminophenyl-glucuronide (Levvy and Storey, 1948).

With slices, Levvy and Storey found 0·0025 per cent (w/v) to be the optimal final concentration of o-aminophenol for a 2-hour incubation, and added ascorbic acid to 0·001M concentration to prevent oxidation of the phenol to a yellow and possibly toxic substance. Since there was no reason to suppose that this concentration was the most suitable for homogenates, with their lack of diffusion difficulties and the greater access of substrate (and possible inhibitors) to the liberated enzymes, experiments were performed to find out the best concentrations of o-aminophenol and ascorbic acid.

0·005 per cent substrate seemed definitely inhibitory and gave unsatisfactory colours after diazotisation, whereas 0·0005 per cent appeared to be too low for effective utilisation; 0·0015 per cent was the concentration of o-aminophenol eventually chosen. The substrate stock solution, stored at - 20° and of which 0·2 ml. was used in 3 ml. reaction mixture, contained 6 mg. o-aminophenol and 50 mg. ascorbic acid in 25 ml. water.

l-menthol was also used occasionally as a substrate; the final concentration employed was 0·1 per cent (w/v).

<u>Medium</u>.- The enzymes contained in the homogenate were intended to act on the substrate in a medium best suited for the purpose. As a general principle /

principle, potassium ions were used instead of sodium, and calcium excluded, to resemble more closely the intracellular environment (Potter,1948). Sulphate ion was also excluded in the earlier work, since Storey had shown its inhibitory action with slices.

To achieve consistent results the pH of the medium must not vary and a buffer was essential. The usually-accepted homogenate buffers were in the region of 0·015M final concentration of phosphate for a pH of about 7·4. It was found that this concentration was insufficient for pH constancy during the reaction (cf. Cohen & McGilvery, 1947), and the following mixture was used instead, giving satisfactory control of pH before and after incubation: 0·15 ml. 0·4M-potassium bicarbonate and 0·15 ml. 0·5M-potassium phosphate, at pH 7·4, in an atmosphere of oxygen containing 5 per cent carbon dioxide. Later, when bicarbonate was found to have no effect on synthesis, the routine buffer was 0·2 ml. 0·5M-potassium phosphate at pH 7·4, in air. The buffers were always adjusted to the final pH with the glass electrode and stored in the refrigerator.

Thus a flask contained 0·5 ml. 10 per cent liver homogenate, 0·2 or 0·3 ml. buffer, and o-aminophenol and ascorbic acid in final concentrations of 0·0015 per cent and 0·015 per cent respectively (w/v), made to a total volume of 3 ml. with distilled water or other /

other added solutions (always brought to pH 7·4 at the glass electrode unless otherwise stated). These other additions will be considered under the relevant experiments; magnesium chloride at a final concentration of 0·015M was added from a very early stage of the work. The homogenate was always placed in the flask last, with the latter standing on ice.

Gassing.- If the medium was to be incubated in an atmosphere of nitrogen, oxygen, or oxygen and carbon dioxide, a stream of the required gas was bubbled through a trap into the flask for 2 minutes; the flask was then stoppered until the homogenate was added, when the gas was again passed for 30 seconds before final stoppering. The solutions to be used were also well gassed with the required atmosphere.

Incubation.- Incubation was conducted at 37° in a Warburg bath, the 3 ml. reaction mixture in its 25 ml. rubber-stoppered flask being shaken sufficiently to ensure continuous mixing of the contents and to permit gaseous exchange with its atmosphere. When once they had reached 37° the flasks were momentarily unstoppered to relieve the gaseous pressure within. The period of incubation was usually 30 minutes.

Estimation of Synthetic Product:

Protein Precipitation.- Precipitation of the protein present was essential for the subsequent colorimetry /

colorimetry, and the resulting mixture had to have a pH of 2·25 - 2·50 if Levvy and Storey's method was to be reliable.

After much trial it was found that 3 ml. of a solution containing sodium trichloroacetate and phosphate was sufficient, if added to the flask, to precipitate all protein, overcome the buffer at pH 7·4, and bring the final pH to 2·25.

This solution was prepared daily by mixing equal amounts of stock 20 per cent trichloroacetate and 2M-phosphate, both at pH 2·10. Immediately after incubation the flask was placed on ice and 3 ml. of the precipitant mixture added. After mixing, the contents of the flask were poured into a 10 ml. centrifuge tube, allowed to stand 15 minutes, and centrifuged. The method is that of Levvy and Storey, modified to suit homogenates.

Colorimetric Estimation:

(a) With o-aminophenol as substrate:

The method used was that of Levvy and Storey (1948), which depends upon the fact that very small amounts of o-aminophenylglucuronide can be measured colorimetrically after diazotisation and subsequent coupling with N-1-naphthylethylenediamine, this process being carried out at pH 2·25 - 2·50 to prevent interference from free o-aminophenol.

4 ml. of the deproteinised solution were added to a tube, followed, at intervals of at least two minutes, with shaking, by 1 ml. 0·05 per cent sodium nitrite /

nitrite, 1 ml. 0·5 per cent ammonium sulphamate, and 1 ml. 0·1 per cent naphthylethylenediamine dihydrochloride (all in aqueous solution).

The tubes were then kept at 25° for 2 hours to allow the colour to develop fully. Any slight turbidity was then centrifuged down, and the colour intensities read with a Hilger Spekker photoelectric absorptiometer against suitable controls, using Ilford yellow-green filter No. 605.

The controls were those most appropriate to the occasion, it being found that because of slight unavoidable interferences a simple reagent blank could not be employed. The pH in the tubes was checked from time to time with the glass electrode.

Interferences: From tissue: With homogenates (if present in amounts greater than those normally used), concentrated muscle, yeast and wheat germ extracts, a pinkness was apparent, having all the characteristics of interference due to p-aminobenzoic acid: the colour developed immediately on addition of the coupling reagent and did not increase thereafter at 25°. The development of the colour due to o-aminophenylglucuronide was slow, and not complete until after 2 hours' incubation at that temperature.

From light: It was noticed that, in tubes left in direct sunlight after colour development, a turbidity and darkening of the solutions occurred. This was traced to the action of light /

light on the naphthylethylenediamine in the presence of the trichloroacetate solution used (the former was from Messrs. British Drug Houses, Ltd.; the latter, of Analytical Reagent quality). All tubes, therefore, were kept in the shade before reading.

(b) With l-menthol as substrate:

The method used was Tollens' naphthoresorcinol procedure, with great improvements due to Dr. J. Paul (private communication). The homogenate and medium were as for o-aminophenol, but the latter contained menthol of final concentration 0·1 per cent as substrate, in a total volume of 2 ml.

After incubation, 2 ml. 3 per cent aqueous salicylsulphonic acid were added, and the precipitated protein centrifuged down. 3 ml. aliquots from both duplicates were combined and the resulting 6 ml. extracted with 10 ml. and then with 5 ml. of ethyl acetate. After separation, the ethyl acetate layers were blown dry at 50°.

3 ml. water, 2 ml. 18N-sulphuric acid, and 2 ml. 0·3 per cent naphthoresorcinol (Messrs. Light & Co.) in 0·01 N-sulphuric acid, were then added to the residue. After heating at 100° for 1 hour, the tubes were cooled, and to each were added 4 ml. ethanol and 6 ml. toluene, the whole being well mixed. The toluene layer was separated, any slight emulsion centrifuged down, and the colours read immediately on the Hilger Spekker absorptiometer, using /

using Ilford yellow-green filter No. 605.

Interference: The only interference noted was a very considerable one due to added α-ketoglutaric acid; it was not investigated further.

Estimation of Phosphorus:

Inorganic Phosphorus.- As Fiske and Subbarow (1925), described by LePage and Umbreit (1945), with a modification for lower concentrations using half the amount of reagents in a total of 6 ml. solution.

'Labile' Phosphorus.- The sample was made normal with HCl in 1 ml. of solution and heated at 100° for 10 - 12 minutes (see Experimental Results). Inorganic phosphorus was then determined as above.

Total Phosphorus.- King's method was used (King, 1946), modified as follows:- 0.3 ml. sample was placed in 1·2 ml. 60 per cent perchloric acid, and boiled until white fumes had evolved for 3 minutes. 6 ml. water were added and the solution heated at 100° for 15 minutes to destroy any possible pyrophosphates (no evidence for these ever found). 0·8 ml. ammonium molybdate (2·5 per cent w/v, aqueous) and 0·4 ml. Fiske and Subbarow reagent (quoted by LePage and Umbreit, 1945) were then added with mixing, followed by water to a total volume of 10 ml. Again, a modification for lower phosphorus concentrations was employed, using half the amount of reagents in a total volume of 6 ml.

Every /

Every estimation was accompanied by at least one standard of comparable phosphorus content to cover the conditions, checked from a standard graph.

30 minutes were allowed for colour development, which was then measured with a Hilger Spekker absorptiometer, using Ilford orange filter No. 607.

Attempted Preparation of β-D-Glucose-Phosphate and β-D-Glucuronic Acid-1-Phosphate.- Acetobromoglucose was prepared by the method of Barczai-Martos & Köròsy (1950) and treated with monosilver phosphate to form β-D-glucose-1-phosphate by the method of Reithel (1945). The solution of the product contained a phosphate ester and did not reduce Fehling's solution unless previously hydrolysed by boiling with N-HCl.

Acetobromoglucuronic acid was prepared, using glucurone (Messrs. Corn Products, Ltd.) in the above method instead of glucose; after treatment according to Reithel's method for the glucose derivative, a yellowish powder was obtained which may have been potassium β-D-glucuronic acid-1-phosphate. Its solution contained a phosphate ester, gave a positive Tollens naphthoresorcinol test for a uronic acid (Tollens & Rorive, 1908), and only reduced Fehling's solution after acid hydrolysis.

EXPERIMENTAL RESULTS

SECTION I: Attempts to induce Glucuronide Synthesis in Liver Homogenates:

Interference from β-glucuronidase.- An effective scheme of protein precipitation and pH control over the reaction mixture and its subsequent colour development having been satisfactorily evolved (see Experimental Methods), it was necessary to find out whether any glucuronide formed in that reaction mixture would be destroyed at pH 7·4 by the enzyme β-glucuronidase known to be present in such liver homogenates.

Known amounts of o-aminophenylglucuronide were added to the system, with and without various known concentrations of potassium saccharate to act as a specific inhibitor for β-glucuronidase (Karunairatnam & Levvy, 1949). After one hour's incubation it was found that only slight hydrolysis (about 5 per cent) had taken place, and that this could be inhibited by saccharate concentrations of 0·0001M and upwards. Obviously, then, β-glucuronidase had very little hydrolytic action at the pH chosen, but for the preliminary experiments 0·0001M-saccharate was present in control tubes as a precaution.

Preliminary Synthesis Experiments.- The system as described above (see Experimental Methods), containing mixed bicarbonate and phosphate buffer, o-aminophenol, ascorbic acid, and homogenate, all in /

in a volume of 3 ml. and gassed with oxygen and carbon dioxide, was incubated at 37° for varying periods of time with and without added saccharate. The sole evidence for the presence of o-aminophenylglucuronide after such incubations was the development of a faint pinkness in the colour reaction, absent from similar unincubated mixtures, those with boiled homogenate, and those without substrate. Addition of magnesium ion to a concentration of 0·015M increased the colour somewhat, but at no time did it correspond to more than 10-15 per cent of the amount of o-aminophenol conjugated by an equivalent weight of liver in the form of slices; the presence of saccharate did not seem to affect the colour production and it was therefore discontinued. Increasing the amount of homogenate seemed to increase the synthesis relative to tissue weight, but gave rise to precipitation difficulties and interference with the colour reaction.

Whole slices, even in this designedly intracellular medium, synthesised 50-70 per cent of the o-aminophenylglucuronide produced in their more usual Ringer, but slices deliberately crushed with a glass rod gave no trace of synthesis at all in either medium. It was plain that something had to be added to the homogenate system to make up for the disorganisation and dilution of the enzymes of the slices.

<u>Addition</u> /

Addition of Metabolites and known Co-factors.- In an effort to raise the level of synthesis, both in these preliminary experiments and later when it was desirable to see if the effect of the unknown factor could be reproduced, very many substances were added, singly or in combination, to the medium (Table I). Various systems believed to support tissue phosphorylation *in vitro* (Potter, 1945,1948), containing dicarboxylic acids, cytochrome c, adenylic acid and adenosine triphosphate were tried, and the effect of varying the homogenising medium and increasing its tonicity with sucrose or potassium chloride (Hogeboom, Schneider & Pallade, 1947) was also investigated. In no case was the degree of synthesis raised more than 5-10 per cent of its meagre total, and it became clear that the only clue to progress lay in the homogenate itself. This obviously contained sufficient of some substance to produce the slight colour mentioned, which seemed as if it might be due to o-aminophenylglucuronide.

Addition of Boiled Homogenate Supernatant.- The whole homogenate could not be increased very much because of precipitation difficulties, and it was decided to add the supernatant of excess boiled homogenate to the system, in the hope that some overlooked essential substance was contained in it. This experiment, performed with guinea-pig liver, gave a result exceeding expectations, and was carried /

TABLE I.

Compounds Added to System to Induce Appreciable Synthesis without Added Factor.

Reference No.	Compound, with final concentration.
1	Manganese sulphate, 0.0001M
2	Adenylate, 0.001 and 0.005M
3	Saccharate, 0.001M
4	Acetate, 0.001 and 0.005M
5	Lactate, 0.001 " 0.005M
6	Fumarate, 0.001 " 0.005M
7	Pyruvate, 0.001 " 0.005M
8	α-Ketoglutarate, 0.001 and 0.005M
9	Glutamate, 0.005M
10	Folate, various concentrations
11	Pantothenate, 0.0005M and 0.005M
12	Pyridoxine, 0.0005M " 0.005M
13	Riboflavine, 0.0005M " 0.005M
14	Aneurin, 0.0005M and 0.005M
15	Biotin, 1 μg. (as solution)
16	Co-carboxylase, 0.002M
17	Nicotinamide, various concentrations
18	Coenzyme I, various concentrations
19	Adenosine triphosphate, 0.001 and 0.004M
20	Flavine-adenine dinucleotide, various concentrations
21	Dihydroxyacetone, 0.005M
22	D-Xylose, 0.005M
23	D-Ribose, 0.005M
24	D-Glucose, 0.0005M
25 /	

TABLE I (continued):

Reference No.	Compound, with final concentration
25	Hexosediphosphate, 0.005M
26	∝-Glucose-1-phosphate, 0.0001 - 0.002M
27	Cytochrome c, various concentrations
28	Cysteine, 0.02M
29	Coenzyme A, 0.04 and 4 μg. pantothenate added

Combinations of above:

2, 9; 6, 7, 5; 6, 7, 5, 27; 6, 7, 27, 18;

(all ± 3). 6, 2, 5, 4, 22, 27, 19; 4, 5, 6,27,21;

6, 7, 2, 9, 27; 19, 7, 16; 19, 7, 6; 19, 7, 6, 27.

Notes on above:

8: interferes with colour reaction for l-menthylglucuronide.

10: diazotisable impurity present in preparation, interfering in Levvy and Storey colour reaction.

17: results, when added, in formation of brown (oxidation?) product from o-aminophenol.

18: various preparations from yeast, by method of LePage (1949).

19: the quoted concentrations were also added at intervals during incubation. Obtained as Ca salt; Ca ions precipitated with oxalate.

26: kindly given by Dr. A. B. Roy.

27: reduced cytochrome c spectrum apparent in presence /

TABLE I (continued):

Notes (continued):

presence of ascorbic acid and o-aminophenol.

Prepared from horse heart by method of Keilin and Hartree (1945).

29: active preparation, kindly given by Dr. W. E. Balfour.

carried out in the following manner:

12 g. ice-cooled guinea-pig liver was roughly chopped into a homogeniser tube and boiling 0·5 per cent KCl added. The whole was then rapidly homogenised and the mixture poured into excess boiling KCl solution and boiled, with stirring, for 10 minutes. The supernatant was decanted and centrifuged; the resulting 30 ml. of pale green solution, cloudy with glycogen, was stored overnight in the cold.

The boiled extract was then added to the system at three levels of concentration, with 0·015M Mg ion. Controls were identical systems, but not incubated, or incubated and with substrate added later; this allowed for the slight colorimetric interference from the extract itself.

From Table II it will be seen that the amount of o-aminophenol conjugated had been increased eight times, and was comparable with that in slices. Succeeding experiments confirmed that, for the extract to be effective, the unboiled homogenate had to be <u>incubated</u> in the <u>presence</u> of o-aminophenol; the latter did not spontaneously link up with enzymically-released substances from the extract, to give a colour with the coupling reagent.

It seemed, therefore, that an apparently new factor had been discovered, the addition of which to the simple homogenate system resulted in the formation of o-aminophenylglucuronide in the presence of o-aminophenol. Proof, however, was needed that /

that the final colour was indeed due to o-aminophenylglucuronide. In the next section is collected all the evidence obtained, sufficient to show that the enzymic process does result in such a product.

TABLE II.

Effect of Adding Boiled Liver Extract to Unfortified Homogenate System.

ml. Extract	µg. o-aminophenol conjugated (per 50 mg.(wet weight) liver)
0	0.7, 1.0
0.3	5.0, 6.2
0.5	7.0, 7.0
1.0	7.9, 8.3
With slices	10.0, 9.0

SECTION II: Identification of Synthetic Product:

The evidence was gathered from three sources, which are presented below:

Colorimetric Estimations:

(a) It has been mentioned already that o-aminophenol is needed for the synthesis of a substance acting like o-aminophenylglucuronide in Levvy and Storey's very selective method. This substance behaves identically with known o-aminophenylglucuronide throughout the colorimetric estimation, the rate of colour development of the diazotised compound at 25° and pH 2·25 being the same as that of similarly-treated o-aminophenylglucuronide; the final colour in early experiments was slightly more reddish, but this was due to contaminating green compounds, and later colourless extract preparations gave rise to a reddish-purple solution which, when studied on the Hilger Spekker absorptiometer with a range of Ilford spectrum filters, was indistinguishable from that due to o-aminophenylglucuronide.

(b) Using the method of Paul (see Experimental Results), the product obtained when the boiled extract was incubated in the system with l-menthol gave a solution indistinguishable in colour and conditions of colour development from that due to known l-menthylglucuronide.

Paper Chromatography:- Because of the probable similarity in chromatographic behaviour of compounds both containing the naphthylethylenediamine moiety, too /

too much reliance cannot be placed on the fact that the final coloured substances (after Levvy & Storey's colorimetric development), from the synthesis and from known o-aminophenylglucuronide, had identical rates of travel in the butanol layer of a solvent containing 40 per cent n-butanol, 10 per cent glacial acetic acid, and 50 per cent water. It does, however, confirm the findings of the other experiments.

<u>Enzymatic Hydrolysis</u>.- If the product were indeed a glucuronide, then it should be susceptible to the action of β-glucuronidase; and such destruction as does occur should be inhibited by saccharate, the specific inhibitor of that enzyme (Karunairatnam & Levvy, 1949). The following experiment was designed to investigate this. In outline, the procedure was to synthesise the product and expose it, before colorimetric estimation, to the action of β-glucuronidase; the difficulties were pH governance and the provision of suitable controls. The enzyme had to be capable of hydrolysing a significant amount of o-aminophenylglucuronide, and the saccharate of noticeably inhibiting that hydrolysis; nothing was to interfere with the colour development and the volumes had to be kept conveniently low.

Preparation of β-glucuronidase: (Talalay, Fishman & Huggins, 1946; Kerr, Graham & Levvy, 1948). Four fresh mouse livers were each added to 5 ml. water and homogenised; then to each were added 3 ml. water /

water and 1 ml. 0·3M-potassium citrate buffer at pH 5·1. The mixtures were then combined and incubated at 37° for 30 minutes, after which they were centrifuged for 15 minutes and an equal volume of saturated ammonium sulphate added to the supernatant. The resulting precipitate was centrifuged for 30 minutes, and then partially dissolved and suspended in 12 ml. water. 0·4 ml. aliquots were used in the experiment.

Procedure: (see Table III for scheme). The synthetic system was set up in a 2 ml. volume with the 0·5M-phosphate buffer at pH 7·4 reduced to 0·1 ml. This was incubated for 30 minutes at 37° for synthesis to take place. It was then cooled on ice, and 0·05 ml. 1N-HCl was accurately added to all flasks; this brought the pH down to 3-4 units, and rendered the task of the citrate buffer easier; it also precipitated most of the homogenate protein.

β-glucuronidase in its 1 ml. of buffered system was then added and this was incubated for $2\frac{1}{2}$ hours at pH 5·25 and at 37°. Subsequently, 4 ml. precipitant was added and a 5 ml. aliquot taken for colorimetric estimation of the synthetic product remaining in the solution. Known o-aminophenylglucuronide was exposed to hydrolysis also. The final pH in the tubes was checked and found to be within 2·36 - 2·40.

The results of this experiment are shown simply in Table IV and indicate that the synthetic product is /

TABLE III.

Scheme of Procedure for Enzymatic Hydrolysis of Synthetic Product.

Key		Design	µg. o-aminophenol conjugated
1 and 2.			
H E I	G" I'	Normal synthesis; glucuronidase added after hydrolytic incubation to check on 'tissue blank'.	6.8, 7.2
3 and 4.			
H E I	G I'	As above, but hydrolysed with glucuronidase.	4.9, 4.7
5 and 6.			
H E N	G I'	Effect of glucuronidase on unincubated synthetic system.	0, 0
7 and 8.			
H E I	GS I'	Effect of saccharate on 3 and 4.	6.5, 7.0
9 and 10.			
H E N	GS I'	Effect of saccharate on 5 and 6.	0, 0
11 and 12.			
H E A N	G" I'	As 1 and 2, but unincubated synthetic system with added APG.	8.5, 8.4

13 /

TABLE III (continued).

Key	Design	µg. o-aminophenol conjugated
13 and 14.		
H E A G I' N	Effect of glucuronidase on 11 and 12.	5.3, 4.7
15 and 16.		
H E A GS I' N	Effect of saccharate on 13 and 14.	7.5, 7.5

Synthetic System: 0.1 ml. phosphate buffer, 0.5 ml. homogenate, 0.5 ml. extract, 0.2 ml. ascorbic acid and o-aminophenol mixture, 0.1 ml. 0.015M-$MgCl_2$, water to 2 ml.

Hydrolytic System: 0.3 ml. citrate buffer of final molarity 0.03 at pH 5.1, 0.4 ml. glucuronidase (if added), 0.3 ml. saccharate of final molarity 0.0015 (if added), water to 1.0 ml.

Explanation of Key: H: 10 per cent mouse liver homogenate.

E: crude extract, 10 mg/ml., pH 7.

G: glucuronidase (see text).

G": glucuronidase added after hydrolytic incubation.

S: potassium hydrogen saccharate 0.0015M (final), pH 5.1.

A: (or APG) fresh aqueous o-aminophenyl-glucuronide.

I: synthetic incubation, 37°for 30 minutes, pH 7.4;

I' /

TABLE III (continued).

Explanation of Key (continued).

I': hydrolytic incubation, 37° for $2\frac{1}{2}$ hours at pH 5.2.

N: no synthetic incubation.

is almost certainly o-aminophenylglucuronide, for it is destroyed by a preparation containing β-glucuronidase and this destruction is inhibited when the specific inhibitor for β-glucuronidase is present in the system. Moreover, its degree of destruction in these two cases is of the same order as for a comparable amount of known o-aminophenylglucuronide.

This experiment was repeated with ox spleen glucuronidase (Cox, 1952), kindly given by Dr.R.I.Cox, as the hydrolysing enzyme, and in spite of difficulties owing to pigment liberation on incubation of this preparation, the results obtained led to a similar conclusion as did those above. (Table V).

The above evidence was sufficient to show, in default of the actual isolation of the products themselves, that the action of the unknown factor on the system resulted in the formation of the glucuronide of o-aminophenol or l-menthol. Because of the minute amounts of glucuronide produced, it was not at that time possible to surmount the very great technical difficulty of isolating sufficient of the pure product for a chemical characterisation.

TABLE IV.

Hydrolysis of Synthetic Product by Mouse-liver β-glucuronidase.

	µg. o-aminophenol conjugated
Control synthesis	6.8, 7.2
Synthetic product treated with β-glucuronidase	4.9, 4.7
Synthetic product treated with β-glucuronidase and saccharate	6.5, 7.0
Known o-aminophenylglucuronide	8.5, 8.4
" " " treated with β-glucuronidase	5.3, 4.7
Known o-aminophenylglucuronide treated with β-glucuronidase and saccharate	7.5, 7.5

TABLE V.

Hydrolysis of Synthetic Product by Ox-spleen β-glucuronidase.

	μg. o-aminophenol conjugated
Control synthesis	6.5, 6.0
Synthetic product treated with β-glucuronidase	5.2, 5.6
As above, in 0.0015M-saccharate	5.9, 6.0
Known o-aminophenylglucuronide	8.3, 8.4
Above, treated with β-glucuronidase	5.9, 6.3
" " " " in 0.0015M-saccharate	7.9, 8.4

SECTION III: Examination of Homogenate:

To facilitate subsequent experiments, the enzyme present in the homogenate was examined in the hope that a stable preparation of it could be produced, capable of being standardised and stored. It was found, however, to be a rather unstable system, sensitive to the usual precipitation and storage techniques, and further investigation was deferred until the unknown factor itself could be purified. The results of the few preliminary experiments on the homogenate enzyme are given below.

Stability of Fresh Homogenate.- 10 per cent homogenate from two mouse livers was kept for various periods at room temperature, at 0° in ice-water, and in the frozen state at -20°. Results obtained indicated that there was little falling-off in activity up to an hour when kept cold, but if left overnight most of the potency of the enzyme disappeared. (Table VI).

Acetone Powder Preparation.- It was thought that if the enzyme were precipitated from the homogenate by acetone and dried, the powder would retain the same level of activity on storage. However, such a process destroyed the conjugating power of the preparation.

The method was that described by Kaplan and Lipmann (1948) for two experiments; in the third the drying of the powder was done *in vacuo*. All these /

these preparations were rosy-pink, not brown, indicating that no denaturation of haemoglobin had occurred, a condition that Kaplan and Lipmann found essential for the activity of their powders. In spite of this, no synthesis at all was detected, at several levels of the acetone powder of the homogenate.

Low-speed Centrifugation of Homogenate.- This was performed to see whether the enzymic activity was associated with the gross cellular particles, or was present unadsorbed and apparently soluble. The activity survived the centrifuging and subsequent recombination, all experiments showing that the enzyme was present only in the supernatant, although the presence of the residue seemed to enhance its power somewhat.

The 10 per cent homogenate was spun in the cold for 5 minutes at 3300 r.p.m. and the supernatant poured into a cooled tube. The residue was washed once with the original volume of fresh cold alkaline isotonic KCl, then resuspended in a similar volume. To make a recombined preparation, a portion of the residue was suspended in an equivalent portion of the supernatant. The results are shown in Table VII.

TABLE VI.

Stability of Fresh Homogenate.

Treatment of Homogenate	µg. o-aminophenol conjugated
Fresh, untreated	4.1, 4.5
15° for 15 minutes	4.2, 3.8
0° for 15 minutes	4.2, 3.9
0° for 30 minutes	3.7, 3.7
0° for 60 minutes	3.8, 3.7
-20° for 30 minutes	4.0, 3.7
0° for 24 hours	1.8, 1.6
-20° for 24 hours	1.1, 1.4

TABLE VII.

Low-speed Centrifugation of Homogenate.

Homogenate Fraction	µg. o-aminophenol conjugated (a)	(b)
Whole homogenate, not spun	4.3, 4.2;	4.1, 4.1
Supernatant only	3.4, 3.4;	2.6, -
Residue only	1.2, 1.1;	0.7, -
Supernatant and residue recombined	3.4, 3.8;	- -

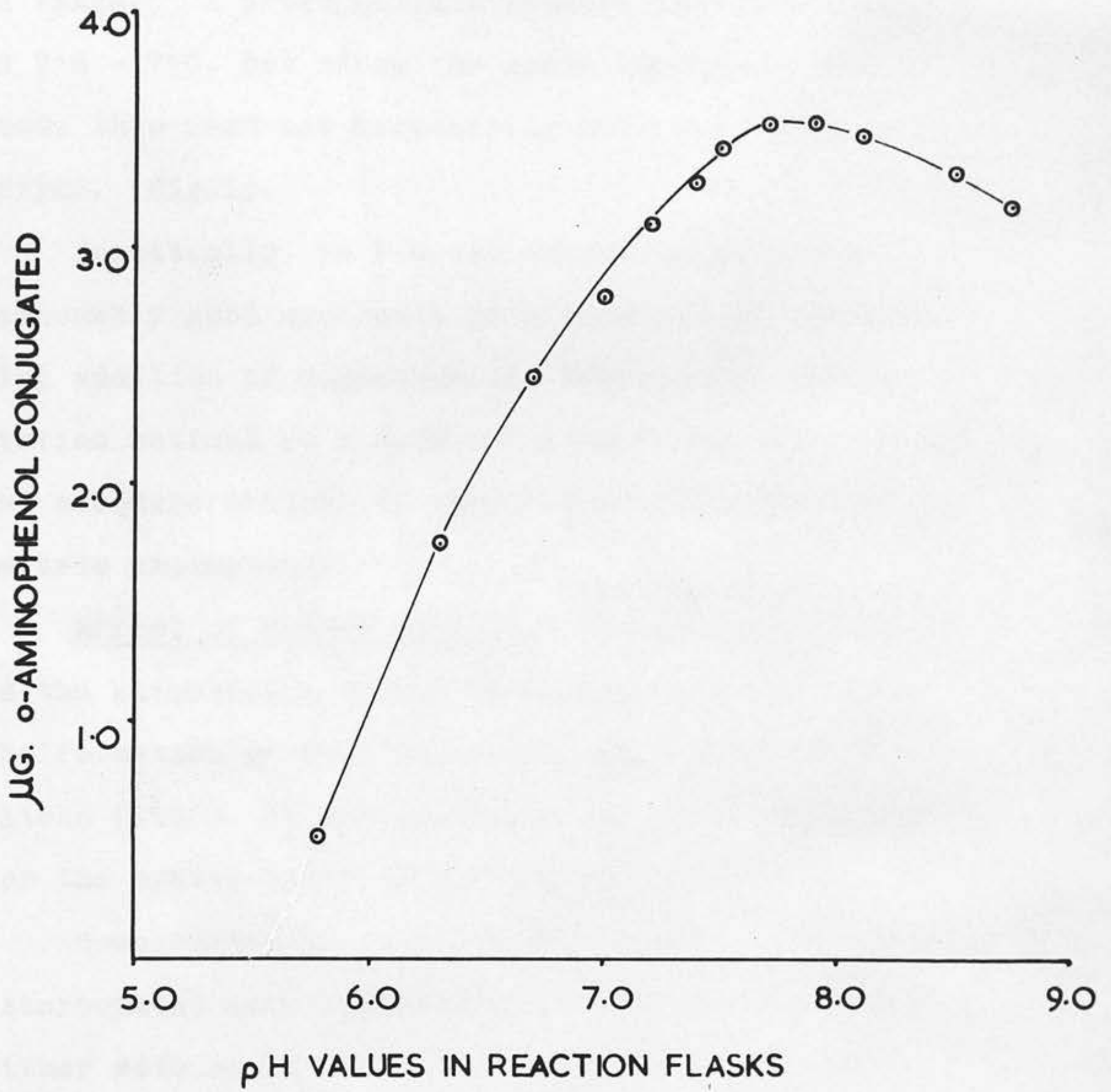
(Two experiments, (a) and (b)).

SECTION IV: Examination of Synthetic Process:

A method having been found for the formation of glucuronides in liver homogenates, it was decided to investigate briefly the nature of the process as a whole before proceeding with the laborious task of purifying, isolating and identifying the essential factor in the boiled liver extract.

Presence in Other Tissues.- 10 and 20 per cent homogenates of other tissues besides liver were employed, but in no case was there any obvious synthesis, with or without the added extract. Tissues studied were mouse kidney, spleen, brain and intestine, and rat kidney, and the procedure was exactly as that giving good conjugation in liver preparations.

Effect of pH.- The effect of pH was next investigated. Using phosphate buffer only, of final molarity 0·025M, it was found that the buffering power above pH 7·6 was insufficient to prevent the pH value from falling to 7·4 during incubation. Since increasing the phosphate concentration would have little effect in the alkaline range, and caused precipitation with the added magnesium ion, veronal buffers were tried; to obviate differences due to variation in cations added with the buffer, the Michaelis fixed-ion buffer (Michaelis, 1931) was used, with potassium replacing sodium. To prevent any gradual change of pH, the time of incubation was cut to 15 minutes and the pH checked immediately on /



EFFECT OF pH ON SYNTHETIC PROCESS

FIG. I

on cooling.

Readings with veronal were never as high as with phosphate buffer, but the graph gives an indication of the conjugation of the system over a wide pH range. A broad maximum appears in the region pH 7·6 - 7·9, but since the crude homogenate was used, this need not necessarily hold for the pure enzyme. (Fig.1).

Practically, pH 7·4 was chosen as giving a reasonably good synthesis at a constant pH, permitting addition of magnesium ion without the precipitation noticed at a more alkaline value, and allowing adequate control of the pH for subsequent colorimetric estimation.

Effect of Carbon Dioxide.- Storey having reported the stimulating effect of bicarbonate ion upon the formation of o-aminophenylglucuronide in liver slices (1950), it was necessary to see if this held for the system under investigation.

Homogenates in isotonic KCl (without the usual bicarbonate) were employed and flasks were gassed either with oxygen or with oxygen and 5 per cent carbon dioxide; for the latter, bicarbonate buffer was used together with phosphate, the final pH after equilibration being 7·3. No difference could be detected between the amounts of o-aminophenol conjugated in the two atmospheres (Table VIII).

When the experiment was repeated, with cups containing 20 per cent KOH and small wicks of filter paper /

TABLE VIII.

Effect of CO_2 on Synthetic Process.

Gas Phase	Buffer	µg. o-aminophenol conjugated
Oxygen	0.0040M-phosphate	4.1, 4.0
Oxygen	0.0025M-phosphate	3.9, 4.0
Oxygen and Carbon Dioxide	0.0025M-phosphate and 0.0020M-bicarbonate	3.7, 3.8, 4.0

TABLE IX.

Effect of CO_2 on Synthetic Process (continued).

Gas Phase	Buffer	µg. o-aminophenol conjugated
Oxygen, (KOH cups)	0.0040M-phosphate	3.1, 3.4, 3.6
Oxygen and Carbon Dioxide	0.0025M-phosphate and 0.0020M-bicarbonate	3.5, 3.8, 3.6

paper present in the oxygen-gassed flasks to absorb any carbon dioxide formed during the incubation, there was again no detectable difference between the two sets. (Table IX).

Effect of Nitrogen.- From the above results it was suspected that the conjugating system was dissimilar to that obtaining with whole tissue preparations. These suspicions were proved by its behaviour under anaerobic conditions. Adenosine triphosphate (Messrs. Light & Co.) was present in some of the flasks to see whether it increased the synthesis in these circumstances by providing energy for synthetic processes under anaerobic conditions. No provision was made for absorbing the last traces of oxygen, but the anaerobic flasks were well flushed out with nitrogen; all solutions used were likewise gassed.

It will be seen from Table X that the absence of oxygen made no difference to the degree of conjugation attained and that adenosine triphosphate had no effect on the anaerobic results.

The small synthesis obtained without added extract (the 'endogenous' synthesis) had a like behaviour, being unaffected by anaerobic conditions (Table X). Advantage was taken of the absence of oxygen to incubate one of these flasks without added ascorbic acid; no increase in the small conjugation was obtained, showing that ascorbic acid was no inhibitor of the unfortified process.

These /

TABLE X.

Effect of Nitrogen and Adenosine Triphosphate on Synthetic Process.

	µg. o-aminophenol conjugated
(a) Gassed O_2	5.8, 6.2
Gassed N_2	6.0, 5.8
" " with 0.004M ATP	6.1, 5.9
(b) Gassed O_2	5.8, 5.7
" " with no extract	1.3, 1,3
Gassed N_2	5.7, 5.8
" " with no extract	1.3, 1.1
Gassed N_2 " " " and no ascorbic acid	1.2, -

TABLE XI.

Effect of Sulphate Ion upon Synthetic Process.

Added Compound	µg. o-aminophenol conjugated
0.005M-potassium sulphate (final)	4.2, 3.9
0.010M-potassium chloride (final)	4.2, 4.1

These anaerobic results could be repeated with a pure solution of the factor and were therefore not due to any excess of contaminating adenosine triphosphate.

Effect of Sulphate.- In the cases of both carbon dioxide and nitrogen treatment, this system differed from slices and it was therefore interesting to see whether sulphate ion inhibited to the same extent as found by Storey (1950) in those preparations.

In Storey's experiments, 0·0024M-sulphate gave 32-55 per cent inhibition; 0·0050M-potassium sulphate was used here to ensure that any reasonable inhibition would be noticeable and an equivalent amount of potassium ions was added to the control to counter any cation effect (see Table XI). No trace of any inhibition was found at this concentration.

Effect of Glucuronate.- Storey (1950) found that glucuronide synthesis in slices was inhibited by glucuronate ion. Using various concentrations of glucurone (Messrs. Roche Products, Ltd.),brought to neutrality with $KHCO_3$ solution (Storey, 1950), some significant inhibitionsof the extract-fortified conjugation were obtained (Table XII), but they were not so striking as those found in the slice experiments. While the inhibition due to 0·005M-glucuronate was 18 per cent, being the same for this system as for slices, the higher concentrations of /

TABLE XII.

Effect of Glucuronate (Messrs. Roche Products Ltd.) on Synthetic Process.

Final concn. glucuronate	µg. o-aminophenol conjugated and degree of inhibition		
	(a)	(b)	(c)
0	5.0, 4.8	6.2, 6.6	5.5, 5.4
0.0005M	4.4, 4.6	- -	- -
0.001M	4.5, 4.8	- -	- -
0.005M	3.8, 4.0(19%)	5.4, 5.3(18%)	- -
0.01M	- -	4.8, 4.6(27%)	- -
0.02M	- -	4.1, 4.2(38%)	3.5, 3.4(37%)

TABLE XIII.

Effect of 'Potassium Glucuronate' on Synthetic Process.

Final concn. glucuronate	µg. o-aminophenol conjugated
0	7.9, 7.8
0.006M	8.0, -
0.012M	7.7, -
0.02M	8.1, 8.0

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of 0·01M and 0·02M gave inhibitions of only 27 per cent and 38 per cent respectively, against 56 per cent and 85 per cent obtained with slices.

With the 'potassium glucuronate' supplied by Messrs. Corn Products, Ltd. (and kindly given by Dr. J. Paul), used as the untreated neutral solution, no inhibition was obtained at all (Table XIII), and this preparation behaved similarly with slices (Dr. J. Paul, private communication).

The chemistry of glucuronic acid having been so little studied, all that can be deduced is that treatment of the Roche glucurone as described above results in an inhibition of about half that occurring with slices, and that this inhibition is not apparent with the substance sold as 'potassium glucuronate'. The matter was not further investigated at this stage, it being judged sufficient to show how the crude homogenate compared with Storey's slice preparations.

Effect of Saccharate.- The effect of saccharate was studied for two reasons: firstly, because Storey (1950) had found it inhibitory, a concentration of 0·01M giving a 30 per cent inhibition with slices, and secondly, because, in spite of the recent work of Levvy and his co-workers, the idea that β-glucuronidase might have a synthetic role was still encountered. The results (Table XIV) show that on this system saccharate had no effect at all and illustrate yet another difference from the slice experiments;/

TABLE XIV.

Effect of Saccharate on Synthetic Process.

Final concn. saccharate	μg. o-aminophenol conjugated (a)	(b)
0	4.7, 5.0	5.5, 5.4
0.0015M	4.9, 4.7	-
0.005M	- -	5.4, 5.0
0.010M	- -	5.4, 5.3
0.015M	- -	5,7, 5.8

TABLE XV.

Effect of Sodium and Potassium Ions on Synthetic Process.

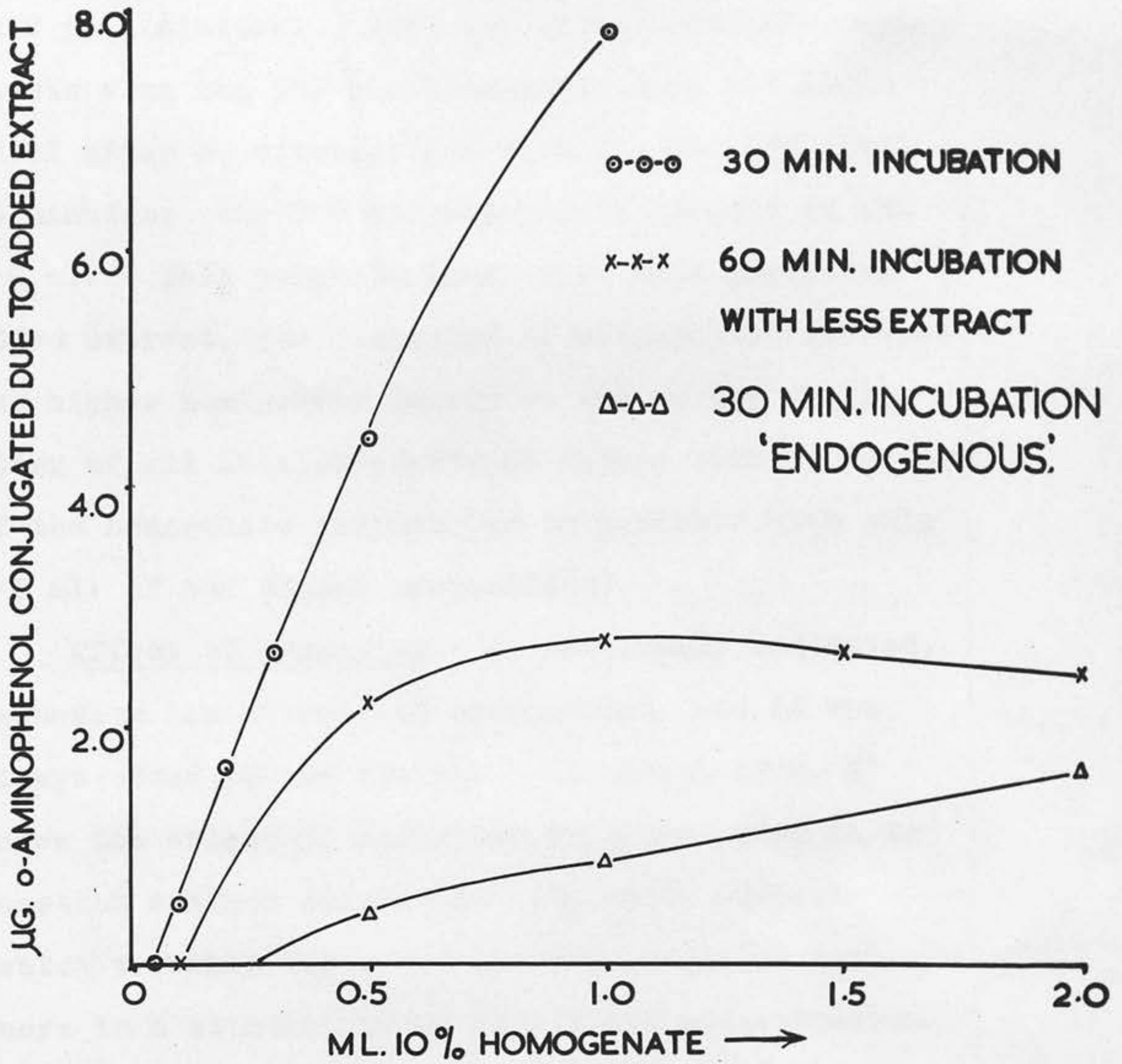
Treatment of system	μg. o-aminophenol conjugated	
(i) Buffered with K phosphate	8.7, 8.6	
(ii) Buffered with Na phosphate	7.8, 8.2	
	(K ion)	(Na ion)
0.0015M ion added to (ii)	7.5, 7.6	7.5, 7.6
0.009M ion added to (ii)	7.6, -	7.7, -
0.015M ion added to (ii)	7.7, -	7.5, 7.5

experiments; they also suggest that β-glucuronidase, known to be inhibited by saccharate, plays no part in this particular process (though a synthesis by an enzyme at this pH might not require the same inhibitor as a hydrolysis by it at an acid pH). Since this work, Levvy (1952) has reported that saccharo-1:4-lactone present in the saccharate solution is the actual inhibitor of β-glucuronidase hydrolysis, but a preparation of this also has no effect on the reaction studied (Dr. I. D. E. Storey, private communication).

Effect of Homogenate Concentration.- With the amount of extract employed, increasing the amount of 10 per cent homogenate also increased the conjugation, over the range 0·05 to 1·0 ml.; beyond this the only effect observed was a slight decrease with 2·0 ml. homogenate. Greater concentrations of the tissue brought difficulties with protein precipitation and in the colorimetric estimation. Controls covered each level for both 'endogenous' synthesis and tissue blanks, and the incubation was for 30 minutes at 37°. (Fig. 2).

The upper curves on the graph concern the conjugation due to added extract and the lower that due to the 'endogenous' conjugating activity of the homogenate, which increases fairly regularly with the concentration of tissue.

Time of Incubation.- The amount of conjugation over various time intervals was studied in systems with /



THE EFFECT OF HOMOGENATE CONCENTRATION UPON SYNTHESIS

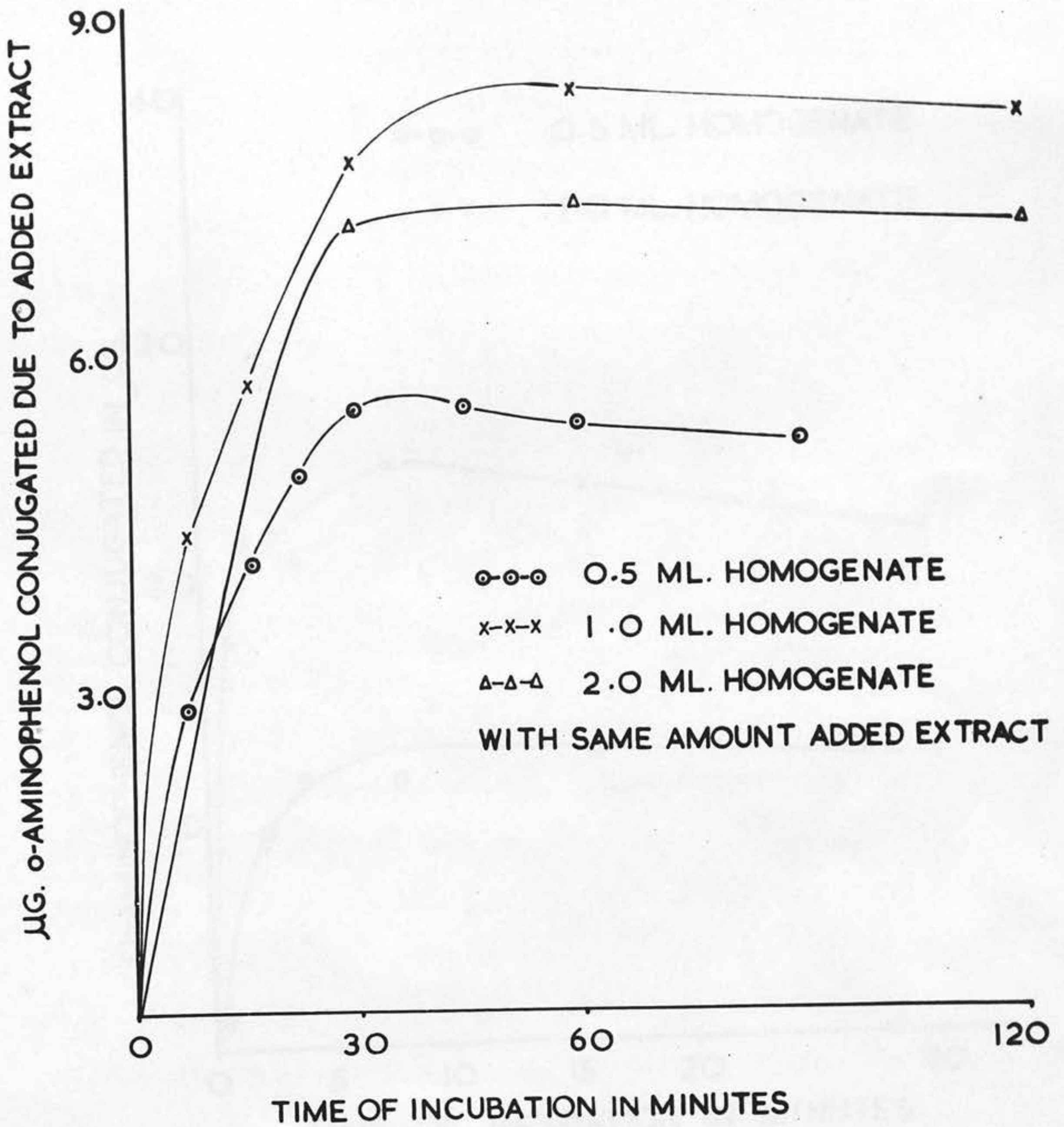
FIG. 2

with and without added extract and at several levels of homogenate.

It will be seen (Fig. 3) that most of the measurable conjugation in unfortified systems is complete at 2 to 4 minutes. With the extract present, synthesis with the 0·5 ml. homogenate does not cease until after 30 minutes, and with 1·0 ml. not until 60 minutes; the 2·0 ml. homogenate behaves as the 1·0 ml. This suggests that, with this amount of added extract, the cessation of conjugation at these two higher homogenate levels is due to the utilisation of all available extract rather than to failure of the homogenate enzymes (as is probable with only 0·5 ml. of the tissue preparation).

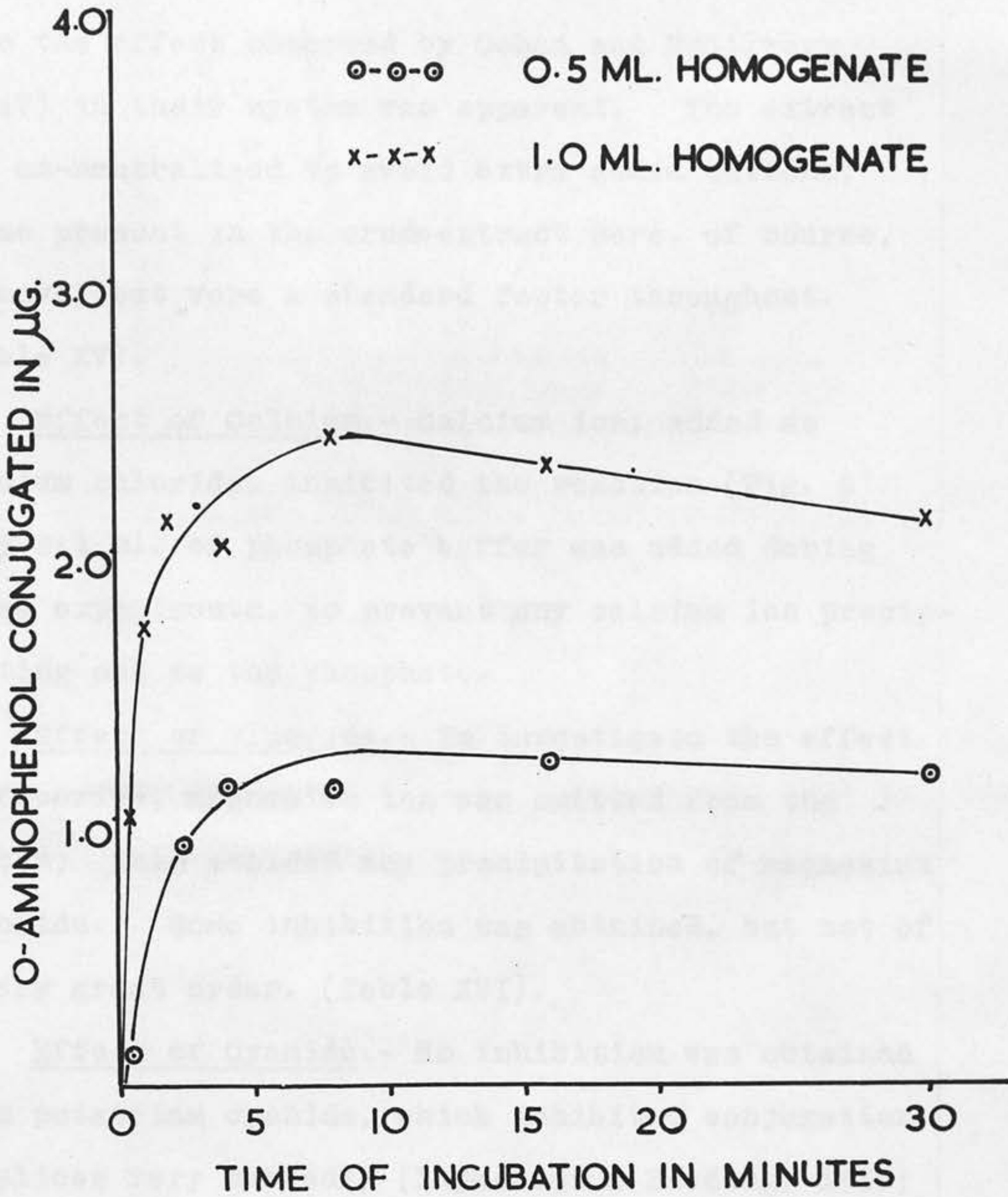
Effect of Magnesium.- As previously indicated, magnesium ion stimulated conjugation, and it was always added to the system. The graph (Fig. 4) shows the effect of magnesium chloride added to the reaction mixture reinforced with crude extract (which probably contained its own magnesium ion). There is a stimulation up to a final concentration of 0·013M, after which a slight inhibition is apparent. An added concentration of 0·015M (final) was always present in subsequent systems.

Effect of Sodium and Potassium.- Since a homogenate system usually tries to reproduce intracellular conditions, potassium ions were always used in preference to sodium ions. However, Table XV shows that both have almost the same effect when added /



TIME OF INCUBATION ON EXTRACT-FORTIFIED SYSTEM

FIG. 3a



<u>TIME OF INCUBATION ON UNFORTIFIED SYSTEM</u>

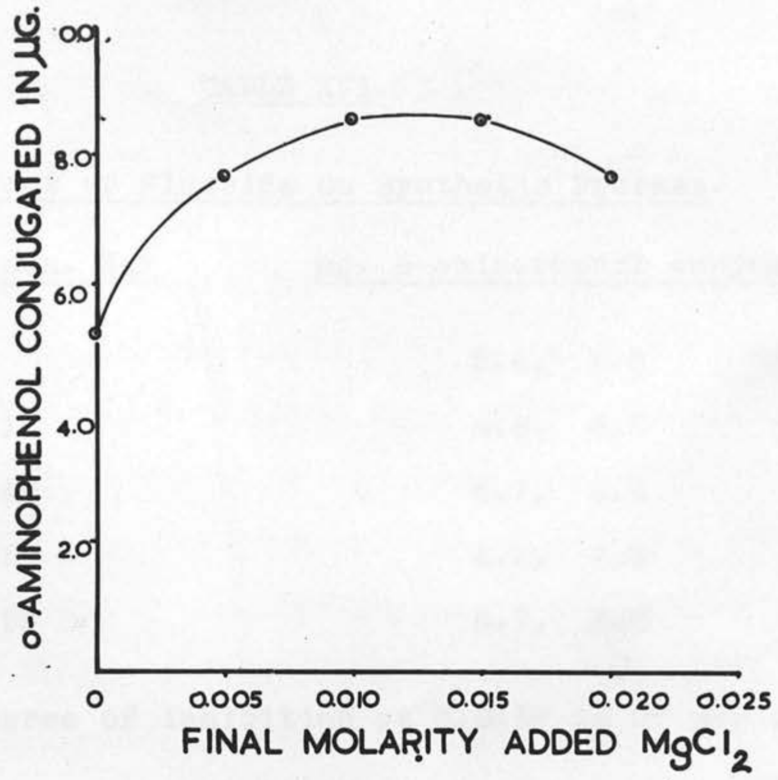
<u>FIG. 3b</u>

added to the medium buffered with sodium phosphate at pH 7·4. Synthesis by an identical system buffered with potassium phosphate at the same pH was a little higher, but not significantly, and nothing like the effect observed by Cohen and McGilvery (1947) in their system was apparent. The extract was un-neutralised to avoid extra added cations; those present in the crude extract were, of course, unknown, but were a standard factor throughout. (Table XV).

Effect of Calcium.- Calcium ion, added as calcium chloride, inhibited the reaction (Fig. 5). Only 0·1 ml. of phosphate buffer was added during these experiments, to prevent any calcium ion precipitating out as the phosphate.

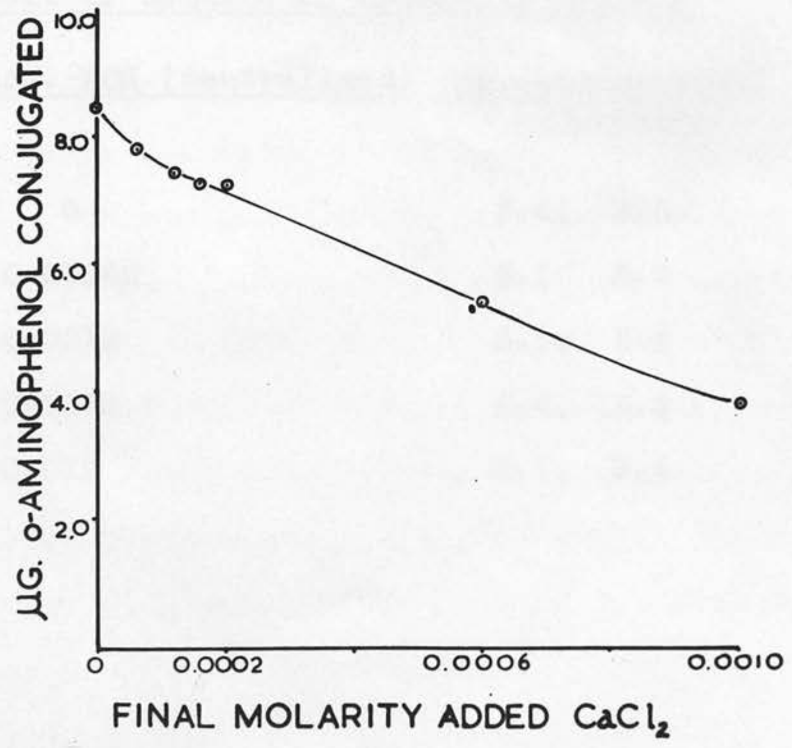
Effect of Fluoride.- To investigate the effect of fluoride, magnesium ion was omitted from the system; this avoided any precipitation of magnesium fluoride. Some inhibition was obtained, but not of a very great order. (Table XVI).

Effect of Cyanide.- No inhibition was obtained with potassium cyanide, which inhibited conjugation by slices very markedly (Lipschitz & Bueding, 1939; Storey, 1950). Since cyanide poisons the oxidative mechanisms of tissue and this system has been shown to be anaerobic, such a result is not surprising. The KCN used was neutralised before addition. The results in Table XVII were obtained with a fairly pure solution of the unknown factor.



EFFECT OF MAGNESIUM ION ON SYNTHESIS

FIG. 4



EFFECT OF CALCIUM ION ON SYNTHESIS

FIG. 5

TABLE XVI.

Effect of Fluoride on Synthetic Process.

Final concn. NaF	µg. o-aminophenol conjugated
0	5.4, 5.0
0.001M	4.8, 4.5
0.004M	4.7, 4.5
0.008M	4.3, 4.5
0.015M	3.7, 3.8

(Degree of inhibition at 0.015M is 27 per cent)

TABLE XVII

Effect of Cyanide on Synthetic Process.

Final concn. KCN (neutralised)	µg. o-aminophenol conjugated
0	3.4, 3.3.
0.0004M	3.1, 3.2
0.001M	3.2, 3.3
0.004M	3.4, 3.3
0.01M	3.1, 3.1

Summary.- The above experiments were designed, as has been stated, to throw some light on the nature of the reaction whereby glucuronides were produced in the system on incubation with the crude unknown factor. Such crudeness of both factor and enzyme system utilising it ensured that these experiments could only be preliminary ones, yet the results obtained were so striking that there could be no doubt as to the marked difference between the reaction studied and that occurring with intact slices.

The anaerobic nature of the process and the lack of any effect from added adenosine triphosphate suggested that no energy derived from oxidative reactions was needed for the production of o-aminophenylglucuronide in this system. The absence of stimulation from carbon dioxide or inhibition from cyanide and fluoride further suggested that a much simpler process was being studied than that observed in slices. It was considered that some compound was present in the crude extract which was readily used by the homogenate to form glucuronides with the substrates and which might very well be an intermediate in the complete synthesis of conjugated glucuronic acid and one occurring at a late stage in that synthesis; its identification was therefore to be regarded as most important.

SECTION V: Examination and Purification of Extract:

The above brief examination of the synthetic system and its crude enzymes having been completed, attention was turned to the unknown factor whose addition was necessary for the production of glucuronides. In this section will be found an investigation into its sources and stability, followed by the development of a method for its purification, isolation, and preliminary identification.

Sources:

Kidney.- A boiled kidney preparation was added to the system in place of the crude extract from boiled liver. 8 fresh mouse kidneys were homogenised in 3·5 ml. of boiling water, and the supernatant added at two levels, 0·3 and 0·8 ml. Colorimetric estimation failed to reveal any synthesis above that of the unfortified system.

Muscle.- This was added in the same manner as the kidney preparation. 10g. fresh rat muscle was dropped slowly into 20 ml. boiling water and disrupted in the homogeniser, giving rise to a cloudy supernatant. Nothing more than a trace of extra conjugation could be detected at the highest level (1.0 ml.) added.

Yeast.- Yeast was examined as a possibly convenient source of the factor. Great interference with the Levvy and Storey colour estimation was found, presumably from p-aminobenzoic acid, necessitating an acetone precipitation of the yeast extract. /

extract.

500 g. baker's yeast was made into a paste with 50 ml. of cold water; 30 g. trichloroacetic acid in 100 ml. of ice-cold water was added, and the mixture stirred vigorously for 20 minutes at 0°; 0·8 volume of ethanol at -20° precipitated the starch, which was then centrifuged down. The supernatant was treated with 4 volumes of acetone at -20°, and the resulting precipitate washed with acetone and dried. This method (which preserves the greater part of the activity from fresh liver) failed to result in an active preparation in three attempts, although interference was very much reduced.

<u>Wheat Germ</u>.- 1 g. of a dry preparation of wheat germ was disrupted in 5 ml. boiling water, giving a cloudy green supernatant which, when added at levels of 0·2 to 1·0 ml. to the system, did not induce any extra conjugation.

Thus it was considered that fresh kidney or muscle, yeast or dried wheat germ did not contain appreciable amounts, if any, of the unknown factor, and hence liver would have to remain the source of any future preparation.

<u>First Stage of Purification</u>:

(a) <u>Boiled Extract</u>.- As first prepared, the crude boiled extract was a pale green solution, cloudy with glycogen; this cloudiness remained in unincubated mixtures, and diminished by varying amounts with the length of incubation, interfering greatly /

greatly with colorimetric estimations. Activity lessened on storage at 0° (lower temperatures were not then available).

(b) Acetone Precipitation.- It was found that if ten times its volume of acetone were added to the above solution, a precipitate was obtained which, after washing with more acetone and drying *in vacuo*, gave an active solution when redissolved in water. Thus the factor was insoluble in acetone and not destroyed by this process, which removed p-aminobenzoic acid as a source of interference.

This precipitate could be stored as a creamy-white powder over calcium chloride or phosphorus pentoxide *in vacuo* at 0° for several weeks without losing appreciable activity. However, glycogen was also precipitated by acetone and remained an embarrassment.

(c) Removal of Glycogen.- Following the method of LePage and Umbreit (1945), glycogen was precipitated from the solution by adding 1 volume of ethanol at an acid pH; this acidity was most conveniently achieved by extracting the liver originally with ice-cold trichloroacetic acid instead of boiling water. According to LePage and Umbreit, phosphate esters remain in solution under these conditions. From the clear green solution resulting, the active factor could be precipitated as before by more acetone, but this time unaccompanied by glycogen. The following is a typical preparation of /

of the period.

120 g. liver, rapidly excised from three rabbits and cooled in ice, were roughly sliced with scissors and placed in an Atomix blendor (Messrs. Measuring & Scientific Equipment Co., Ltd., London). After disruption in 50 ml. ice-cold water for 1 minute at high speed, the mash was poured into a cooled beaker and 18 g. trichloroacetic acid (Analytical Reagent Quality) added gradually in 40 ml. water, with constant stirring, all the vessels being immersed in ice-water. The exact concentration of acid required seemed to vary with the condition of the livers, but was about 20 per cent final volume and had to be sufficient to cause a slight granulation of the precipitated protein, without which the subsequent operation, that of squeezing the mash in two layers of muslin, was made impossible; if conditions were correct, only a cloudy yellow solution came through the muslin and was collected in a cooled vessel, the granular protein remaining behind.

After this straining, an equal volume (100 ml.) of cold ethanol was added and the mixture stirred and centrifuged in the cold. The supernatant was then poured into 10 volumes of acetone at the lowest available temperature, stirred, and allowed to flocculate for an hour. The yellow precipitate was washed well with acetone to remove all traces of trichloroacetic acid and then with peroxide-free ether, finally being dried *in vacuo*. Kept at -20° in /

in vacuo over P_2O_5, the resulting cream powder (subsequently to be referred to as the 'crude extract' preparation) remained active for many months.

Stability on Storage.- The solution obtained from the above cream powder had a pH of 3·5 and lost its activity over a period of days at 0° unless neutralised. Neutralising the preparation immediately after glycogen precipitation was then adopted and prolohged the activity considerably. In this condition the powder, stored as above, had retained 80 per cent of its original activity after 5 months.

The concentration generally used in the earlier experiments was 10 mg. per ml. at pH 7·4; 5 mg. of the powder was employed per 3 ml. reaction mixture, colour interference being apparent only when more than 20 mg. was added.

Stability in Liver.- For convenience, the livers of pig, ox and sheep were tried in place of those from the rabbit, but at no time could an active preparation be obtained from them; even when bought warm at the slaughter-house and allegedly 'fresh from the animal', immediate slicing and cooling failed to reproduce the effect of fresh rabbit livers. That this might have been due to rapid destruction in the dead organ was indicated by the fact that any delay in excision and cooling of the rabbit livers themselves inevitably resulted in a partial or total loss of activity in the subsequent preparations; 5 to 10 minutes' delay gave poor to negative /

negative results, immediate excision from the animal seeming to be essential. Freshly-excised liver stored frozen overnight also failed to yield an active extract.

Stability in Acid and Alkali.- Suspicions that the crude factor was labile to acid and alkali were confirmed by its behaviour in 0·1N-HCl and KOH at 100°. (Fig. 6). Allowing for the small synthetic powers of the unfortified homogenate (the 'endogenous' synthesis), 90 per cent of the activity of the added factor was destroyed within 4 minutes at 100° in 0·1N-HCl, and total destruction was apparent by 8 minutes. Approximately the same destruction (80 per cent after 4 minutes) was observed with 0·1N-KOH.

At this stage there was no evidence to suggest that the unknown factor could not be a simple phosphate ester. It was thought possible that glucuronic acid-1-phosphate itself (never, up to then, synthesised), whose participation in glucuronide synthesis had been postulated by Storey (1950), might be the required compound. Accordingly, many experiments were performed to test this likelihood; these are outlined below and consisted of tests for glucuronic acid in the extract, behaviour of the extract with phosphatases, and attempted synthesis and trial of α- and β-glucuronic acid-1-phosphate.

Tests for Glucuronic Acid.- Tollens' naphthoresorcinol test for uronic acids (Tollens and Rorive, 1908 /

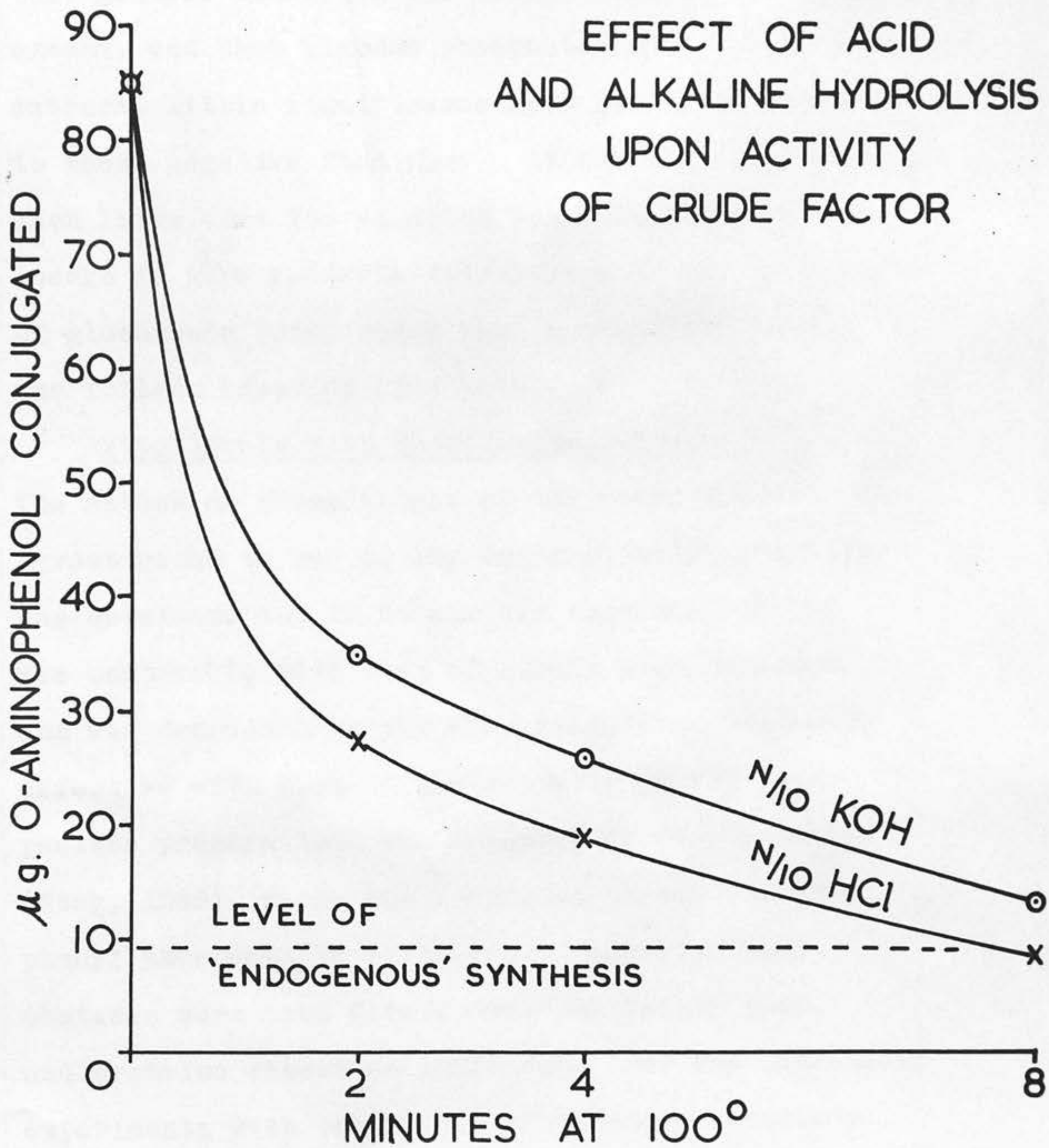


FIG. 6

1908) was carried out several times, but never with any true positive result; the colour obtained was nearer to the browny-pink from glucose than to the purple from glucuronic acid. Since it was known that glucose inhibited the colour reaction to some extent, and that glucose phosphates would be in the extract, little significance need have been attached to these negative findings. It was not till very much later that the solution was obtained pure enough to give reliable indications of the presence of glucuronic acid, using Paul's modification of the Tollens reaction (see Methods).

Experiments with Phosphatase Preparations.- The action of phosphatases on the crude extract was investigated to see if any destruction of activity was obtained, and if so whether this destruction was comparable with that of simple phosphate esters and was decreased by the same inhibitors that were effective with them. The activity of the phosphatase preparations was measured by King's method (King, 1946), where the liberated phenol from diphenyl phosphate is estimated. Alkaline phosphatases were used first, crude or fairly pure, and cysteine chosen as inhibitor; for the subsequent experiments with crude acid phosphatase, attempts at inhibition were made with molybdate ion.

(a) Crude Alkaline Phosphatases:

Crude Mouse Kidney Phosphatase.- This was a 10 per cent water homogenate of fresh mouse kidney /

kidney, and was tested by King's method; it was diluted to 1 per cent and 0·1 per cent solutions. 0·2 ml. of a 0·1 per cent solution liberated 0·0045 mg. phenol under King's conditions. The experimental procedure was as follows and serves to illustrate the method for all phosphatase experiments (Table XVIII).

The extract was incubated with the phosphatase preparation and this mixture subsequently heated at 100°, driving off the carbon dioxide of the buffer and destroying the enzyme; the phosphate buffer and the remainder of the synthetic system were then added to the treated extract and further incubation took place as usual. 4 ml. of precipitant were added and 5 ml. aliquots taken for colorimetric estimation of the glucuronide produced. The pH was checked at each stage by the glass electrode. Controls (see Table) were employed to cover various possible interferences in the synthesis and its estimation, and the effect of pH on the extract.

The results showed that the activity of the extract was destroyed by the crude mouse kidney phosphatase at pH 9·2, and were confirmed in another experiment.

Crude Mouse Intestinal Phosphatase.- A 10 per cent water homogenate was made with the first 4 to 6 inches of fresh mouse small intestine, slit and washed gently in saline. 0·2 ml. of a 10 per cent solution liberated 0·015 mg. phenol. Results were /

TABLE XVIII.

Scheme of Procedure for Hydrolysis of Extract by Crude Mouse Kidney Phosphatase.

Key		Design	µg. o-aminophenol conjugated
$\frac{E\ C}{I}$	I'	Synthesis with unhydrolysed extract.	4.1, 4.0
$\frac{E\ C\ P}{I}$	I'	Synthesis with hydrolysed extract.	0.8, 1.0
$\frac{E\ C}{N}$	I'	Synthesis with extract not hydrolytically incubated.	4.0, -
$\frac{E\ C\ P}{N}$	I'	Check on blank of unincubated phosphatase.	4.2, -
$\frac{C}{I}$	I'	Synthesis with no extract.	0.6, -
$\frac{E\ C\ P}{I}$	N'	Tissue blank of incubated hydrolytic system.	0, -
$\frac{E\ C}{N}$	N'	Extract blank.	(Control)

Hydrolytic System: 1.0 ml. carbonate-bicarbonate buffer, 1.0 ml. crude extract (if added), 0.1 ml. phosphatase preparation (if added), water to 2.1 ml.

Synthetic System: (Made up in bulk, and added to all flasks on ice). 0.2 ml. phosphate buffer, 0.2 ml. ascorbic acid /

TABLE XVIII (continued):

acid and o-aminophenol mixture, 0.1 ml. 0.015M $MgCl_2$, 0.3 ml. 20 per cent mouse liver homogenate; this added to each flask after hydrolytic incubation, addition of 0.1 ml. N-HCl, and heating at 100° for 2 minutes.

Explanation of Key:
- E: crude extract solution, 10 mg./ml.
- C: M/10 Na_2CO_3 and $NaHCO_3$ buffer at pH 9.2, at 37° (Delory and King, 1945). (Final molarity 0.05).
- P: 10 per cent crude mouse kidney phosphatase.
- I: hydrolytic incubation, 45 minutes at 37°.
- N: no hydrolytic incubation.
- I': synthetic incubation, 30 minutes at 37°.
- N': no synthetic incubation.

were obtained which showed that this preparation also destroyed the unknown factor. It was found that 0·1 ml. of a 10 per cent concentration almost completely destroyed the activity after 20 minutes' incubation, but only to about 25 per cent after 10 minutes. To study the action of inhibitors a 15 minute incubation was used.

Effect of Cysteineon Crude Mouse Intestinal Phosphatase.- According to Schmidt and Thannhauser (1943), cysteine inhibits alkaline phosphatase. With this preparation no inhibition of extract destruction was found at all (Table XIX(a)), using cysteine of final molarity 0·005.

(b) Purer Alkaline Phosphatases:

Purer Dog Intestinal Phosphatase.- It was thought that purer preparations of phosphatase might yield more conclusive information.

From 30 g. dog intestinal mucosa a solution of phosphatase was prepared by the method of Abul-Fadl, King, Roche and Thoai (1948). After the second acetone precipitation, a flaky powder was obtained which had considerable phosphatase activity in the King test; however, whilst having identical power in the King test with the crude mouse kidney and intestine phosphatases, this purer preparation was much less capable of destroying the extract's activity. For example, a solution of purified dog intestinal phosphatase splitting the same amount of diphenyl phosphate as 0·1 ml. 1 per cent crude mouse intestinal /

TABLE XIX.

Effect of Phosphatase Preparations on Crude Extract.

Showing conjugation attained with variously-treated solutions of the crude extract.

	µg. o-aminophenol conjugated
(a)	
Extract unhydrolysed.	3.4, 3.1
Extract hydrolysed with crude mouse intestinal phosphatase.	1.7, 1.7
Ditto, but 0.005M-cysteine added.	1.5, 1.8
(b)	
Extract unhydrolysed.	5.3, 5.6
Extract hydrolysed with purer dog intestinal phosphatase.	4.5, 4.1
Ditto, but 0.005M-cysteine added.	5.0, 5.2
(c)	
Extract unhydrolysed.	8.0, 8.2
Extract hydrolysed with phosphatase from Dr. Klenow.	5.8, 6.1
Ditto, but 0.005M-cysteine added.	7.0, 6.7
(d)	
Extract unhydrolysed.	3.7, 3.7
Extract hydrolysed with crude mouse liver acid phosphatase.	1.3, 1.3
Ditto, with 20×10^{-6} M-molybdate.	1.5, 1.3
Ditto, but with 200×10^{-6} M-molybdate added instead.	1.6, 1.6

Effect of cysteine and ammonium molybdate solutions on synthesis and colour estimation controlled.

intestinal phosphatase, needed 50 times greater a concentration to produce the same destruction of the unknown factor as did that crude enzyme. This effect was also noticed with a preparation of fairly pure alkaline phosphatase, kindly given by Dr. H. Klenow, which was prepared from calf intestinal mucosa by the method of Schmidt and Tannhauser (1943) (up to the beginning of the adsorption stages).

However, with these purer enzymes there was some indication of inhibition of extract destruction by cysteine (Table XIX (b) and (c)).

All this evidence seemed to suggest that purer phosphatases did attack the unknown factor, but only slowly; the rapid inactivation of the extract by cruder preparations was probably due to the presence of a greater excess of other enzymes. The unknown factor, then, might not be the simple phosphate ester it was first thought, though it might very well be one of a more complex kind.

(c) <u>Acid Phosphatases</u>.- A few experiments were done with crude mouse liver phosphatase at pH 5·0, using molybdate ion (Bossard, 1947) as inhibitor. Acetate buffer, 0·03M, ph 5·0, was used, with 20 per cent liver homogenate as the source of phosphatase; the incubation was for 30 minutes.

The results (Table XIX (d)) suggested some slight inhibition of extract destruction, but of such a degree as to support the conclusions already reached from the alkaline phosphatase experiments.

<u>Experiments</u> /

Experiments with Glucuronic Acid-1-Phosphate Preparations.- Although the results with phosphatase discussed above had made identification with the unknown factor unlikely, the availability of methods for the preparation of glucuronic acid-1-phosphate allowed a direct test of this compound to be possible. It should be noted that none of the preparations actually made was purified or characterised, it being felt that if glucuronic acid-1-phosphate was indeed the factor, then even a very impure and possibly inhibitory preparation should show some effect. None whatever was observed, and even from pure and characterised α- and β-glucuronic acid-1-phosphates the same negative results were obtained. Because of this the experiments will be dealt with briefly.

α-glucose-1-phosphate was treated with oxidising agents such as potassium permanganate, or hydrogen peroxide and platinised charcoal (c.f. Marsh, 1952), under very mild conditions. The resulting solutions gave a positive Tollens reaction and no reaction for free reducing groups (though this is insufficient evidence for the presence of α-glucuronic acid-1-phosphate). No effect was observed when this solution was added to the system, beyond a very slight inhibition. β-glucose-1-phosphate was prepared (see Experimental Methods) and treated with hydrogen peroxide and platinised charcoal to give a solution positive in the Tollens naphthoresorcinol /

naphthoresorcinol test, only slightly reducing Fehling's solution before hydrolysis and containing ester phosphate. This preparation was likewise ineffective in increasing conjugation of o-aminophenol. The 'β-D-glucuronic acid-1-phosphate' synthesised by a new procedure (see Experimental Methods) also failed to give rise to any effect on the system.

No stimulatory or inhibitory action was found on the unfortified system with α-glucuronic acid-1-phosphate prepared by the method of Marsh (1952), or with the β-isomer, made by the method of Touster and Reynolds (1952), kindly given by Dr. Marsh and Dr. Touster respectively. These two compounds had no effect, stimulatory or inhibitory, when added to the extract-fortified system at a final concentration of 0·0015M.

α- and β-glucose-1-phosphates themselves had a slight inhibitory action (for both of the order of 8 per cent at 0·005M), but insufficient to suggest that it was a competitive one interfering with any utilisation of α- or β-glucuronic acid-1-phosphate.

These results confirm the previous conclusions, that glucuronic acid-1-phosphate probably plays no part in the reaction studied, though, of course, they do not necessarily indicate that it does not participate in the complete synthesis of conjugated glucuronic acid as observed in slices (cf. Touster and Reynolds, 1952; Levvy and Marsh, 1952).

<u>Evidence</u> /

Evidence of Phosphate by Chromatography.- As has been seen, the evidence so far available suggested that the factor could very well be a phosphate ester. Further information was sought from chromatography, it being hoped to prove the presence of phosphate in that separated part of the crude extract which contained the active factor.

Paper Chromatography.- For paper chromatography the method was that of Hanes and Isherwood (1949); the solvent chosen was 60 per cent n-propanol, 30 per cent ammonium hydroxide (sp. gr. 0·880) and 10 per cent water, to avoid probable destruction by the acid mixtures. The paper was Whatman No.541, not further washed, and the tank was all-glass, with a polythene trough for downward chromatography; the upward method (Williams and Kirby, 1948) gave similar results and, being more convenient, was more often used. Detection of phosphate compounds was by the Hanes and Isherwood method, spraying with a molybdate-HCl-perchloric acid mixture, heating at 80°, and hanging in a moist atmosphere. Exposure to hydrogen sulphide gave a rapid and sensitive method of developing the characteristic blue colour of liberated phosphate on the paper. Storage, however, was impossible for after a few days the whole chromatogram became blue; subsequent exposure to ammonia or hydrogen chloride removed the general colour, but left certain areas bright blue which, on ashing, did not contain phosphorus and which might have been due /

due to salt effects.

Early chromatograms of the crude extract showed very considerable smearing, and following the example of Walker and Warren (1951), 'Versene'(ethylene-diamine tetra-acetic acid) was added, but to the trough liquid rather than to the solutions to be chromatographed; this ensured uniform conditions. 20 mg. of the sodium salt were added for each 10 ml. solvent, reducing the smearing very greatly.

Inorganic phosphate (becoming yellow on spraying), was detected in the crude extracts, which gave evidence of at least five other phosphorus-containing compounds, two being very fast-moving; a substance moving like α-glucose-1-phosphate was also very evident. The main purpose of the method, however, could not be achieved for it was found that, under these conditions, the activity of the crude extract was destroyed within 2 hours at 20°, and at least 12 hours were needed for a reasonable separation of the spots. Although activity was retained when ammonia was absent from the solvent system, or was present in much reduced concentration, there appeared then to be little or no movement of the crude extract; or if solvents acidic enough to give separation were used, these destroyed the activity.

Attempts were also made using bands of the extract instead of spots, spraying sufficient of the paper to show the phosphate-containing zones, and /

and eluting the chromatogram in parallel strips; none of these strips, however, containing phosphorus or not, gave any activity. (Versene inhibited the conjugation slightly, but its effect could be overcome by the addition of more magnesium ions.) Because of these repeated failures, the method was discontinued; later it will be seen that under different conditions paper chromatography played a major part in the isolatinn of the pure factor.

Column Chromatography.- Since it was exposure to an alkaline solvent for many hours which destroyed the unknown factor in the preceding experiments, recourse was had to a gentler method of finding whether the activity was associated with phosphate. Columns of Whatman's cellulose powder (acid-washed) were used, with water as solvent. In early experiments the column, after solvent had forced the extract into it for 1 to 2 hours, was extruded and sliced longitudinally into two; one side was sprayed for phosphate and the other divided into sections and incubated in a series of flasks. It was found that the activity was associated with part of the phosphate and quite distinct from the yellow-green pigment of the crude extract, which moved more slowly down the column. In later experiments the eluate itself was collected in fractions and examined for phosphorus and activity, as in the following example:

A column of cellulose powder, 18 x 1 cm.^{2}, was packed /

packed by slight suction, and washed with 50 ml. water by pressure. 20 mg. crude extract were added in 1 ml. water and pressed into the column, when more water was added and pressed through with 25 cm. mercury. 1 ml. fractions were collected, of which half was used for activity tests and the remainder for phosphorus estimations. (Table XX).

With solvents other than water the fractions were not so distinct and the activity came out with the first few drops. The most obvious solution would have been to employ longer columns with a slower run, but apart from collection difficulties the possibility of inactivation was to be feared, and it was doubtful whether a neutral solvent would have been good enough for the separation of such a crude preparation.

Experiments with Ion-exchange Resins.- The use of columns of ion-exchange resin was then considered as a means of inducing a more rapid and complete separation of the mixed phosphate esters of the crude extract. Preliminary experiments indicated that the active factor was bound on to an anion exchange resin and that it could be eluted undestroyed by adding the loaded resin to the reaction flask.

The work of Cohn and Carter (1950) was chosen as a model and a 1 x 1 $cm.^2$ column of Dowex-1 anion exchange resin (Messrs. Dow Chemical Co.) was set up. The resin was swirled in, washed with 2N-HCl, then with /

TABLE XX.

Cellulose Column Experiment.

Activity and phosphate content of successive fractions eluted from a cellulose powder column 18 x 1 cm^2. Solvent used was water; 20 mg. crude extract on column.

Fraction No.	Volume	Inorganic P per 0.15 ml.	Total P per 0.15 ml.	o-aminophenol conjugated per 0.5 ml.
0	8 ml.	0.4 µg.	-	1.6 µg.
1	4 "	0.6 "	-	2.0 "
2	1 "	0.8 "	-	1.7 "
3	" "	1.0 "	-	2.1 "
4	" "	2.5 "	8.0 µg.	2.6 "
5	" "	7.5 "	40.5 "	4.1 "
6	" "	10.5 "	56.0 "	3.2 "
7 Y	" "	5.0 "	40.0 "	2.3 "
8 Y	" "	2.0 "	9.8 "	1.4 "
9 Y	" "	0.5 "	-	1.5 "
10	" "	0 "	-	0.5 "

Standards for inorganic P and total P were 4 and 20 µg. P respectively. Linear relationships for colorimetric P estimation held down to 2.0 µg.; beneath this, quoted figures can only be approximate.

Y denotes fractions containing yellow pigment.

with a large excess of water, and subsequently, 4N-ammonium hydroxide until free from chloride ion. (In most of the experiments the free base form of the resin was used; conversion to the chloride form did not seem to improve or alter the results at all.) Since it was not known what substances were present in the crude extract, a fresh column was prepared for each experiment.

The dilute crude extract solution (35 mg. in 20 ml. N-ammonium hydroxide) was added to the column, which was then washed with 10 ml. water, succeeded by 35 ml. each of 0·01M-NH_4Cl in 0·1M-NH_4OH, 0·01M-NH_4Cl in water, 0·003M-HCl, 0·02M-NaCl in 0·01M-HCl, 0·2M-NaCl in 0·01M-HCl, and M-HCl. (With the first change to acid eluant, the issuing solution was not collected until its pH fell below 7). The effluents from each of these washings were collected in 5 or 10 ml. volumes and immediately neutralised. They were subsequently tested for activity and inorganic and total phosphate. The results showed that the active factor, though in greatly diminished amount, had for the first time been separated from all the inorganic and much of the ester phosphate present in the crude extract; it had been eluted with the 0·2M-NaCl in 0·01M-HCl, while those impurities appeared in the fractions eluted with the preceding solutions. There was still, however, much contamination present; the activity (measured as weight of o-aminophenol conjugated) per atom of 'ester' phosphorus /

phosphorus (as described by LePage and Umbreit,1945) was only about two-thirds higher than that of the crude extract, and the solution was intensely green. The greatest impurity was probably adenosine triphosphate, for this, according to Cohn and Carter, would have been eluted with the same solution that washed out the activity.

Experiments with Apyrase.- It was decided to remove adenosine triphosphate, not by fractional precipitations of the solution with barium and ethanol (Le Page and Umbreit, 1945), which up to that time had been consistently unsuccessful,resulting as they did in the concentration of substances interfering with the Levvy and Storey colour reaction, but rather through the action of apyrase. This enzyme was prepared from potato by the method of Krishnan (1949) in both the soluble and insoluble forms.

It was found that the soluble, unlike the insoluble, form did not attack the active factor in concentrations which were sufficient to destroy a large excess of adenosine triphosphate. That all the latter was destroyed was confirmed by showing the amount of inorganic phosphate freed by acid hydrolysis to be identical with that liberated by exposure to the enzyme. The apyrase-treated extract was then put through the column and the five-fold increased purity of the active fraction was revealed by subsequent estimations (Table XXI).

TABLE XXI.

Relative Purity of Active Solution Preparations.

Relative purity judged by total ester phosphorus needed to conjugate 5.8 ug. o-aminophenol in the standard system.

Preparation	µg. Total Ester P required
Crude acetone-precipitated extract:	150
Ditto, after cellulose column elution:	122
Crude extract after resin treatment:	95
Ditto, but treated with apyrase first:	30
Crude extract after Ba fractionation, resin treatment and further Ba fractionation:	45

Effect of Apyrase on Extract.- 0·5 ml. 0·1M-succinate buffer at pH 6·5, 5 mg. crude extract and soluble apyrase solution in a total volume of 2 ml., were incubated 30 minutes at 30°. After 2 minutes at 100° to destroy the enzyme, the synthetic system (using 0·3 ml. of 20 per cent homogenate) was added, to give a final volume of 3 ml. and the usual incubation performed. The results indicated no destruction of the factor by the soluble apyrase at a five-fold dilution of the stock enzyme.

Effect of Apyrase on Adenosine Triphosphate.- Using commercial adenosine triphosphate (Messrs. Light & Co.), one-third of which was judged, by behaviour on hydrolysis, to be actually the triphosphate, a sufficient amount was added to the above apyrase system to account for all the ester phosphate found in the active fractions of the resin eluate. Its total destruction to adenosine monophosphate and inorganic phosphate was checked by acid hydrolysis (10 minutes at 100° in N-HCl) and subsequent phosphate estimations.

Purification of Extract by Apyrase.- Five 250 ml. flasks were set up, each with 5 ml. succinate buffer, 5 ml. (50 mg.) crude extract, and 10 ml. of a five-fold dilution of the stock solution of soluble apyrase; the contents were incubated for 30 minutes at 30°, heated to destroy the enzyme, centrifuged, and the supernatant placed on the column and eluted as described.

Preparation /

<u>Preparation of Barium Salts of the Crude Extract</u>.- Although the apyrase treatment had been very successful in the purification of the extract, it was felt that fractionation with barium acetate and ethanol, as described by LePage and Umbreit (1945), would be more convenient for removing the interfering adenosine triphosphate and that the method, unsatisfactory up till then, ought to be further investigated. It was found that the activity was precipitable in the 'water-soluble ethanol-insoluble' fraction of the barium salts, not in the 'water-insoluble' fraction as had been previously thought; in the latter group were precipitated the misleading substance that interfered with the colour reaction and, of course, the unwanted adenosine triphosphate. No trace of activity was found in the other fractions, singly or in combination (Table XXII). This was a notable advance, and the purification procedure as it stood subsequently may be outlined as follows:

After extraction of the disrupted tissue with boiling water (this gave more reproducible results than the use of trichloroacetic acid), the strained solution was brought to pH 1·5 with trichloroacetic acid, 1 volume ethanol added, and the glycogen spun down (all operations being conducted in the cold). The barium-ethanol fractionation was then performed, and the active fraction, freed of barium ion and ethanol, added to the resin and eluted as described. The /

TABLE XXII.

Activity of Barium-ethanol Fractions.

A: Water-insoluble fraction of Ba salts of extract

B: Water-soluble, ethanol-insoluble fraction

C: Water- and ethanol-soluble fraction

A, B and C were concentrated to comparable final volumes, ethanol sucked off and Ba ion precipitated as $BaSO_4$. Separate controls were used for incubation and for colour reaction (at both added volumes), for the fractions singly or in combination.

Fraction	Added Volume in ml.	µg. o-aminophenol conj. by added solution
A	0.1, 0.5	0, 0
B	0.1, 0.5	1.3, 6.4
C	0.1, 0.5	0, 0
A & C	0.5, -	0, -
A & B	0.5, -	6.5, -
A & B & C	0.5, -	6.9, -

The active eluate was concentrated *in vacuo* at 60° and the barium-ethanol fractionation repeated.

The final barium precipitation was necessary because the high concentration of salt in the active resin eluate resulted in smearing whenever paper chromatography was attempted. No precipitate was observed in the water-insoluble fraction, suggesting complete removal of adenosine triphosphate in the first fractionation; the activity-total ester phosphate relationship also indicated this (Table XXI).

Purification of the Factor by Paper Chromatography.- Since a reasonably pure solution of the active factor had been obtained, it seemed that paper chromatography, could a suitable solvent be found, had a better chance of separating the activity from the few remaining components of the extract. After many trials, the alkaline solvent of Bandurski and Axelrod (1951), which consisted of 60 per cent methanol, 30 per cent water, and 10 per cent concentrated ammonia solution, was found to give good travel and separation; and when run at 2°, it preserved the activity of the factor for at least 18 hours. The results of the purification attained up to that time may be seen from Fig. 7, which depicts a chromatogram of the variously-purified solutions of the factor run under these conditions, and treated to show phosphate-containing areas. It will be noticed how the crude extract is split into /

20

15

TRAVEL IN CM. →

10

5

ZONE OF ACTIVITY

INORGANIC PHOSPHATE

CRUDE EXTRACT

A

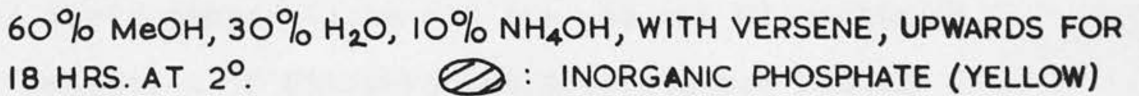
C

B

E

Ba-EtOH FRACTIONS

(B AFTER RESIN TREATMENT)



CHROMATOGRAM OF VARIOUSLY-PURIFIED SOLUTIONS OF EXTRACT SHEWING PHOSPHATE-CONTAINING AREAS

FIG. 7

into the three fractions A, B, and C, by the barium-ethanol treatment, and how B is further purified to E after the resin procedure.

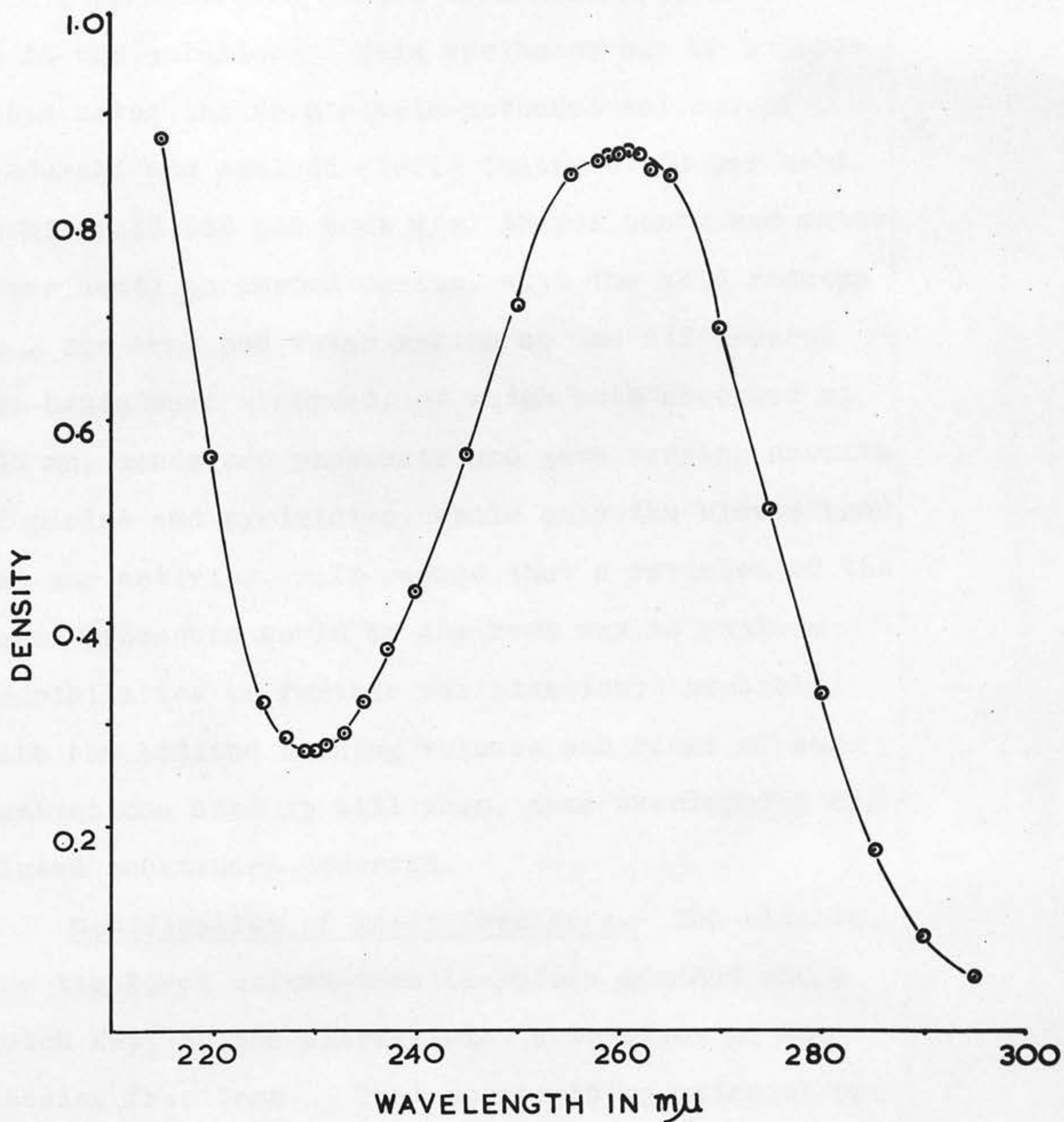
Location of Activity on Chromatograms.- One phosphate spot only was to be seen with E and this travelled just a little slower than the inorganic phosphate marker. From many experiments it was found that the activity could be located only in that area, coincident with the phosphate spot of E. This was a further suggestion that the unknown factor was a phosphate-containing compound.

For all experiments where location of activity was desired, a band of active solution was chromatographed and the dried chromatogram cut latitudinally into narrow equal strips; these strips were shredded and added to the reaction mixture in a complementary series of flasks. The strip or strips giving rise to o-aminophenol conjugation were regarded as having borne the activity. In every case such activity was exactly coincident with the phosphate zone. Versene was found unnecessary with these purer preparations and its use was discontinued.

Detection of Ultra-violet Absorption.- Preliminary investigations had revealed that the purified solution, after the barium precipitations and resin treatment, possessed absorption in the ultra-violet with, in acid or neutral solution, a maximum at 261 mμ and a minimum at 231 mμ (measured on the Unicam SP 500 Quartz Spectrophotometer). Ultra-violet /

violet photography, after the method of Markham and Smith (1949, 1951), of band chromatograms of this solution, revealed that the absorption coincided exactly with the phosphate-containing and active zone. Elution of this zone gave a colourless solution with the same absorption curve (Fig. 8). The fact that there was no green (flavine?) compound present suggested that the absorption curve was due to the active factor; the type of absorption curve obtained, the occurrence of phosphate, and the behaviour with Dowex-1, indicated that a purine or pyrimidine nucleotide was probably present and it became necessary to liberate the base itself so that a more characteristic type of curve might be obtained.

At that time, the work of Park (1950, 1952) and of Caputto, Leloir, Cardini and Paladini (1950) had focussed attention on the uridine-containing compounds and it was thought probable that the most likely purine or pyrimidine would be adenine or uracil respectively. According to Smith and Markham (1950), hydrolysis for 1 hour at 100° with N-HCl resulted in the liberation of purines from their ribonucleotides, whereas pyrimidines remain attached. Hydrolysis of the so-far purified factor for 1 to 3 hours under these conditions and subsequent chromatography with a solvent of iso-propanol and HCl, adjusted to give maximum separation of purines and pyrimidines (Wyatt, 1951), revealed the presence of both a free purine and a pyrimidine nucleotide in /



ULTRA-VIOLET ABSORPTION CURVE OF PARTIALLY-PURE ACTIVE FACTOR SOLUTION (ACID)

FIG. 8

in varying amounts.

Although the active factor might have contained both a purine and a pyrimidine, it was suspected that a considerable amount of impurity still remained in the solution. This was borne out by experiments using the formic acid-methanol solvent of Bandurski and Axelrod (1951) (methanol 80 per cent, formic acid (88 per cent w/v) 15 per cent, and water 5 per cent) on chromatograms, with the acid reduced to 1 per cent and water making up the difference; two bands were observed, of which both absorbed at 260 mμ, contained phosphate and gave varying amounts of purine and pyrimidine, while only the slower band had any activity. It seemed that a revision of the resin procedure would be the best way to explore possibilities of further purification; probably, with the limited washing volumes and range of concentrations used up till then, some overlapping of eluted substances occurred.

<u>Modification of Resin Procedure</u>.- The eluants for the Dowex column were therefore amended and a watch kept on the ultra-violet absorption of the issuing fractions. That no absorbing material was being washed off the resin itself was also checked. The following experiment illustrates the result:

The solution from barium fractionation (freed of Ba ion and ethanol) was added to the usual size of column in 0·1M-ammonium hydroxide (total volume 150 ml.); the column was then washed with 20 ml. water /

water. 0·02M-NaCl in 0·01M-HCl was added till the issuing solution was acid; continuing with this eluant, 4 x 25 ml. were collected and neutralised as usual; 8 x 10 ml. collected with 0·05M-NaCl in 0·01M-HCl, 6 x 10 ml. with 0·1M-NaCl in 0·01M-HCl, and 4 x 10 ml. with 0·2M-NaCl in 0·01M-HCl, followed.

The absorption of all these eluates at 260 mμ was determined and 0·4 ml. of each added to the reaction system to test its activity. The relationship of absorption and activity (the latter expressed as readings on the absorptiometer from the Levvy and Storey colorimetric method) can be seen from Fig. 9 and may be summarised as follows: P is the fraction group containing no activity but much absorption, which falls off rapidly at first, rising a little to A, where it maintains a plateau for the first half (Aa), falling steeply in the second (Ab). The activity in A rises to a plateau and stays there, indicating a constant elution of a small amount of the active factor during the rapid washing out of an inactive contaminant. In B, both activity and absorption rise to a sharp peak and fall steeply together, maintaining a fairly constant ratio. In E, both activity and absorption fall away together.

Thus it seems that the active factor does indeed absorb at 260 mμ and is the last such absorber to be washed off the resin under these conditions. Considerable interference from overlapping P and A would explain the impurity of previous preparations, and /

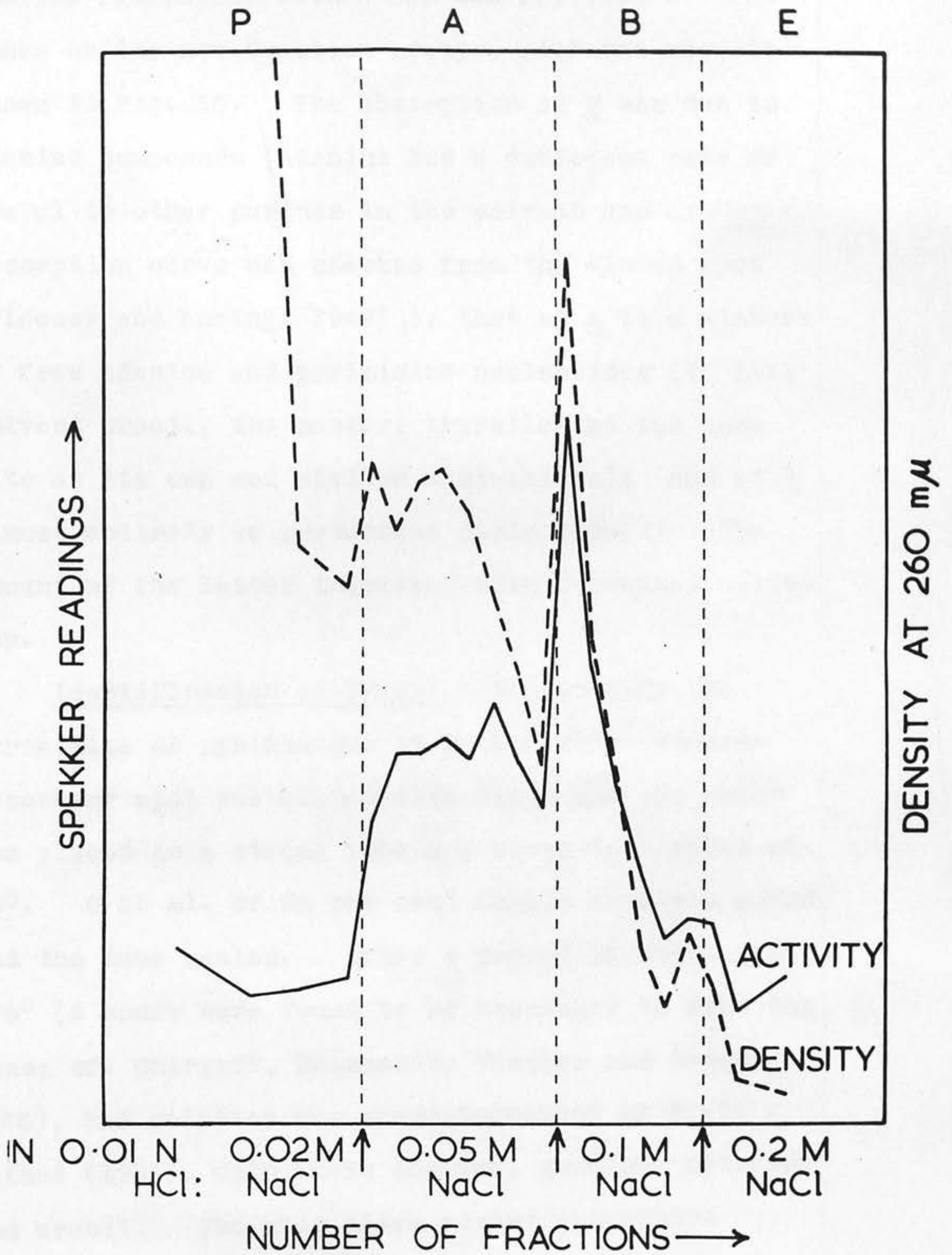
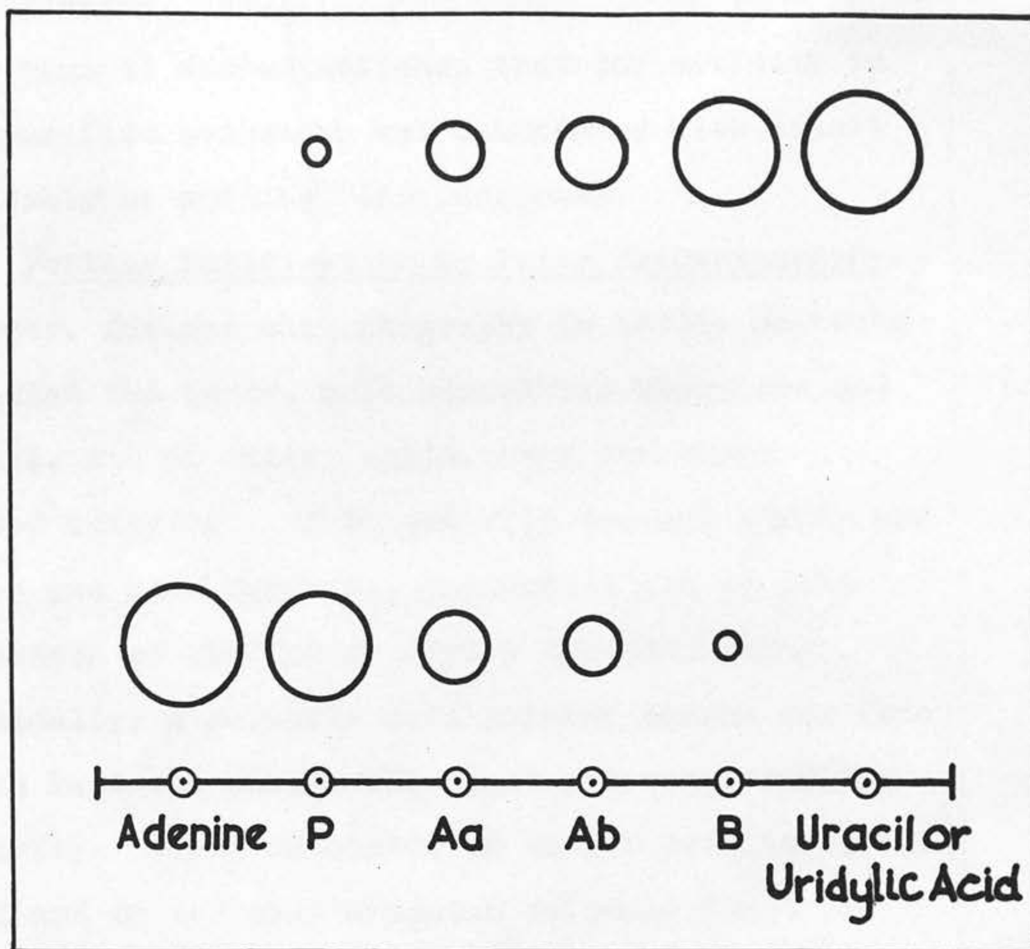


DIAGRAM SHEWING ACTIVITY & U/V ABSORBENCY OF RESIN ELUATES

FIG. 9

and the hydrolysis with N-HCl was repeated several times on the new fraction groups, with the results shown in Fig. 10. The absorption at P was due to adenine compounds (adenine had a different rate of travel to other purines in the solvent and its absorption curve was checked from the eluted spot (Ploeser and Loring, 1949)); that at A to a mixture of free adenine and pyrimidine nucleotides (in this solvent uracil, the marker, travelled at the same rate as its own and similar nucleotides); and at B almost entirely to pyrimidine nucleotide(s). The amount of the latter increased with increased activity.

Identification of Uracil.- To identify the pyrimidine or pyrimidines in B, the ultra-violet-absorbing spot was eluted with water and the solution placed in a strong tube and blown to dryness at 70°. 0·35 ml. of 98 per cent formic acid was added and the tube sealed. After a period of heating at 175° (5 hours were found to be necessary to free the base; cf. Chargaff, Magasanik, Vischer and Green, 1950), the solution was chromatographed by Wyatt's method (1951), with known adenine, guanine, cytosine and uracil. The only ultra-violet absorption present was at the uracil level. This absorbing area was eluted and gave the characteristic uracil absorption curve in the ultra-violet (Hotchkiss, 1948; Ploeser and Loring, 1949), with identical maximum and minimum, in both acid and alkaline solution,/



A CHROMATOGRAM OF RESIN FRACTION HYDROLYSIS PRODUCTS, SHEWING U/V ABSORBING AREAS

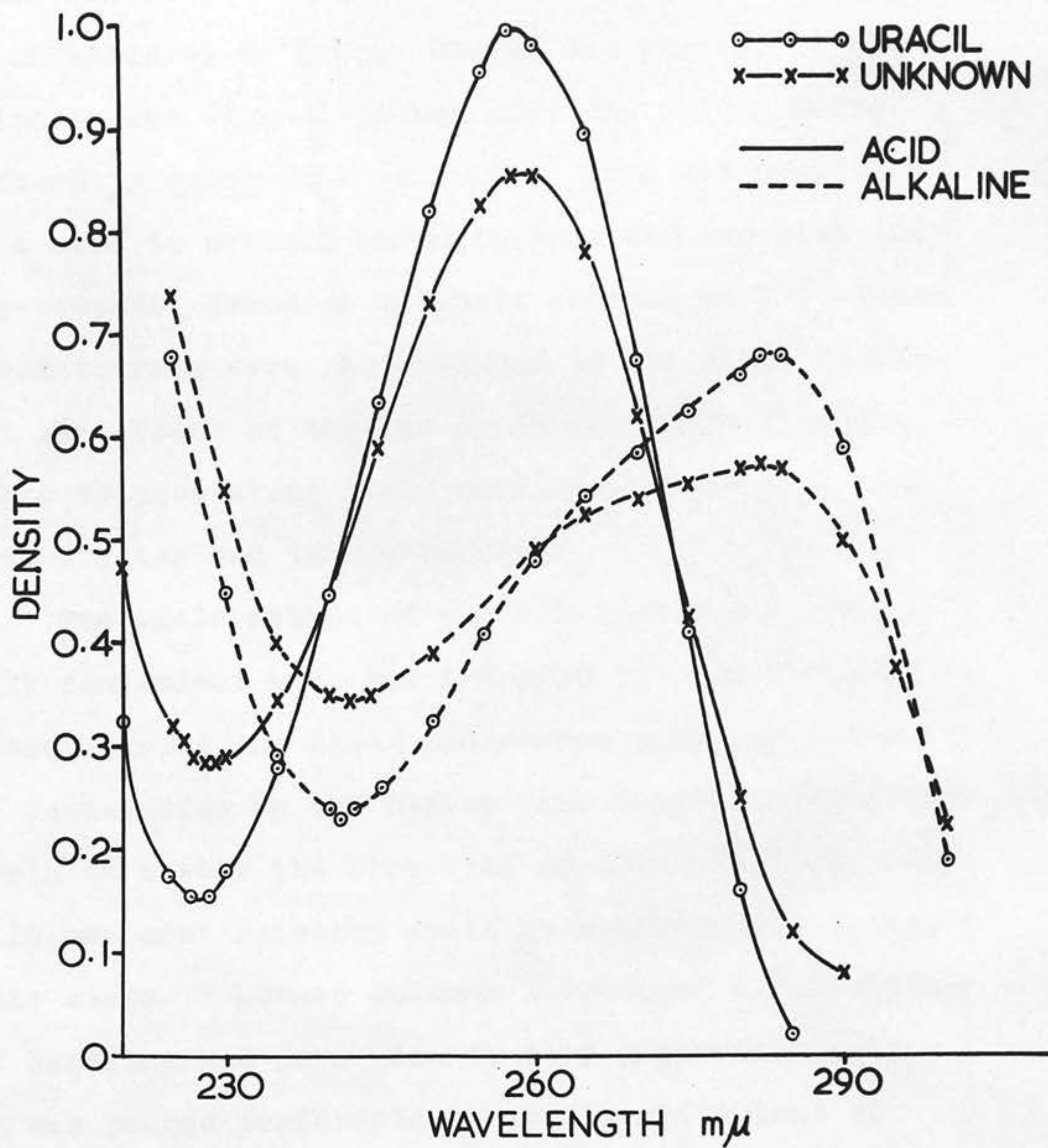
FIG. 10

solution, with those from known uracil (Fig. 11). The partially-hydrolysed substance gave an absorption spectrum similar to that for uridine anduridine-containing nucleotides (Caputto, Leloir, Cardini and Paladine, 1950).

Thus it was established that the activity in the purified solutions was associated with uracil (probably as uridine) and phosphate.

Further Purification by Paper Chromatography.- However, further chromatography in acidic solvents revealed two bands, both containing phosphate and uracil, and of which, again, only the slower contained activity. This activity was not great, and there was some smearing, presumably due to acid breakdown of the factor during chromatography. Eventually, a suitably mild solvent system was found which kept the bands well apart and preserved the activity. This consisted of 60 per cent iso-propanol and 40 per cent ammonium sulphate (in 1 per cent aqueous solution) run on Whatman No. 541 paper previously soaked in the salt solution and dried. (Anand, Clark, Hall and Todd, 1952).

The slower-moving band here also ran as a single substance at 2° in 60 per cent n-propanol, 10 per cent water, 30 per cent concentrated ammonia solution; in 60 per cent methanol, 30 per cent water, 10 per cent concentrated ammonia solution; 60 per cent ethanol, 40 per cent water; 60 per cent ethanol, 40 per cent aqueous M-ammonium acetate solution /



U/V ABSORPTION CURVES OF PYRIMIDINE FROM FACTOR AND KNOWN URACIL

FIG. 11

solution at pH 4·0; 80 per cent methanol, 15 per cent water, 5 per cent formic acid (88 per cent w/v). The mode of preparation of the pure factor, then, after the second barium-ethanol fractionation, can be outlined as follows: Barium was removed as the sulphate and ethanol sucked off, the volume being reduced _in vacuo_ to 2 or 3 ml. This was applied as a band to several chromatograms and run with the iso-propanol-ammonium sulphate solvent at 2°. These chromatograms were photographed in the ultra-violet, and the slower of the two bands eluted by allowing water to pass along it by capillary attraction and drip off the end into a receiver.

The whole method of purification was a reasonably convenient one, but attended by considerable losses during the resin procedure, probably because of destruction by the strong base Dowex-1; with the resin in either the free base or chloride form, only a 10 per cent recovery could be counted upon during this stage. Longer columns introduced difficulties of handling and gave rise to poor separation, and it was judged preferable to obtain sufficient of the factor by the present method for characterisation purposes than to spend time searching for more economical conditions. Only a very small amount of the pure factor could be obtained at a time and examination of it had to be performed on a small scale.

SECTION VI: Analysis and Structure of the Pure Factor.

The factor as eluted from the slower band being considered an isolated pure single compound, its detailed examination could be carried out. Experiments on the active solution at earlier stages of purification had revealed the presence of acid-labile phosphate, relatively acid-stable phosphate, a reducing substance, and a substance giving a positive test for a uronic acid in the Tollens naphthoresorcinol reaction. Since uracil was known to be the only ultra-violet absorbing component present, almost certainly existing as a uridylic acid in the compound, all molar ratios for the other constituents were based on the assumption that the unknown factor contained 1 molecule of uridylic acid; the concentration of this uridylic acid was calculated from the ultra-violet absorption at 260 mμ, taking it all to be from that source.

Below are given estimations so far carried out on the components of the substance of the slower band; the faster band contained uracil and phosphate and had some reducing properties on hydrolysis, but did not yield a positive Tollens test, nor, of course, did it give rise to activity.

Each estimation was conducted on amounts of the estimated material lying within the range for which a linear relationship held between the amount of material present and the colour produced, as checked by standard calibration graphs.

Solution /

Solution A: <u>Uridylic Acid</u>.- This was estimated by the absorption of the band eluate at 260 mμ, being read against a control solution containing the same amount of ammonium sulphate; both were 0.1N with respect to HCl.

0.2 ml. eluate in 3 ml. gave a density of 0.326. The density of the undiluted solution was therefore 4.90.

Now the density of a 0.001M solution of uridylic acid under these conditions is 9.89 (Ploeser and Loring, 1949). So the concentration of uridylic acid in solution A is . . . . <u>0.50 mM</u>

<u>Total Phosphorus</u>.- Solution A has 9.8 μg. phosphorus in 0.3 ml. So concentration of total phosphorus in solution A is . . . . <u>1.06mM</u>

<u>Labile Phosphorus</u>.- When heated in N-HCl at 100°, half of the total phosphorus is rapidly split off within 15 minutes, while the remainder is released more slowly (see Fig. 12); the former is the 'labile' phosphorus.

Solution A has 5.1 μg. phosphorus in 0.3 ml. So concentration of labile phosphorus in solution A is . . . . <u>0.55mM</u>

In solution A the molar ratios of uridylic acid, total phosphorus, and labile phosphorus are 1.0 : 2.1 : 1.1.

Solution B: Uridylic Acid : 0.21mM

Total Phosphorus : 0.42mM

Labile Phosphorus: 0.19mM

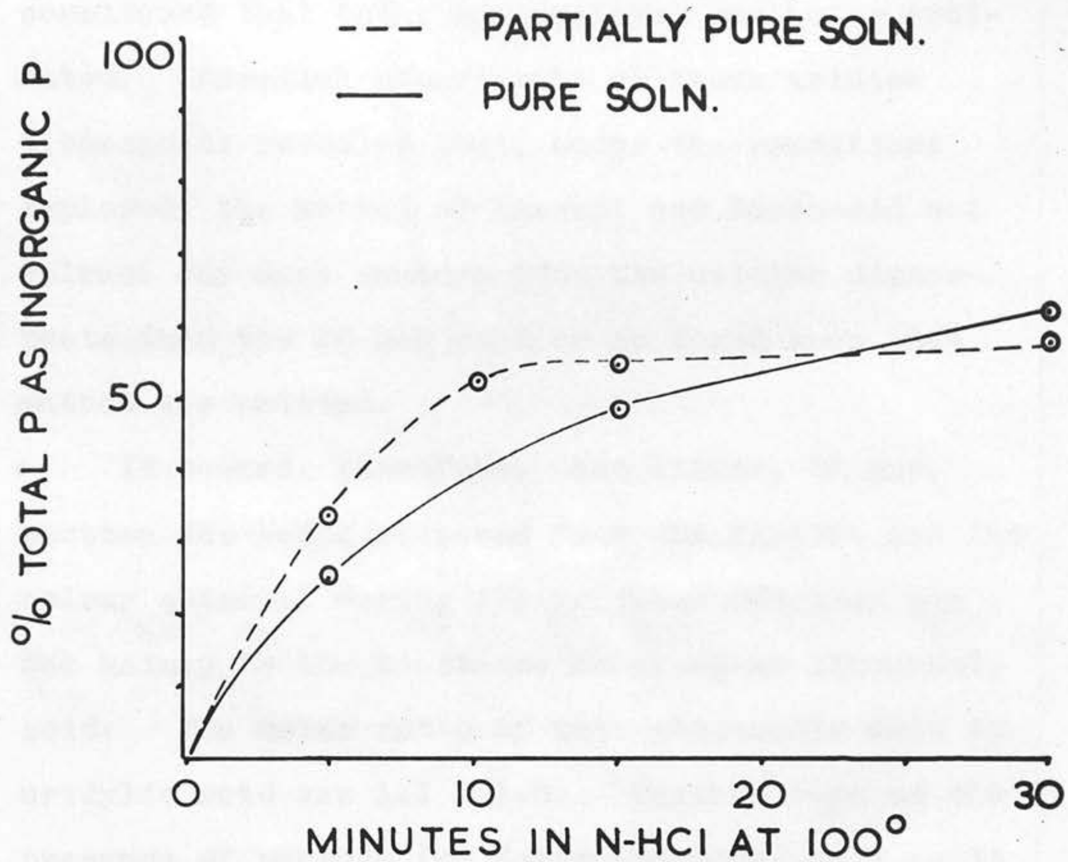
The /

The ratios here are 1.0 : 2.0 : 0.9 for uridylic acid, total phosphorus, and labile phosphorus respectively.

The graph (Fig. 12) illustrates the amount of phosphorus liberated from the pure compound at 100° in N-HCl over a period of 1 hour. It will be seen how 50 per cent of the total phosphorus is split off fairly rapidly and the remainder much more slowly; this was also observed by Caputto, Leloir, Cardini and Paladini (1950) with their uridine-diphosphate-glucose.

It will be gathered that conditions sufficient to destroy the activity of the compound (4 to 8 minutes at 100° in 0.1N-HCl) would not mean the splitting off of anything but a small amount of inorganic phosphorus; it was found that 4 minutes of these conditions (resulting in 90 per cent loss of activity) only so removed about one twelfth of the total phosphorus. Inactivation by this means would not therefore seem to involve the permanent loss of an inorganic phosphate group.

Pentose.- Since the pyrimidine-sugar link is notoriously difficult to break, the presence of pentose in the pure factor was tested for by the method of Massart and Hoste (1947), where the uracil nucleus is claimed to be destroyed with bromine under carefully-controlled conditions, followed by heating with orcinol and concentrated HCl (Meijbaum), 1943) for the colour estimation. Results obtained suggested /



RELEASE OF INORGANIC PHOSPHATE FROM ACTIVE FACTOR SOLUTIONS ON N-ACID HYDROLYSIS

FIG. 12

suggested one molecule of pentose to be present in each molecule of factor, but it was found that glucuronic acid also gave a green coloration with the Meijbaum treatment. Since the factor contained a substance behaving like glucuronic acid it was considered that this, not pentose, was being estimated. Parallel experiments on known uridine diphosphate revealed that, under the conditions employed, the method of Massart and Hoste did not release any more pentose from the uridine diphosphate than the 20 per cent or so found when that method was omitted.

It seemed, therefore, that little, if any, pentose was being released from the factor, and the colour obtained during the Meijbaum reaction was due mainly to the substance behaving as glucuronic acid. The molar ratio of this glucuronic acid to uridylic acid was 1.1 : 1.0. Further work on the presence of pentose (or desoxy-pentose) must await larger-scale isolation of the hydrolysis products of the factor, when the interfering glucuronic acid can be satisfactorily removed.

Tollens-positive Substance.- This was calculated as glucuronic acid and estimated by the Tollens method, modified by Paul (see Experimental Methods). The slower band was eluted with 5 ml. 0.1N-HCl; the density at 260 mµ of 3 ml. was read and the solution washed into a tube and estimated for glucuronic acid, along with a standard solution checked with /

with a standard graph and reagent controls.

Solution C: Uridylic Acid: 0.024mM

Glucuronic Acid: 12 µg. in 3 ml.

were found to be present, giving a concentration of 0.021mM

This gives a ratio of uridylic to glucuronic acid of 1.0 : 0.9.

Solution D: Uridylic Acid: 0.022mM

Glucuronic Acid: 0.020mM

This gives a ratio of 1.0 : 0.9 again.

Reducing Substance.- In impure preparations a reducing substance had been noted and its behaviour resembled that occurring with uridine-diphosphate-glucose (Caputto, Leloir, Cardini and Paladine, 1950), in that most of it was liberated after a mild acid hydrolysis. However, the pure compound exhibited 75 per cent of its total reducing properties without any further treatment than the alkaline heating necessary during the estimation of those properties. The method used was that of Folin and Malmros (1929), regarded by LePage and Umbreit (1945) as a relatively mild one; nevertheless, this treatment removed about 75 per cent of the activity of the factor as well, and there thus appeared to be some connection between the rate of appearance of reducing power and the rate of disappearance of activity (as with uridine-diphosphate-glucose). A method of determining whether the intact molecule possesses any reducing property has not yet been found practicable, but the results /

results below show the evidence from the Folin-Malmros estimations of the reducing substance, calculated as glucuronic acid. Reagent controls and standards checked from a standard graph were used with each determination.

0.2 ml. quantities of the pure solution (containing 10-20 µg. glucuronic acid) were heated with 1.0 ml. of 0.1N-HCl for various periods of time at 100°, neutralised with 0.1N-KOH (checked with paper), and the reducing substance present estimated by the Folin-Malmros method, using half the quantities quoted by Umbreit (1945), the colours being read on the Hilger Spekker absorptiometer using Ilford filter No. 604 (green). Since the eluate contained ammonium sulphate, there was liberation of ammonia during this reaction, but standards also containing an equivalent amount of the salt were not affected by this process. The liberation of reducing substance appeared to be practically complete after 10 minutes' heating with 0.1N-HCl, not increasing appreciably after 30 minutes.

Solution E: (i) Uridylic Acid: . . . . .. <u>0.37mM</u>

Glucuronic Acid: With no previous acid hydrolysis 0.27mM

With 10 minutes' hydrolysis . . . . <u>0.35mM</u>

With 20 minutes' hydrolysis 0.39mM

(ii) Uridylic Acid . . . . . <u>0.37mM</u>

Glucuronic Acid: With no previous acid hydrolysis 0.26mM

With /

With 10 minutes' hydrolysis 0.34mM

With 30 minutes' hydrolysis 0.35mM

Solution F: Uridylic Acid: 0.57mM

Glucuronic Acid: With

12 minutes' acid hydrolysis 0.54mM

In all these solutions the ratio of uridylic to glucuronic acid is 1.0 : 0.9.

The effect apparent above may be compared with the loss of activity due to the Folin-Malmros alkaline heating where the conjugation due to added factor fell by 75 per cent (from 4.4 to 1.1 µg. o-aminophenol), when the factor was previously subjected to 8 minutes at 100° with the Folin-Malmros alkaline reagents.

Effect of Alkaline Hydrolysis.- Although the effect of alkaline hydrolysis on the pure factor has not yet been fully investigated, it seemed to be similar to that occurring with uridine-diphosphate-glucose (Caputto, Leloir, Cardini and Paladini, 1950).

After 10 minutes at 100° with 0.1N-KOH, the amount of reducing substance apparent was much less than that after acid hydrolysis, or even after the milder alkaline conditions of the Folin-Malmros heating of the unhydrolysed substance.

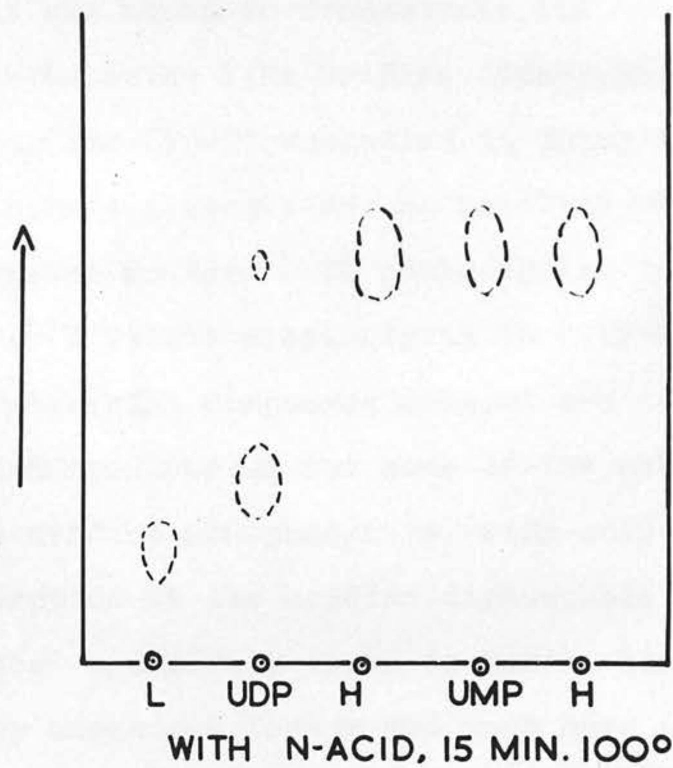
Products of Hydrolysis of Factor.- Because of the small amounts of the pure factor so far available, the preliminary investigation into its breakdown products has been conducted by paper chromatography /

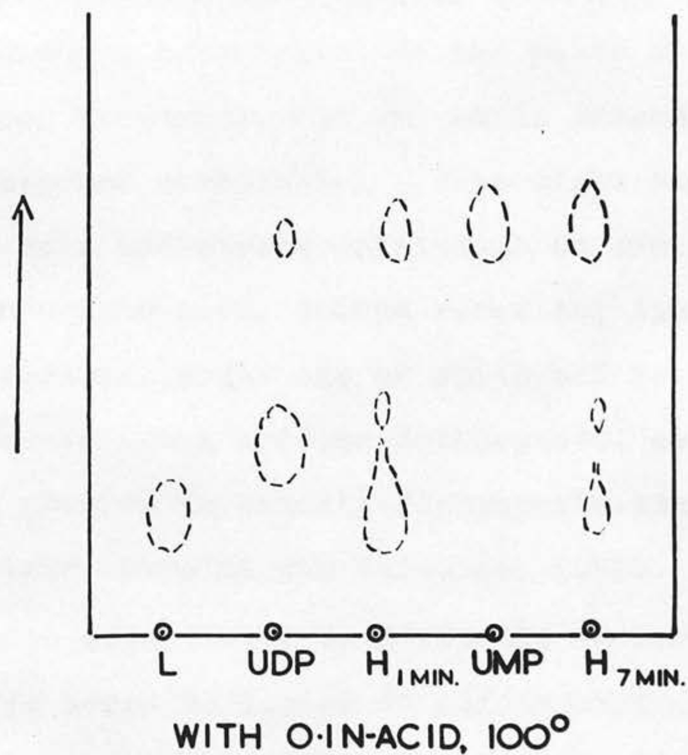
chromatography, with ultra-violet photography by Markham and Smith's method to reveal the absorbing compounds.

If hydrolysed with HCl, considerable smearing occurred, and it was not until H_2SO_4 was employed for hydrolysis that sufficient resolution was obtained, using the 60 per cent iso-propanol, 40 per cent ammonium sulphate solution and a 60 per cent ethanol, 40 per cent M-ammonium acetate solution (aqueous, at pH 4.0) as solvent systems.

0.5 ml. of the factor solution was heated with 0.5 ml. 2N-H_2SO_4 at 100° for 12 minutes, cooled, and neutralised with NH_4OH (checked by indicator paper). The solution was then blown down to a volume of 0.25 ml. at 50° and chromatographed in the two solvents, with known uridine diphosphate (kindly given by Professor A. R. Todd, F.R.S.) similarly treated (to give uridine-5'-phosphate), unhydrolysed uridine diphosphate, and unhydrolysed factor, as markers. The results are shown in Fig. 13. The product of hydrolysis of the factor under these conditions, which has absorption in the ultra-violet, is a substance travelling at the same rate as uridine-5'-phosphate in the two solvent mixtures. The solution of the similarly hydrolysed factor, it will be remembered, contained one mole 'inorganic' phosphorus, one mole 'organic' (stable) phosphorus, and one mole of a uracil compound.

By using 0.1N acid and reducing the time of hydrolysis /





CHROMATOGRAM OF HYDROLYSIS PRODUCTS (H) OF PURE FACTOR (L) IN RELATION TO URIDINE-5'-PHOSPHATE (UMP) & URIDINE DIPHOSPHATE (UDP), SHEWING U/V ABSORPTION

FIG. 13

hydrolysis, it was hoped to demonstrate the formation of a substance behaving like uridine diphosphate, as Paladini and Leloir (1952) succeeded in doing with uridine-diphosphate-glucose, but no positive results have been obtained so far. It would appear that, even after only 1 minute's hydrolysis in 0.1N-H_2SO_4 at 100°, the absorbing compounds present are the apparently unchanged factor and some of the substance travelling as uridine monophosphate, with only a trace of absorption at the uridine diphosphate level; after 8 minutes' hydrolysis there is hardly any of the apparently unchanged factor and much more of the uridine monophosphate-like substance (inorganic phosphate seemed to be revealed on the paper by the Hanes-Isherwood treatment, but the salts present made this procedure unreliable). This might suggest that in 0.1N acid hydrolysis sufficient to destroy activity, glucuronic acid, though revealing its full reducing properties, might not be split off from the compound so as to leave uridine diphosphate, as happens with glucose in uridine-diphosphate-glucose (Caputto, Leloir, Cardini and Paladine, 1950).

Glucuronic acid itself is difficult to chromatograph, for it seems to appear in different forms, depending on the solvent system (Partridge, 1948). If a solution of glucurone were chromatographed in a slightly acid solvent, two spots appeared on treating with aniline hydrogen oxalate as described by Partridge (1949); the upper was rosy-pink, and the /

the lower, fainter, one an orange-brown. Amounts of less than 10 µg. were very hard to detect. If the glucurone were treated with 2N-NH_4OH in the cold, the solution became yellow, and if this were chromatographed, only the lower of the two spots was visible. The upper may be due to the lactone and the lower to the free acid (Partridge, 1948). When N and 0.1N-H_2SO_4 hydrolysates of the pure factor were chromatographed, an orange-brown smear was noticeable (in slightly acid solvents), but it was difficult to correlate it with known glucuronic acid, which also smeared in the same chromatogram region when present in small amounts. If no hydrolysis had been performed, the orange-brown coloration (only apparent in this case on prolonged heating) was exactly coincident with the factor itself, but hydrolysis, with its attendant salt concentrations, introduced the smearing. Before any definite results can be presented, either the solvent systems must be altered or, better still, sufficient of the factor isolated to enable separation of the barium salts of the different hydrolysis products to be carried out.

<u>Amount of Glucuronic Acid Conjugated per Molecule Factor</u>.- 0.2 ml. pure factor solution, containing a known amount of uridylic acid (calculated from the ultra-violet absorption), was incubated for one hour with 1.0 ml. homogenate at 37° (conditions under which maximum utilisation of the active substance /

substance by the homogenate was noticed: see Fig.3). The amount of glucuronic acid conjugated with o-aminophenol was estimated, being read against an identically treated control without the added factor.

Assuming there to be 1 molecule each of uridylic acid and glucuronic acid in 1 molecule of the active factor, then, since the concentration of uridylic acid in the factor solution was 0.75mM, the concentration of glucuronic acid should also have been 0.75mM, or 14.5 µg. per 0.1 ml.

After incubation, the reaction mixture contained 5.8 µg. of glucuronic acid conjugated with o-aminophenol for each 0.1 ml. of active factor solution added, that is, for each 14.5 µg. of glucuronic acid added in the factor molecule.

This represents a 'transference' of glucuronic acid from the factor to the substrate of 40 per cent. Taking into account destruction of the factor by the homogenate and probable absence of exactly optimal conditions, it would seem that this is a minimum figure.

DISCUSSION OF RESULTS

It has been stated how the aim of the work presented was to study the formation of glucuronides by attempting to induce such a process in homogenates of liver and to relate this to previous work on the biosynthesis of glucuronic acid. The 'Experimental Results' have shown that the products of the reaction studied are almost certainly glucuronides. Their behaviour throughout the whole colorimetric technique corresponds exactly with that of known glucuronides, using two completely unrelated methods of estimation; the product considered to be o-aminophenylglucuronide has the same chromatographic travel as known o-aminophenylglucuronide, when coupled to the naphthylethylenediamine; and the supposed o-aminophenylglucuronide is attacked by β-glucuronidase unless there is present the specific inhibitor for that enzyme, when its destruction is lessened to the same extent as that of a comparable amount of known o-aminophenylglucuronide. Furthermore, the presence of a substance behaving like glucuronic acid in the active factor needed for the synthesis, offers further evidence that we have been studying a process resulting in the formation of the glucuronides of o-aminophenol and l-menthol in liver homogenates. Before, however, the /

the significance of this work can be discussed, the composition and possible structure of the factor, as revealed by the work so far performed upon it, must first receive attention.

The experimental results have shown that production of glucuronides in the liver homogenate systems employed was only appreciable when a certain substance, present in the liver, was added to the reaction flask. The course of purification and isolation of this factor has been followed and the results of analysis of the pure substance put forward. That the final solution obtained was that of a pure single compound may be gathered from its travel as a single spot in several widely-different chromatographic solvents, and from the analyses of several preparations of the compound, which showed the presence of certain substances, invariably occurring in a constant ratio to one another. The active factor appears to contain uracil, phosphate, a uronic acid and a reducing substance, in the molar ratios 1 : 2 : 1 : 1, assuming that the uronic acid is glucuronic acid and that the reducing properties belong to it.

The ultra-violet absorption spectrum of the partially-hydrolysed pure factor resembles that of a uridine nucleotide and this, together with the fact that a substance behaving chromatographically like uridine-5'-phosphate has been detected among the hydrolysis products, would suggest that uracil and /

and one of the phosphate groups (together with as yet unidentified pentose) are present in the form of such a nucleotide. The difficulty observed in splitting off the pentose by acid hydrolysis is in agreement with this conclusion, for pyrimidine nucleotides are resistant to such treatment. The recent work of Leloir and his co-workers, and of Park, provides a precedent, for they have demonstrated beyond doubt that uridine nucleotides, associated with other substances, occur in living tissues, uridine-diphosphate-glucose, for example, being present in liver (Caputto, Leloir, Cardini and Paladini, 1950). Preliminary attempts to characterise further the substance behaving like uridine-5'-phosphate by its copper-binding properties (cf. Caputto, Leloir, Cardini and Paladini,1950) have so far been unsuccessful because of interference from the chromatographic solvent salts. Once, however, sufficient of this substance is available for practicable barium precipitation, it should be possible to state its constitution more confidently. The possibility that pentose is in reality absent from the factor and that glucuronic acid occupies its position in the nucleotide is most unlikely; it is difficult to reconcile this with the ready appearance of reducing properties and the extreme reluctance of the supposed uracil-glucuronic acid link to break on acid hydrolysis and yield detectable uracil. This reluctance would also argue against /

against any pentose present being a desoxy-pentose.

It is difficult to avoid the conclusion that the uronic acid found is indeed glucuronic acid; the molar ratios suggest a hexuronic acid, apart from the alleged specificity of the Tollens reaction for such compounds (Artz and Osman, 1950) (although the course of this investigation revealed that α-ketoglutaric acid is also estimated as a 'uronic acid' in the reaction) and galacturonic acid does not seem to have been reported as yet from animal tissues. The chromatographic evidence is unsatisfactory, but in spite of smearing, the colour obtained with aniline hydrogen oxalate from the hydrolysed factor was identical with that from known (smeared) glucuronic acid. If pre-formed glucuronic acid were present in the factor molecule, it is more likely that a relatively simple enzymatic process, like the one studied, would suffice for its transfer to a substrate. The reducing properties would seem to belong to this glucuronic acid for they could not be from any of the other components; the pentose would not exhibit them under such mild conditions and no evidence of any further substance present having reducing properties has been discovered.

There remains the problem of the structure of the factor. As we have seen, it is very probable that uridine-5'-phosphate exists in the molecule, but the placing of the other phosphate group and of glucuronic acid remains to be determined. On hydrolysis /

hydrolysis with N-acid for 12 - 15 minutes, there is chromatographic evidence that uridine-5'-phosphate is produced and 50 per cent of the total phosphate appears as inorganic phosphate. This behaviour is almost identical with that of uridine-diphosphate-glucose as reported by Caputto, Leloir, Cardini and Paladini (1950) or of Park's various uridine diphosphate-containing substances (1951); in uridine-diphosphate-glucose the glucose appears and then the pyrophosphate link is split in 12 minutes in N-acid to give 50 per cent of the total phosphate as inorganic phosphate. With uridine-diphosphate-glucose also, mild (0.01N) acid hydrolysis causes inactivation without much phosphate loss; and at the same time, the reducing powers of glucose are unmasked. This is due to the splitting off of the glucose to leave uridine diphosphate. The behaviour of the studied factor is so similar in these respects that it is tempting to call the substance not merely a compound of uridine diphosphate and glucuronic acid, but uridine-diphosphate-glucuronic acid, taking the glucuronic acid to be attached to the uridine diphosphate by a pyrophosphate link. The inactivation by 0.1 N-acid would then be explicable by the splitting off of the glucuronic acid to leave uridine diphosphate, thus unmasking the reducing properties of glucuronic acid. N-acid hydrolysis would result in the breakdown of uridine diphosphate to the monophosphate /

monophosphate, liberating 50 per cent of the phosphate as inorganic phosphate in the required 12 - 15 minutes.

Unfortunately for this point of view, it has not yet been possible, as we have seen, to produce convincing evidence that uridine diphosphate is formed from this compound on mild acid hydrolysis. 0.1 N-acid hydrolysis sufficient to destroy activity results in only about one-sixth of the labile phosphate appearing as inorganic phosphate, as was also observed in uridine-diphosphate-glucose; however, it does seem to result in the formation of uridine monophosphate (whether corresponding to the amount of inorganic phosphate liberated, it has not yet been possible to determine), with only a trace, if any, of uridine diphosphate and a little of the apparently unchanged factor remaining. What may be happening is that, on 0.1 N-acid inactivation, some internal re-arrangement of the glucuronic acid or its mode of attachment occurs, resulting in the appearance of full reducing powers and it subsequently splits off as a glucuronic acid-phosphate, leaving uridine monophosphate; the glucuronic acid-phosphate is then hydrolysed on further (normal) acid treatment, producing inorganic phosphate. This last hydrolysis would have to occur at approximately the same rate as that for the loss of a phosphate group from uridine diphosphate.

From the experiments on the reducing properties it /

it is apparent that these are very readily exhibited by the compound. Possibly, as suggested above, an internal re-arrangement of the molecule due to mild acid treatment results in the unmasking of the potentially-reducing group of glucuronic acid. The mild alkaline treatment of the Folin-Malmros method,which results in loss of activity, might also cause some re-arrangement and such unmasking,but it is puzzling that similar alkaline treatment, but with 0.1 N-KOH, would seem to lessen the reducing power displayed. Paladini and Leloir (1952) have made a detailed investigation of the products of alkaline hydrolysis of uridine-diphosphate-glucose and have found evidence for the formation of a cyclic glucose-phosphate ester which subsequently breaks down into a mixture of glucose-2- and glucose-1-phosphates. That something similar may be occurring with this compound is very probable, and until more is known about the hydrolysis products and, equally important, about the pure chemistry of glucuronic acid and its derivatives, it is perhaps best to leave the matter open, although it has been found in preliminary experiments that α- and β-glucuronic acid-1-phosphates, treated with the Folin-Malmros method, do not show reducing properties. What can be said is that the factor very probably contains uracil, a phosphate group and an unidentified pentose arranged as uridine-5'-phosphate, with the positions of the other phosphate group and of glucuronic acid unsettled at the moment.

With the problem of the exact structure of the factor as yet incompletely solved, but with the directions of future investigation very evident, we may turn to a consideration of the reaction itself. It may be fairly assumed that the glucuronic acid of the factor is the glucuronic acid which appears in the glucuronide of the substrate. This may be deduced, as has been noted, from the relative simplicity of an enzymic process not dependent on oxidative reactions or any of the accepted sources of energy employed. There is no evidence that the conjugated glucuronic acid is synthesised here from any three-carbon precursor, hexose, free glucuronic acid or its phosphate. There is equal lack of evidence that sulphate ion, free glucuronic acid, the glucuronic acid-1-phosphates, or glucose and the glucose-1-phosphates inhibit to any great extent; this would again suggest that no 'intermediates' are present to give rise to inhibition on a competitive basis.

If we accept, then, this 'transferring' of glucuronic acid, it is of interest to note that the overall transference is only some 40 per cent. The factor is, in the reaction studied, not a co-enzyme in the sense that it accepts and passes on some substance continuously from one set of enzymes to another; indeed, the apparent simplicity of the observed reaction might preclude such a role. This 40 per cent is a minimum figure, as has been indicated, for /

for apart from the absence of exactly optimal conditions with regard to pH or other unknown requirements and the possible presence of undetected competitive inhibitors introduced with the homogenate, the latter is known to destroy the factor on incubation without added substrate; because of this, it is impossible to say whether the value of 40 per cent represents just a fraction of an optimal 100 per cent conversion of factor-glucuronic acid to substrate-glucuronic acid, or whether it is not far short of the actual equilibrium obtainable between the factor and substrate glucuronide.

It remains to be proved that in this reaction investigated we are observing the last stages of the synthesis of conjugated glucuronic acid in the liver, that overall reaction which has been studied in the intact animal and in sliced tissue. The very presence of the factor in liver, however, makes such an event likely and its extremely rapid destruction in the dead organ would perhaps indicate its being a labile intermediate in some process. It might be as well to point out here that there is nothing in the evidence presented which precludes the participation of a glucuronic acid-1-phosphate in the biosynthesis of glucuronic acid; it merely does not seem to enter into the process studied, and if Storey's postulate be correct and it does play a part, it must be at an earlier stage in the process, possibly during the formation of the factor itself.

This /

This reaction would seem to be the first wherein a uridine compound has been shown to participate in the metabolism of animal tissue, previous work having been concerned with micro-organisms; and the enzyme system, though as yet in a crude state, the first shown to produce conjugated glucuronides and, apart from β-glucuronidase, the only one so far shown to involve any phase of glucuronic acid metabolism. There seems to be no likelihood of β-glucuronidase itself being concerned in the process studied. Apart from the failure of a β-glucuronidase inhibitor like saccharo-1:4-lactone to affect this enzyme system, there was no evidence of its occurrence in organs such as the spleen, rich in β-glucuronidase; a highly-purified spleen glucuronidase preparation replacing the homogenate failed to induce any conjugation in the system. β-glucuronidase is a fairly stable enzyme, whereas the enzymic process studied would seem to be very labile, being destroyed on conversion to an acetone powder, on prolonged freezing, or on autolytic incubation. Also, according to Dr. G. A. Levvy (private communication), a 0.00004M solution of the factor did not influence the hydrolysis of 0.0000625 M-phenolphthalein glucuronide by mouse liver β-glucuronidase; he concludes from this that this last enzyme is not concerned in the 'transference' of glucuronic acid investigated above.

Whether this factor is concerned only with the glucuronic /

glucuronic acid conjugation in the liver of substances like o-aminophenol and l-menthol, or whether by adding such substrates we are interfering in a normal metabolic process and intercepting glucuronic acid destined for another purpose, must so far remain unanswered. None of the other tissues tried has yielded any measurable amount of either factor or enzyme system, but the amount of both needed for the synthesis of conjugated glucuronic acid at any other sites in the body may be too small to be noticeable by the present techniques; or the factor may be elaborated in the liver and conveyed in small amounts to these sites, where a different enzyme system to the one discovered may incorporate its glucuronic acid into another molecule. It would be tempting to speculate whether this substance is an 'active' form of glucuronic acid, taking part in the metabolism of mucopolysaccharides and other large glucuronic acid-containing molecules so widespread in the tissues. Certainly, whatever the solution may be, it is clear that a fruitful field for investigation has been opened up by the discovery, for the first time, of a substance with a chemical identity which produces conjugation of glucuronides in a homogenised tissue preparation.

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ACKNOWLEDGMENTS

As well as to those whose help is acknowledged in the text, thanks are due to Professor G. F. Marrian, F.R.S. for an appointment to his staff, and to Professor Sir James Learmonth for the kind provision of laboratory facilities, without which this work would have been impossible; to Dr. C. M. Mauritzen for generously making available his ultra-violet photographic equipment and for much advice regarding it; and to the Medical Research Council for a maintenance and expenses grant in the year 1952-53.

I wish also to express my indebtedness to Dr. I. D. E. Storey for his continual helpful criticism and advice throughout the course of the whole investigation.

APPENDIX

Overleaf will be found copies of the papers so far published on the results of the investigation reported on the preceding pages.

[*Reprinted from the Proceedings of the Biochemical Society*, 17 *November* 1950.
Biochem. J., 1951, Vol. 48, p. xxix.]

Glucuronide Synthesis in Liver Homogenates. By G. J. Dutton and I. D. E. Storey. (*Departments of Biochemistry and of Surgery, University of Edinburgh*)

Although the synthesis of glucuronides by liver slices may be readily demonstrated, various workers have been unable to show such a reaction in broken liver preparations. In the present study, mouse liver was homogenized in alkaline isotonic KCl, and incubations were carried out in a medium containing phosphate buffer and Mg^{++}, but no Na^{+}, and in presence of *o*-aminophenol and ascorbic acid (Levvy & Storey, 1949). A synthesis of *o*-aminophenylglucuronide could not, however, be detected under these conditions unless a boiled extract of liver was also added.

The activity of such extracts is rapidly lost when they are stored at 0°, but an active powder may be prepared by extracting finely minced rabbit liver with trichloroacetic acid in the cold. Glycogen is precipitated from the acid solution by ethanol, and the clear supernatant liquid after centrifugation is poured into excess acetone. When 3·6 mg. of the most active preparation so far obtained was added to a homogenate containing 50 mg. (wet weight) liver in 3 ml. medium, 11·7 μg. *o*-aminophenol were conjugated as glucuronide. This rate of synthesis is of the same order as that found with liver slices (Storey, 1950). The powder loses activity slowly even when stored *in vacuo* over $CaCl_2$ in the refrigerator, and rapidly when warmed in acid solution.

No synthesis was observed when the powder was replaced by any of the following added singly: adenylic acid, adenosinetriphosphate (ATP), cozymase, flavin-adenine dinucleotide, aneurin, riboflavin, pyridoxin, nicotinamide, pantothenate, folic acid, coenzyme *A*, cytochrome *c*, boiled extracts of yeast and wheat germ, D-ribose, D-xylose, glucose-1-phosphate, acetate, pyruvate, succinate, fumarate, L-glutamate or Mn^{++}. Various combinations of dicarboxylic acids, adenylic acid, ATP and cytochrome *c* likewise failed to reproduce the effect of the extracts.

In phosphate or veronal buffers the system shows a broad pH maximum in the region pH 7·6–7·8. Mg^{++} ions exert a stimulating effect on the synthesis, but saccharate (0·001 or 0·0001 M), added to inhibit β-glucuronidase activity (Karunairatnam & Levvy, 1949), did not increase *o*-aminophenylglucuronide formation.

We are indebted to Mr W. E. Balfour, Department of Physiology, University of Edinburgh, for samples of flavin-adenine dinucleotide and an active coenzyme *A* preparation, and to Dr A. B. Roy for glucose-1-phosphate.

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[*Reprinted from the Proceedings of the Biochemical Society*, 17 *January* 1953.
Biochem. J., 1953, Vol. 53, p. xxxvii.]

The Isolation of a Compound of Uridine Diphosphate and Glucuronic Acid from Liver. By

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We have previously reported (Dutton & Storey, 1951) that liver contains a thermostable factor necessary for the formation of *o*-aminophenylglucuronide from *o*-aminophenol in liver homogenates, and that the factor cannot be replaced by a wide variety of co-factors or metabolic intermediates. Further studies have shown that β-D-glucuronic acid-1-phosphate is without effect (cf. Touster & Reynolds, 1952), and that the reaction differs greatly from the formation of *o*-aminophenylglucuronide by liver slices (Storey, 1950) in that it is anaerobic, and uninfluenced by bicarbonate or sulphate ions.

The factor has been obtained in a highly purified form from a trichloroacetic acid extract of rabbit liver by precipitation of the ethanol-insoluble barium salts, ion-exchange chromatography and re-precipitation with barium. At this stage paper chromatography showed that the activity was always associated with phosphate and with ultraviolet absorption, the latter being due almost entirely to a uracil derivative. Mild acid hydrolysis liberated a reducing agent, its rate of release running parallel to the loss of activity of the substance. A positive Tollens naphthoresorcinol reaction was also obtained, the material responsible being approximately equivalent in amount to that of the reducing agent, if calculated as glucuronic acid.

Further purification could be effected by paper chromatography in isopropanol—1% ammonium sulphate solution (Anand, Clark, Hall & Todd, 1952) when two components were observed. Both contained uracil and phosphorus, but only the slower-moving component was active and contained the naphthoresorcinol-positive substance. In the active compound half the total phosphorus was split off in 12 min. in N-HCl at 100°, the remainder proving much more resistant. Uridylic acid, total P, 'labile' P and the naphthoresorcinol-positive substance (calculated as glucuronic acid) were present in the ratios **1:2:1:1**.

When incubated with liver homogenates and *o*-aminophenol or (−) menthol, approximately one equivalent of the corresponding glucuronide is formed for each equivalent of uridylic acid in the purest preparation, the glucuronides being characterized by enzymic and chromatographic methods.

Recent work (Caputto, Leloir, Cardini & Paladini, 1950; Park, 1952) has demonstrated the natural occurrence of various derivatives or uridine diphosphate. The marked resemblance of these compounds to the newly isolated factor suggests that it may have the structure 'uridine-diphosphate-glucuronic acid'. Further evidence will be presented.

We wish to thank Dr O. Touster for the gift of the barium salt of β-D-glucuronic acid-1-phosphate.

* In receipt of a grant from the Medical Research Council.
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