

THE UNIVERSITY OF GLASGOW

Glucuronic Acid

A Study of its Chemistry and Role in Animal  
Metabolism.

by

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## ISOLATION AND DEMONSTRATION OF GLUCURONIC ACID.

The widespread occurrence of glucuronic acid has stood as a direct challenge to the biochemist for over half a century. It is one of the most commonly occurring organic compounds and it has proved to be one of the most difficult to investigate.

The earliest traceable clue to the existence of glucuronic acid appears to be contained in a paper by W. Schmid (1855), entitled 'Ueber das Mangostin'. Schmid was investigating the chemistry of antifebrile materials in the husks of the fruit of the mangosteen tree (*Mangostana mangifer*) and in the course of his investigations he isolated a material which he called mangostin. Considering that this material might be altered in the body and excreted as euxanthic acid he investigated the yellow dye, purree, which used to be prepared by drying the urine of camels and other animals which had fed on the leaves and fruits of the mangosteen tree and which consists largely of the magnesium salt of euxanthic acid. Schmid found that, on acid hydrolysis, euxanthic acid yielded euxanthone and another substance which had the property of reducing alkaline copper oxide, a property possessed by neither euxanthone nor euxanthic acid. We now know that euxanthic acid is the glucuronide of euxanthone and that Schmid had actually observed the presence of glucuronic acid.

The/

The nature of this material was not, however, investigated for many years, and in fact, it was not until 1870 that Bayer published his paper 'Ueber das Euxanthon und die Euxanthinsäure'. On the basis of rather scanty evidence Bayer made some quite remarkable deductions as to the nature of the reducing material released by hydrolysis of euxanthic acid. He suggested for it the formula  $C_6H_{10}O_7$  and suggested that it was 'a sort of saccharic acid'. He also observed that, if this were the case, euxanthone was probably linked to it by a glycosidic linkage to form euxanthic acid.

Unfortunately, the significance of Bayer's paper was not appreciated at the time and in the next decade there appeared only a few rather disconnected observations relating to the occurrence of laevorotatory or reducing substances in the urine after the administration of a variety of materials. In particular Mering and Musculus (1875) demonstrated the existence of such a substance in the urine of patients receiving chloral. They managed to isolate and purify this material which they called 'Urochloralssäure' and which had the formula  $C_7H_{12}Cl_2O_6$ . These workers also observed that the administration of crotonchloralhydrate and morphine produced the same phenomenon in the urine. Baumann (1877) observed that indole gave rise to the appearance of an indigo-forming compound in the urine in addition to indoxyl sulphate.

Von Wiedemann (1877), after feeding camphor, isolated the barium salt of an organic acid which could be decomposed to yield a camphor derivative and reducing material. He considered his material to be a glycoside.

The position began to crystallise when Jaffe (1878-9) isolated and purified 'uronitrotoluolsäure', a material which yielded on hydrolysis nitrobenzylalcohol and a syrup. His attempts to purify the syrupy material failed but it had the properties of a sugar, was a reducing material, rotated the plane of polarised light to the left and was not fermented by yeast. He deduced that its formula was  $C_6H_{10}O_7$  and considered that it might be an aldehyde acid produced by oxidation of the primary alcohol group of a sugar to a carboxyl group. He made the important observation that there were similarities between his material, 'urochloralsäure', and the material isolated by von Wiedemann, but was apparently unaware of the very similar deductions made by Bayer in relation to euxanthic acid some eight years earlier.

Shortly after the appearance of Jaffe's paper Baumann and Preusse (1879) contributed the information that after feeding bromobenzol the urine contained in addition to phenol sulphate/

sulphate a laevorotatory material which on hydrolysis yielded a reducing substance. This material they considered to be similar to uronitrotoluolsäure.

Later in the same year the nature of glucuronic acid was finally established and its lactone isolated in the pure state as a result of the excellent work of Schmiedeberg and Meyer (1879). Following the technique of v. Wiedemann they fed dogs with camphor and isolated an acid syrup from the urine. From this they isolated three substances for which they proposed the names  $\alpha$ -camphoglykuronsäure,  $\beta$ - camphoglykuronsäure and uramidocamphoglykuronsaure. By further hydrolysis of camphoglucuronic acid they eventually obtained campherol and an acid for which they proposed the name glykuronsäure. In its anhydrous state this material had the formula  $C_6H_8O_6$  while its salts indicated a formula of  $C_6H_{10}O_7$ . From its properties they suggested that it was a carbohydrate acid midway between gluconic acid and saccharic acid, having the formula

$$\begin{array}{r} \text{(CHOH)} \\ 4 \\ \text{(COOH)} \end{array} \quad \begin{array}{r} \text{(CHO} \\ \text{(COOH)} \end{array}$$

and being linked to campherol by the aldehyde group. They considered it was probably the same material as the 'hypothetical acid' described by Jaffe but that Jaffe's material had been contaminated with 'uronitrotoluolsäure', a fact which explained why he had found his material to be laevorotatory whereas they found glykuronsäure to be dextrorotatory.



Soon papers began to appear in increasing numbers on the appearance of glucuronic acid conjugates in the urine. Kossel (1880) described a compound 'chinäthonsäure' in the urine after feeding phenetol. On hydrolysis this gave an ether-soluble material, apparently an oxidation product of phenetol, and an ether and alcohol insoluble reducing material which he suggested might be closely similar to if not identical with glucuronic acid. Baumann and Preusse (1881) observed in the course of a paper on the formation of bromphenylmercapturic acid following the administration of brombenzol that there also appeared in the urine laevorotatory, non-sulphate-containing, reducing material. Schmiedeburg (1881) isolated phenylglucuronide. v. Mering (1882) and Külz (1882) independently investigated urochloralsäure and found it to be a compound of trichlorethyl alcohol and glucuronic acid. Hoppe-Seyler (1882-3) suggested that indoxyl might be conjugated with glucuronic acid as well as with sulphuric acid. Külz (1883) isolated phenylglucuronide in the pure state. Pellacani (1883) described the excretion of glucuronides following the administration of borneol and menthol. Zeller (1883) suggested that the laevorotatory, reducing material appearing in the urine after the administration of chloroform might be the glucuronide of trichlormethylalcohol. Thierfelder and v. Mering (1885) isolated the glucuronides of tertiary butyl and tertiary amyl alcohol, which differed from/

from glucuronides so far discovered, in not being precipitable by basic lead salts. They also adduced evidence for the excretion of a glucuronide on the administration of tertiary hexylene-glycol. Mester (1888) suggested the existence of a skatoxyl glucuronide. Külz (1890) either isolated and characterised or provided evidence for the existence of phenylglucuronide, hydroquinone-glucuronide, resorcin-glucuronide, thymolglucuronide and terpenolglucuronide. Blum (1892) isolated thymolglucuronide as a dichloro compound and characterised it. Nencki (1894, 1895) isolated glucuronides of resacetophenone and gallacetophenone. Brahm (1899) isolated o-oxyquinoline-glucuronide after feeding quinosol, and v. Fenyvessy (1900) isolated carbostyrylglucuronide.

Thus, by the turn of the century, a considerable literature on the subject of glucuronides had begun to accumulate and continues to expand to the present day, the traditions of the early German workers now being carried on mainly by Williams and his school (Williams 1947). It will be discussed further in Section II

Up to this time only ether-linked glucuronides had been discovered, although Salkowski (1877) had observed the occurrence of reducing material in the urine of rabbits which had been fed benzoic acid and Kossel (1880) had suggested that the reducing material might be glucuronic acid. Siebert (1901) confirmed Salkowski's early observation and managed to isolate glucuronic acid/

acid from the urine of the animals but he was unable to isolate the aglycone and suggested that it was either an oxybenzoic acid or benzyl alcohol. Finally Jaffe (1904) isolated a dimethylaminobenzoylglucuronide, which was undoubtedly an ester glucuronide, and three years later Magnus-Levy (1907) reported the isolation of benzoylglucuronide itself. To it he ascribed the structure of a glucuronide in which benzoic acid was linked to the glycosidic hydroxyl of glucuronic acid by an ester linkage. Fryde and Williams (1933-6) reinvestigated benzoylglucuronide and confirmed Magnus-Levey's suggestion. Quick (1926, 1934) argued that, since the glucuronide underwent mutarotation spontaneously and combined directly with cyanide to form a cyanhydrin compound, benzoic acid must be linked to a hydroxyl group other than the glycosidic one. Apparently Quick did not appreciate the instability of the ester-compounds and Magnus-Levy's originally proposed structure was established as correct by the beautiful study of Goebel (1938) in which he demonstrated that the methyl ester of the acetylated natural product was identical with 1-benzoyl-2:3:4-triacetylglucuronide methyl ester prepared synthetically.

The chemistry of glucuronic acid itself did not receive a great deal of attention in the early years but Fischer and Piloty/

Piloty (1891) successfully established its relationship to glucose by isolating glucuronic acid, along with gulonic acid and gulose, from the products of progressive reduction of glucosaccharic acid by sodium amalgam. In general the chemistry of glucuronic acid from that time has been regarded as analogous to that of glucose and in general this has proved to be true.

The glycosidic structure of the glucuronides, which had been suggested before glucuronic acid was known, was eventually established by Neuberg and Niemann(1905a ) who synthesised euxanthic acid and phenylglucuronide by employing diacetyl bromoglucurone. Salkowski and Neuberg (1907) demonstrated that natural and synthetic phenyl-glucuronides were identical.

#### Analytical Methods.

Early workers attempting to investigate the metabolic significance of glucuronic acid were greatly handicapped by the lack of suitably specific methods for the determination of glucuronic acid. They had either to attempt sometimes difficult and frequently non-quantitative isolations of the conjugated material from the urine or else had to rely on such non-specific methods as the degree of laevorotation of the urine or the reducing material released by acid hydrolysis (Thierfelder, 1886). We therefore owe a great deal to the observation of Tollens//

Tollens and his school that the ready degradation of glucuronic acid by strong mineral acids to yield furfural and carbon dioxide could be employed as a method for its determination. In the first paper on the subject (Günther, de Chalmot and Tollens, 1892) a method was described based on determination of the furfural distilled from a solution of glucuronic acid undergoing this degradation. The yield of furfural was, however, far from theoretical and tended moreover to be inconstant, particularly in urine. On these grounds Neuberg and Niemann (1905 b) objected to the method and proposed their own which consisted of oxidising glucuronic acid to saccharic acid and isolating the latter as the silver salt. Unfortunately the method was only approximately accurate for glucuronic acid and phenylglucuronide whilst it did not work with other glucuronides tried. Tollens (1905) quite rightly replied to this attack and the original furfural method remained the most satisfactory until Lefèvre and Tollens (1907) described a method for the determination of glucuronic acid by the carbon dioxide released in the reaction. This has now become a standard tool of the carbohydrate chemist (Buston 1932 and see Part II). However, although attempts have been made to apply the method to biochemical estimations (Sauer, 1930) it has proved impractical for serial determinations and lacks the/

lacks the specificity required for even a qualitative test for the presence of glucuronic acid. Such a test had been described by Mayer and Neuberg (1900) who employed the derivative formed with *p*-bromophenylhydrazine for this purpose. Neuberg (1900) also succeeded in producing a highly characteristic thiosemicarbazone. However, even these methods were unsatisfactory, especially when applied to urines (Giemsa, 1900, 1904; Naidus, 1903; Neuberg and Niemann, 1905).

Thus, it appeared to be a very valuable contribution when Tollens and Rorive (1908) described the reaction of a number of carbohydrates with naphthoresorcinol in the presence of hydrochloric acid and claimed (Tollens, 1908) that only the pigment with glucuronic acid was soluble in ether, giving a violet colour. The claim did not go long unchallenged and Mandel and Neuberg (1908) reported that a large number of materials gave the naphthoresorcinol reaction according to Tollens' method. They concluded that any molecule with a carboxyl and carbonyl group would give the reaction. Neuberg and Saneyoshi (1911) claimed that they had rendered the reaction more specific by first forming the osazones of the carbohydrate materials in urine and then causing these to react with naphthoresorcinol. They further claimed that only/

only the pigment formed with glucuronic acid was soluble in benzene and chloroform. That the solubility of the pigment in benzene was specific was confirmed by van der Haar (1918) although he found that it behaved towards chloroform in the same way as ether. Neuberg and Kobel (1931) eventually recommended the use of no less than five different solvents while White and Green (1932) concluded that, due to interference, neither this method nor a method using Bial's reagent were of any use. It was obvious that the reaction had not realised its early promise and attempts were made to develop other specific colour reactions.

Actually, attempts had been made to develop such reactions since near the beginning of the century (Nicolas, 1906; Deniges, 1908; Asbenstedt, 1918; Goldschmiedt, 1910; Thomas, 1925; Scheff, 1927; Dische, 1947a) Most of these reactions were based either on Bial's reaction or reactions using other phenols. They require no more than passing mention since none of these tests have ever come into use. Of more importance was the condensation reaction of carbazole with different sugars, described originally by Dische (1927). Employing a modification of this reaction Egami (1941) developed a method for the estimation of glucuronic/

glucuronic acid. Dische (1947) again investigated the reaction and produced a method for which he claimed a high degree of specificity. This method too has its disadvantages as will be discussed elsewhere and by 1950 a satisfactory method for the determination of very small quantities of glucuronic acid in biological materials was still lacking.

In order to circumvent this difficulty in studying glucuronic acid metabolism attempts had been made to develop specific reactions for individual glucuronides. Neuberg and Schewket (1912) had suggested extracting glucuronides with alcohol-ether before estimating them and since solubilities of the glucuronides vary considerably, extraction methods employing more critical solvents immediately suggested themselves. Lipschitz and Bueding (1939) employed ether extraction to isolate menthylglucuronide before estimating the glucuronic acid by the naphthoresorcinol reaction. Crépy (1946) utilised the solubility of sodium salts of steroid glucuronides in butanol (Venning and Browne, 1936) towards the same end. A very ingenious method for the determination of *o*-aminophenylglucuronide by diazotisation/



diazotisation was developed by Levvy and Storey (1949) from a suggestion of Williams'.

Natural Occurrence of glucuronic acid.

Schmiedeberg (1890-91) was responsible for another great contribution to the biochemistry of glucuronic acid when he adduced evidence for the occurrence of glucuronic acid as one of the components of the aldobiuronide chondrosin, which he had isolated from the chondroitin of cartilage. This opened up an entirely new field and an exceedingly difficult one. Levene (1925) and his co-workers were largely responsible for developing it in the early part of the century and particularly for investigating the mucins and mucoitin-containing polysaccharides, which also contain glucuronic acid. Howell (1928) suggested that heparin probably contained glucuronic acid. The discovery of hyaluronic acid (Meyer and Palmer, 1934), another glucuronic acid-containing polysaccharide, focussed renewed attention on the importance of these compounds.

At first it was thought that the appearance of glucuronides in the urine only followed the administration of toxic or, at least, foreign substances. Flückiger (1885), however, /

however, adduced evidence that the laevorotatory, reducing material, precipitable by basic lead acetate from normal urine was glucuronic acid. At the time this was not an easy matter to prove conclusively and it was not until 1900 that Mayer and Neuberg were able to claim to have done so, for the first time, by isolation of the p-bromophenylhydrazine derivative from hydrolysed urine. The development of more-or-less specific methods for the determination of glucuronic acid by Tollens and his school led to the accumulation of a mounting pile of evidence for the existence of glucuronic acid as a normal urinary constituent. Even so the opinion seemed to be prevalent (as it still is to a considerable extent) that the glucuronides in the urine were probably formed almost entirely to detoxicate phenolic materials absorbed from the gut. Lewin (1901) performed an experiment which suggested that this might not be true, when he demonstrated that increased amounts of glucuronic acid, associated with increased amounts of phenolic material, appeared in the urine in the absence of disturbances of the gut after the administration of phlorhizin. Mayer (1902) could not confirm Lewin's observation and the subject received no further serious consideration until the isolation of pregnanediol glucuronide by Venning and Browne (1936) and oestriol glucuronide by Cohen and Marrian (1936) dramatically focussed/

focussed attention on the glucuronides as a class.

The possibility that glucuronides might be excreted by other channels was considered by Bial (1902) and Bial and Hüber (1902). Their claim to have demonstrated glucuronic acid in the faeces was not substantiated by Mayer (1903), though van Leersum (1903) claimed to have done so. Bial (1905) again claimed to have isolated menthylglucuronide from the bile but the matter seems to have received no direct investigation since.

The possible occurrence of glucuronic acid in one other form has been suggested; Pollecoff (1924) produced evidence that glucuronic acid was a constituent of urochrome and Rangier and de Traverse (1939, 1939a) investigated the matter further and concluded that urochrome consisted of a sulphur-containing peptide to which was attached indoxyl glucuronide.

Simultaneously with these investigations, of course, a large number of workers had established the widespread occurrence of glucuronic acid throughout the entire field of biology, particularly in bacteria and plants.

#### The biological synthesis of glucuronic acid.

##### Original theories.

When Schmiedeberg and Meyer (1879) reported the discovery of glucuronic acid they suggested that it probably occurred/

occurred naturally as an intermediate in the oxidation of glucose. Their theory of glucuronide formation, then, was that, when an aglycone became linked to the aldehyde group of glucuronic acid oxidation could proceed no further and the glucuronide was excreted in the urine. A somewhat different theory was proposed by Sundvick (1886) and independently by Fischer and Piloty (1891) when they reported the synthesis of glucuronic acid. In essence this theory proposed that glucuronic acid did not occur normally as an intermediate of glucose oxidation since the aldehyde group of glucose was more easily oxidised than the primary alcohol group. According to this school a glucoside was first formed and the aldehyde group then being blocked the primary alcohol group of glucose was oxidised to a carboxyl group and thus a glucuronide was formed. It is important to appreciate the subtle difference between the two schools for, although the Sundvick theory must be rejected in the face of the subsequent work reported below, there still remains the possibility that there may be a germ of truth in the Schmiedeberg and Meyer hypothesis.

Much of the earlier work was concerned with elucidating this problem. The obvious way to test the validity of the Sundvick hypothesis was to administer glucosides and to//

to try to isolate the corresponding glucuronides from the urine. This was done for the first time by Brahm (1899) and he reported that the glucosides were excreted unchanged. Subsequently Falck (1902) administered benzyl glucoside and obtained hippuric acid in the urine. He also fed phenylglucoside to dogs and obtained phenylglucuronide and phenylsulphuric acid from the urine. It was obvious from these experiments that the Sundvick theory could hardly be maintained. Hildebrandt (1905, 1909) tried similar experiments, administering the glucosides coniferin, syringin and bornylglucoside. In one series of experiments he injected both bornylglucoside and bornylglucuronide into rabbits. In each case only a small fraction of bornylglucuronide appeared in the urine. He also tried this experiment on frogs and in this case found that free borneol appeared in the urine but no glucose or glucuronic acid. His conclusion was that glucose and glucuronic acid followed the same metabolic pathway. This conclusion could, of course, be held to support either of the above theories. A more feasible explanation for Hildebrandt's results was advanced by Röhmann (1908) who suggested that glucosides and glucuronides were probably broken down in the body and the aglycones then conjugated with glucuronic acid. Further evidence that this/

this was the case was provided by Rohmann himself when he showed that liver contained an enzyme which would hydrolyse glucuronides and by Hofmann (1935) who demonstrated the presence in liver of an enzyme which would hydrolyse glucosides. Fryde and Williams (1936) finally demonstrated convincingly that the administration of phenylglucoside orally or parenterally to rabbits produced the same effect as injecting or feeding the appropriate amount of phenol insofar as the relative amounts of sulphate and glucuronide appearing in the urine were concerned.

None of this work could be held to support the Sundvick theory but in 1913 Hämäläinen produced a startling experiment in which he claimed to have found santenol-glucoside in the portal blood flowing from an isolated loop of intestine into which had been placed santenol and glucose. If Hämäläinen's observation could be verified we should have to reconsider the Sundvick-Fischer-Piloty hypothesis but since it never has been it must remain in doubt. Moreover, two years earlier Schüller (1911) had provided a piece of evidence that the protagonists of the Sundvick theory could not explain when he showed that the administration of phlorhizin led to the excretion of phlorhizin glucuronide, in which the original glucose/

glucose molecule of phlorhizin remained unchanged, glucuronic acid being attached elsewhere. The perfusion experiments of Hemingway, Pryde and Williams (1934) finally disposed of the Sundvick-Fischer-Piloty hypothesis, these authors demonstrating that the inclusion of a glucoside in the perfusion fluid led to no formation of glucuronide although glucuronide could be formed by perfusion with the aglycone alone. Lipschitz and Bueding (1939) confirmed this observation in experiments with surviving liver slices.

A somewhat different dispute had been proceeding since Mayer (1902a) had suggested that the glucuronic acid in the urine provided an indication of the efficiency of the oxidative processes in the body. Blumenthal (1902) and Bial and Hüber (1903) could not agree with this hypothesis and Blumenthal and Wolff (1903) showed that in febrile conditions, although there was apparently an increased excretion of glucuronic acid, there was a concomitant increase in the excretion of skatoxyl and indoxyl and they considered that the increased amount of glucuronic acid was simply formed in order to detoxicate these. Wohlgemuth (1904), as a result of some observations in a case of cocaine poisoning, favoured Mayer's point of view. The methods which these workers had at their disposal were primitive and their evidence/

evidence was of the most circumstantial kind so that we must consider their work as of little more than historic interest.

Precursors of glucuronic acid.

In both the above theories it had been tacitly assumed that glucuronic acid was derived from glucose. A more fundamentally divergent point of view arose from the experiments of Thierfelder (1886) who, paradoxically, assumed the validity of Schmiedeberg and Meyer's hypothesis. In what was probably the first biochemical experiment on glucuronic acid metabolism Thierfelder set out to demonstrate that carbohydrate could be derived from protein and for this purpose he assumed that glucuronic acid, as an oxidation product of glucose, would give an indication of carbohydrate production. He starved a series of rabbits for five to six days when he assumed that the liver was free of glycogen. The administration of chloral hydrate or tertiary amyl alcohol then gave rise to the excretion of glucuronides in the urine in much the same quantities as in normal control animals. Nebelthau (1891) questioned the significance of Thierfelder's findings on the ground that rabbit liver contained sufficient glycogen for glucuronide formation even after six days. Mayer (1902b) repeated Thierfelder's experiments but he found that the ability of rabbits to /



to excrete glucuronides after the administration of camphor was reduced by starvation and restored by the administration of glucose. It should be mentioned that although camphor was used frequently thereafter as a glucuronogenic material it is unsatisfactory since it must be oxidised before it can be conjugated and hence another factor is introduced. v. Fenyvessy (1904) carried out a more carefully controlled series of experiments of this nature. Though he also employed camphor for most of his experiments some were repeated with phenol and chloralhydrate. Rabbits were starved for twelve days, the ability to excrete glucuronides at the beginning and end of this time, and the glycogen content of the liver and muscles being determined. He found that, whereas the liver and muscles were almost completely glycogen-free, glucuronic acid excretion was unimpaired. Moreover, the administration of glucose did not give rise to increased excretion in the starved animals. On the other hand Hildebrandt (1900) demonstrated that the administration of glucose, sucrose and maltose rendered less poisonous some toxic materials which were normally conjugated as glucuronides. Mandel and Jackson (1903) also tried the effect of feeding camphor to fasting dogs and found that the administration of carbohydrate to these animals led to a/

a decrease both in urinary nitrogen and urinary glucuronic acid whereas the addition of protein to the diet led to an increase in both.

Most of these experiments tended to suggest that glucuronic acid had a protein origin.

A different approach to the problem was made by Loewi (1901) who found that in fasting, phlorhizinised dogs glucuronic acid formation gave rise to neither a decrease in glucose excretion nor a marked decrease in nitrogen excretion, a finding which suggested different origins for glucuronic acid and glucose. The results of Benech (1922), who found that the administration of glucose increased the glucuronuria due to camphor, indicated the opposite while the work of Friedemann and Koechig (1926) was indeterminate, these authors reporting that they obtained a decreased production of menthylglucuronide after treating animals by either a) insulin and glucose or b) phlorhizin and starvation.

Quick (1926 b) criticised the earlier experiments on starved animals in view of knowledge which had by then accumulated regarding the tenacity with which the body retained its stores of glycogen and the facility with which protein could be converted to glucose and glycogen. He approached/

approached the problem once more by methods similar to those of Loewi and Mandel and Neuberg. In a first series of experiments (Quick, 1926a) he found that depancreatized dogs were still capable of excreting glucuronic acid and that an increase in glucuronic acid production resulted in a decrease of glucose production. From this he concluded that glucuronic acid was produced at the expense of the potential glucose. Subsequently (1926b) he found that the production of glucuronic acid in response to borneol or benzoic acid led to very little increase in the urinary nitrogen if the animal received adequate supplies of carbohydrate. After a period of fasting, however, the production of glucuronic acid was accompanied by an increase in urinary nitrogen and this rise was not prevented by the simultaneous administration of glucose. From these findings Quick concluded that, while carbohydrate was the normal source of glucuronic acid, protein might be used preferentially when the carbohydrate stores were depleted. Glucose was not an immediate precursor and he suggested that glycogen might be the precursor, or possibly glycolytic amino-acids or lactic acid.

Adeline (1927) tried the effect of feeding different amino-acids to starved rabbits and found that while some glycolytic amino-acids stimulated glucuronic acid production some//

some non-glycogenic ones, such as tryptophan and phenylalanine, had the same effect while some glycogenic ones had no stimulating effect. Makarevich-Galberin (1930) renewed the claim that glucuronic acid production was decreased in starvation and paved the way for two important experiments. Schmid (1936) employed hibernating frogs, in which the glycogen content of the liver sinks progressively to very low levels as the fast proceeds. He found that in well-fed animals the administration of menthol gave rise to no signs of intoxication whilst the surrounding water gave a strong naphthoresorcinol reaction. On the other hand, starved animals in the same conditions died rapidly and no glucuronic acid could be detected. Apart from the objection that other storage materials besides glycogen might be exhausted the experiment suggested convincingly the necessity of glycogen for glucuronic acid in these amphibians. Dziewiatkowski and Lewis (1944) provided a more direct experiment when they estimated the glycogen content of the livers of rats without the administration of glucuronogenic materials and four hours after the administration of glucuronogenic materials (l-menthol and sodium tertiary butyl acetate). They were able to demonstrate a significant fall in the glycogen content of the latter group. Deichmann and Witherup (1945) found that differences in diet made no significant/

significant alteration to the excretion of glucuronic acid but that on fasting rabbits excreted less glucuronic acid and could not conjugate administered materials to the same extent. Mosbach and King (1950) in radioactive tracer studies using  $^{14}\text{C}$  have adduced evidence that glucuronic acid is derived directly from carbohydrate and, indeed, have taken us back to Schmiedeberg and Meyer by suggesting that the direct source of glucuronic acid is glucose. It is proposed to consider these matters in a later section.

#### Other sources of glucuronic acid.

Apart from glucose, glycogen and protein a number of other possible precursors for glucuronic acid have been proposed. As has been mentioned Quick had suggested that lactic acid might be a precursor of glucuronic acid and he returned (Quick, 1932) to investigate this point but found that aceto-acetic acid, lactic acid and glycollic acid led to decreases in glucuronic acid production. The work of Lipschitz and Bueding (1939) on tissue slices in vitro provided a considerable amount of evidence in favour of the existence of 3-carbon compounds as precursors and led them to propose trioside formation as preceding glucuronide formation. A large number of 3-carbon compounds were fed by Martin and Stenzel (1944) in an attempt to find if any of them/

them gave rise to an increase in glucuronic excretion. Among many other compounds of different molecular structure and size they reported that dihydroxyacetone, glycerol, lactic acid and calcium glycerophosphate stimulated glucuronic acid excretion while pyruvic acid caused a decrease or had no effect. It seems likely that these workers and many others placed too much reliance on their methods and on the constancy of normal excretion, as will be discussed later.

Miller, Brazda and Elliot (1933) and Miller and Conner (1933) put forward the suggestion that glucuronic acid for conjugation purposes was derived from the mucin of the diet. Such a suggestion ignores the production of glucuronic acid in starving animals, the fact that glucuronic acid has to be synthesised in order to appear in mucoproteins and mucopolysaccharides in the first place, and the fact that the aldobiuronides of such materials are difficult to split. The effects of hormones, vitamins and other factors on glucuronic acid formation.

Most of the experiments employing hormones were originally designed to indicate whether glucuronic acid arose from protein or carbohydrate. In view of the complex actions that we now know insulin and adrenaline to possess, involving both protein and carbohydrate metabolism, it is difficult/

difficult to draw definite conclusions from the results obtained and consequently it was considered advisable to consider such experiments under this miscellaneous heading.

Hürthle (1925) first tried the effect of insulin and found that it did not give rise to increased glucuronic acid production. Matsumoto (1928) made a similar observation in relation to the conjugation of chloral and he also found adrenaline to have no effect on this conjugation. On the other hand Quick (1932) reported a 50% increase in the excretion of glucuronic acid monobenzoate after insulin injection. Since benzoic acid is preferentially conjugated with glycine in most species these results could be held to indicate an alteration in its metabolism as much as in that of glucuronic acid. However, Lipschitz and Bueding (1939) in their liver-slice experiments also reported a great increase in the formation of menthylglucuronide on the addition of insulin to the medium whilst they found that adrenaline had an inhibitory effect. Vescia (1946) found that in diabetic patients the percentage conjugation of menthol with glucuronic acid almost doubled when insulin was given. Also he reported that whereas the blood glucose of untreated patients fell during the formation of glucuronic acid it remained unaffected in/

in those receiving insulin. The effect of thyroid on glucuronic acid conjugation was also investigated by Stark (1930).

Shortly after Szent-Györgyi described his 'hexuronic acid' some considerable interest developed in glucuronic acid, particularly when Rygh and Rygh (1932) reported that scurvy could be prevented and cured by a mixture of glucuronic acid and methylnornarcotine. However Widmark and Glimstedt (1933) and Peschke (1933) could not confirm their findings and although Rygh maintained his point of view after the discovery of ascorbic acid it fell into disrepute. Quick (1933) investigated the urinary excretion of glucuronic acid after the administration of borneol to scorbutic guinea-pigs and found little evidence of any change. Meyer (1942), however, reported that after ceasing the administration of 200 mg. of ascorbic acid daily both the urinary ascorbic acid and glucuronic acid fell and were uninfluenced by the administration of aspirin, though greatly increased by thymol. In this respect it is noteworthy that ascorbic acid in high concentrations is liable to interfere seriously with the naphthoresorcinol reaction (Section II). However, Mosbach, Jackel and King (1950) have decided to reinvestigate the possible relationship between glucuronic acid and ascorbic acid since they have observed that a number of substances promoting/



promoting the formation of glucuronic acid also promote the excretion of ascorbic acid.

The effects of deficiency of vitamins A, D, E, B<sub>2</sub> and riboflavin were investigated by Martin and Stenzel (1944) while Basu and Ray (1947) investigated the effect of vitamin B<sub>1</sub> deficiency and Manville (1937) considered that vitamin A was probably connected with glucuronic acid metabolism.

The effect of different types of diet was investigated by Palladin and Persova (1928) who found that an acid diet gave rise to greater conjugation of glucuronic acid with menthol than a basic diet. Precisely the opposite conclusion was reached by Kuznetzova (1930) using thymol in place of menthol.

Giordano (1936) suggested that copper stimulated glucuronic acid production by the liver.

#### In vitro studies.

The first of these studies was made by Lipschitz and Bueding (1939; Bueding, 1939) and their work appeared to indicate quite conclusively that the process was strictly aerobic (cf. Hemingway, Pryde and Williams, 1934) was inhibited by inhibitors of phosphorolysis and that glucuronic acid was probably derived from 3-carbon compounds, the first stage in the formation of glucuronides being the formation of/

of triosides. Crépy (1947) agreed that no stimulation of glucuronide production followed the addition of glucose or glucuronic acid to the saline medium, in which liver slices were incubated with a suitable aglycone, but could not confirm that the addition of lactic and pyruvic acids produced such an increase. On the other hand DeMeio and Arnolt (1944) had demonstrated that the addition of glucuronic acid to liver slices in which the conjugation of phenol had been inhibited by iodoacetate, restimulated phenol conjugation, an observation which suggested that glucuronic acid could combine directly with phenol. Storey (1949, 1950) was also unable to confirm Lipschitz and Bueding's findings with 3-carbon compounds but he found that bicarbonate had a powerful stimulating effect on glucuronide synthesis. His suggestion that carbon dioxide fixation was probably involved in glucuronic acid synthesis received no confirmation from the radioactive carbon studies of Mosbach and King (1950). Florkin, Crismer, Duchateau and Houet (1942) provided some evidence for the synthesis of bornyl-glucuronide by glucuronidase in the presence of borneol and glucuronic acid. The degree of synthesis was very small and Karunairatnam and Levvy (1948, 1949) have provided evidence that glucuronidase does not have a synthetic rôle in vivo. In the majority of these experiments the criticism of Fishman (1950) that there has been no attempt to/

to distinguish between the process of conjugation and glucuronic acid synthesis is undoubtedly valid.

Site of formation of glucuronides.

Pick (1894) produced liver lesions by injecting material into the bile-duct and showed that in these animals there was no interference with the conjugation of urochloralic acid. Pohl (1898) achieved somewhat similar results by causing liver lesions by the administration of diamines. He found the conjugation of phenol unaltered but found that euxanthone no longer stimulated glucuronide production. Pick's experiment suggested that the liver was not the site of glucuronic formation while Pohl considered that his results suggested different sites of formation but indicated that the liver alone was not responsible. However, Embden and Glaessner (1902) conducted perfusion experiments of different organs and found that, whereas phenol conjugated in these circumstances could be accounted for as sulphate in kidney and lung, only part of it could be accounted for as such in the liver and Embden (1902) adduced evidence for the remaining material being in the form of phenylglucuronide. These experiments focussed attention on the probability of the liver being the organ in which glucuronides were conjugated and Stejskal and Grünwald (1909) somewhat prematurely/

prematurely suggested the measurement of glucuronic acid excretion as a test of liver function. Subsequently a large number of papers appeared on this subject (Roger, 1915; Roger and Chiray, 1915, 1915a; Clogne and Fiessinger, 1916; Sauer, 1930; Boku and Kin, 1931; Nasarjanz, 1934 Mukerji and Ghose, 1940, 1940a; Ottenberg, Wagreich, Bernstein and Harrow, 1943, Snapper, Greenspan and Saltzman, 1946; Snapper, Saltzman and Greenspan, 1946; Snapper and Saltzman, 1947, 1948; Rosenmund and Esselier, 1947; Borgström, 1949). Schmid (1922) and Brulé, Garban and Amer (1925) suggested in the early years that the methods then in use were unsatisfactory but this did not deter investigators. The results of these experiments were erratic and in many cases inconclusive but, in general, they did suggest that disturbances of liver function were reflected in alterations of glucuronide excretion or in the ability to conjugate administered materials. Rather more useful experiments were performed by Persova (1930) who demonstrated a great decrease in glucuronic acid conjugation following poisoning by arsenic and by Nasarjanz (1932; 1932a) who showed the same for phosphorus poisoning. On the other hand we must set against the significance of these findings the observations of Bueding and Ladewig (1939) who observed that livers poisoned in different ways responded differently to/

to the additions of lactic acid in vitro. In very many of these earlier investigations the methods applied were unsatisfactory and their significance is very dubious.

Of much greater significance was the experiment of Hemingway, Pryde and Williams (1934) in which transfusion of intact organs with a phenol-containing fluid showed that glucuronide formation could occur in the liver but not in the other organs tried, thus confirming the suggestion implicit in the early work of Embden and Glaessner. Nishimura (1936) performed a one-stage hepatectomy on a dog and found that it could no longer conjugate camphor. This experiment is robbed of its potential significance by the unfortunate choice of drug. At the same time Tsutsui (1936) on the same or a similar animal found that removal of the liver had only a slight effect on the lethal dose of o-hydroxybenzoic acid, a finding which might be held as contrary to that of Nishimura.

Contradictory results were also obtained, on the one hand by Loomis and Quick (1944) who found the ability to conjugate anthranilic acid after chloroform anaesthesia temporarily depressed and Basu and Ray (1947) who found the same after carbon tetrachloride injection, and on the other hand by Deichmann, Kitzmiller and Witherup (1945) who found no impairment of conjugation of cyclohexanone after the/

the production of liver injury by phosphorus or a mixture of carbon tetrachloride and chloroform. Schmid (1947) returned to the field with an experiment that was bound to leave serious doubts about the essential rôle of the liver in this conjugation. He performed the same experiment with hibernating frogs as he had performed eleven years earlier but on this occasion used hepatectomised frogs. These behaved in exactly the same way as intact animals i.e. those with low glycogen stores were poisoned by menthol and died while those with high glycogen stores conjugated menthylglucuronide and lived. One can only conclude from this that the liver is not essential for glucuronic acid conjugation in the frog and it may be dangerous to project the conclusion to other species. At the same time some in vitro evidence has been obtained that, although conjugation is most efficient in the liver, it can proceed to a small extent in the kidneys of guinea-pigs (Lipschitz and Bueding, 1939) and mice (Storey, 1950).

Degradation of glucuronic acid compounds.

Neuberg and Niemann (1905a) reported that euxanthic acid and phenylglucuronide were hydrolysed by emulsin and this observation seems to have dominated the field for many years, probably because earlier workers were mainly interested in using it as a tool to prove the glycosidic structure of glucuronides. Hydrolysis by this enzyme of syringagluconic acid was reported/

reported by Hämäläinen (1905), of l-camphoglucuronic acid by Hildebrandt (1910), of camphoglucuronic acid and menthylglucuronide by Ishidate (1929) and of baicalin by Miwa (1936) whilst this latter investigator also reported a specific baicalinase. Some difference of opinion arose on the effectiveness of emulsin and some observers, including Sera (1913) were unable to demonstrate the hydrolysis. The reason for the discrepancy was apparently supplied by Helferich and Sparmberg (1933) who claimed to show that emulsin preparations actually contained two enzymes, only one of which would attack glucuronides.

Röhmman (1908) was the first to claim to have demonstrated the hydrolysis of a glucuronide (menthylglucuronide) by a tissue extract (chloroform extract of dog's liver). He writes "Die Leber enthält auch ein Enzym, welches gepaarte Glykuronsäuren spaltet. Der Chloroformextrakt einer Hundleber spaltete Mentholglykuronsäure. (F. Röhmman)". This observation is referred to by Sera (1914) who is generally credited with making the discovery. Indeed, Sera was the first to provide convincing figures to illustrate the occurrence of this enzymatic hydrolysis, using orcinolglucuronide and phloroglucinolglucuronide as substrates and extracts of several different organs from several different species as enzyme preparations. It is indeed surprising that, despite the considerable amount of research that appeared on /

on problems of glucuronic acid metabolism in the next twenty years, no further investigation of the phenomenon was conducted until Masamune (1934) described the preparation of a semipurified extract from ox-kidney which had the ability to hydrolyse  $\beta$ -menthyl-, naphthol-, bornyl-, phenyl- and phloroglucinolglucuronides but which did not attack  $\alpha$ -menthylglucuronide nor  $\alpha$ - and  $\beta$ -menthyl- and methyl-glucosides. He proposed for it the name  $\beta$ -glucuronosidase, now shortened to  $\beta$ -glucuronidase, and studied some of the kinetics of its action. He also claimed (Masamune, 1934a) to have discovered an enzyme with an optimum pH between 5.4 and 5.8 which hydrolysed ester glucuronides. This enzyme was prepared from chloroform and toluene extracts of tissues and has apparently not been re-investigated with a view to establishing its identity or otherwise with  $\beta$ -glucuronidase. Masamune's original paper was quickly followed by one from Oshima (1934) and these two workers published several papers in the next two years on the subject while Hofmann (1935) also investigated this enzyme briefly.

In the meantime the glucuronides of the sex hormones had been described and Marrian (1937) suggested the hydrolysis of oestriol glucuronide by glucuronidase in the intestines. Fishman published his first papers in 1938 and to him and his co-workers must be accredited most of the



the information on the enzyme obtained in the next ten years. Possibly one of their most valuable contributions was the development of phenolphthalein glucuronide as a substrate for enzyme assays (Talalay, Fishman and Huggins, 1946). Previous to this reductimetric methods had been employed and whilst these were valuable where pure preparations of enzyme and substrate were available they had obvious limitations in the case of such materials as blood or crude tissue extracts. A method employing phenylglucuronide was also developed by Kerr, Graham and Levvy (1948) and has proved similarly useful.

The preparation of active glucuronidase extracts was investigated by Masamune (1934), Oshima (1934, 1936), Fishman (1938), Graham (1946), Mills (1947, 1948) and Kerr & Levvy (1951) and will be discussed in detail elsewhere (part IV & V).

Glucuronidase was at first held to be a single enzyme with different pH optima for different substrates but Mills (1948) demonstrated the presence of two enzymes in glucuronidase preparations while Mills and Paul (1949) claimed to have demonstrated at least three enzymes with different pH optima and velocity constants.

Fishman (1940a) showed that glucuronidase activity was increased in a number of organs following the administration/

administration of menthol and he considered that this probably constituted an adaptive response, the enzyme having a synthetic role. The claim by Florkin et al (1942) to have demonstrated the synthesis of bornyl-glucuronide in vitro from borneol and glucuronic acid in the presence of a preparation of glucuronidase appeared to confirm this view. It also received some confirmation from DeMeio and Arnolt (1944) who demonstrated increased ability to conjugate phenol in organs of animals which had been fed phenol or borneol. Fishman and Fishman (1944) claimed that the administration of oestrogens increased the glucuronidase of animal organs and Fishman (1947) showed that these increases could be obtained with concentrations of oestrogens well within physiological limits. He suggested that the synthesis of oestrogen glucuronides represented a first stage in the utilisation of oestrogens. However, Mitsuba (1927) had obtained evidence to suggest that the spleen probably had a destructive role so far as glucuronides were concerned and Mills (1946) demonstrated not only that the spleen was one of the richest sources of glucuronides in the body but also that its removal gave rise to an increased excretion of glucuronic acid in the urine. These results indicated that either glucuronidase/

glucuronidase did not have a synthetic function at all or possibly that the spleen had a different function from the liver insofar as glucuronidase was concerned, a proposition advanced earlier by Talbot, Ryan and Wolfe (1943) since they had found spleen and liver enzymes to behave differently towards pregnanediol glucuronide. Fishman, Anylan and Gordon (1947) presented a modified theory of the function of glucuronidase in relation to steroid metabolism, suggesting on this occasion that hydrolysis of the glucuronides in the tissues might be important in releasing them at the site of action.

Kerr and Levvy (1947) confirmed many of Fishman's findings in relation to the stimulation of glucuronidase activity while Levvy, Kerr & Campbell (1948,)1948a) reported still other factors which increased the glucuronidase activity of tissues. They proposed a quite different theory to explain these changes, considering that glucuronidase activity was related to cell proliferation. Mills, Paul and Smith (1949) while verifying some of the findings of Kerr et al, who had used mice in their experiments, were unable to reproduce them all in the rat and concluded that there was no direct relationship between glucuronidase activity and cell proliferation.

Karunairatnam and Levvy (1949) and Karuanairatnam, Kerr & Levvy (1949) about this time investigated the /

the relationship between glucuronide synthesis and glucuronidase activity. They found no connection between the two and also observed that whereas glucuronidase was inhibited by saccharic acid glucuronide synthesis was unaffected, thereby suggesting that glucuronidase is not in any way connected with glucuronide synthesis. There would appear to be no satisfactory evidence at the moment as to the nature of the true function of glucuronidase although a few other interesting observations have been made. Fishman & Anlyan (1947, 1947a, 1947b) have made the claim for some time that neoplastic processes are associated with increased glucuronidase activity and although these findings would seem to agree with Levvy's hypothesis it will be seen elsewhere (part V) that judgment should possibly be reserved on this matter for the moment. Other of Fishman's associates (Fishman & Anlyan, 1947) have demonstrated increases in glucuronidase activity in the serum during pregnancy and have claimed to have shown abnormally high figures in the toxæmias of pregnancy (McDonald & Odell, 1947, Odell & McDonald, 1948). Most of these observations await confirmation by independent observers.

There has been some measure of discrepancy regarding the degradation of glucuronides in vivo. Reference has already been made to the work of Hildebrandt (1909) on

on the administration of bornylglucuronide. The most logical interpretation of his work was that glucuronides were hydrolysed in vivo. Koiki and Nagashima (1936) on the other hand found that if glucuronides were administered parenterally they were excreted unchanged, whereas if they were administered orally some hydrolysis occurred and some conjugated sulphates were excreted. Garton and Williams (1949) have also found that the oral administration of phenylglucuronide gives rise to some sulphate excretion. The experiments of Mitsuba (1927) and Mills (1946) on splenectomised animals suggested that glucuronidase functioned as a hydrolytic enzyme in vivo. It is rather important that the work of Koiki and Nagashima with parenterally administered glucuronides should be verified or otherwise since, if glucuronidase does not hydrolyse glucuronides in vivo we have no knowledge whatsoever that might indicate its true function. The hydrolysis of glucuronides administered orally can, of course, be explained by acid hydrolysis in the stomach and bacterial degradation (Buehler, Katzman and Doisy, 1949) in the gut.

The fate of glucuronic acid itself in the body has been the subject of a number of studies but few concrete conclusions are to be drawn from them. The earliest theories/

theories had no experimental backing. Schmiedeberg and Meyer's (1879) original suggestion was that glucuronic acid, if not combined with an aglycone, was oxidised to saccharic acid. Flückiger (1885) suggested, on the other hand, that it might give rise to acetone. Salkowski and Neuberg (1902) made the observation that putrefactive bacteria could convert glucuronic acid to l-xylose and therefore had some justification for suggesting then and later (Salkowski and Neuberg, 1907) that glucuronic acid acted as an intermediate between glucose and the pentoses of nucleoproteins. A more systematic study was made by Pederi (1908) who found that glucuronic acid was not attacked in vitro by blood or pancreas extracts though some was lost in contact with a liver extract. When he injected glucuronic acid he found it excreted mainly unchanged in the urine, although more was retained if it was given orally, and large doses gave rise to an increase of oxalic acid in the urine. Moreover, the toxicity of camphor and chloral were unaffected by its administration. (The author considered his findings to confirm the Sundvick theory rather than the Schmiedeberg and Meyer hypothesis). Biberfeld (1914) also tried incubating glucuronic acid with a liver brei and found it was not attacked. He, incidentally, tried to obtain the synthesis of menthylglucuronide in vitro/

vitro by adding menthol to the brei but was unsuccessful. This author also found that the administration of glucuronic acid led to the appearance of reducing material in the urine almost immediately, the amount corresponding to the amount of glucuronic acid fed. Hürthle (1927) similarly observed that administered glucuronic acid appeared very rapidly in the urine, largely ungangaged, whilst it failed to relieve insulin hypoglycaemia in rabbits, and Quick (1928) confirmed these observations.

Only two positive indications as to the possible route of glucuronic acid breakdown in the body have been obtained. Enklewitz and Lasker (1935) investigating a series of patients with pentosuria found that the administration of glucuronic acid greatly increased the excretion of l-xyloketose. Flaschenträger, Cagianut and Meyer (1945), on the other hand found a greatly increased excretion of furan-2:5-dicarboxylic acid in the urine after the administration of glucuronic or galacturonic acids.

Butkevich, Minzhinskaya and Trafimova (1938), on the basis of studies with micro-organisms, have advanced the theory that glucuronic acid is an intermediate between glucose and citric acid, though Butkevich (1938) considered that the intermediate was more likely to be diketogluconic acid/

acid or aldehydogluconic acid.

Our knowledge of the oxidative processes involved in the degradation of glucose has advanced very considerably in the past twenty years and the discovery of the glycolysis cycle has diverted interest from glucuronic acid. Although the existence of the well-known glycolysis pathway does not exclude the possibility of alternative routes of oxidation the observations which have been mentioned indicate that glucuronic acid cannot replace glucose and that its subsequent degradation is probably quite different. The persistent indications that it may be in some way connected with pentose metabolism may stimulate some overdue research into the matter.

In reviewing the literature on this entire subject one is struck by the remarkable lack of agreement on almost every point and by the lack of concrete information on many aspects of the subject. In the subsequent sections it is proposed to deal with these matters in greater detail, to attempt to illuminate the reasons for the many discrepancies and to elucidate some current controversies.



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## The Chemistry of Glucuronic Acid and its Compounds.

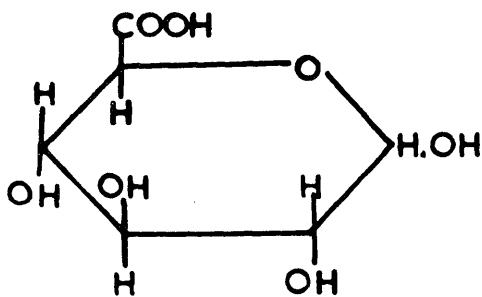
The alduronic acids are sugar acids characterised by the simultaneous presence in the molecule of an aldehyde and a carboxyl group. The most important of these biologically are those derived from the hexoses and in the case of animals the most important by far is glucuronic acid, which is the alduric<sup>on</sup> acid derived from glucose,

### Nomenclature.

Artz and Osman (1950) in a recent publication object to the use of glucurone to designate the lactone of glucuronic acid and glucuronide to designate a glycoside of glucuronic acid, suggesting that the terms glucuronolactone and glucuronoside are more in accord with current practice in carbohydrate chemistry. The former two terms have, however, become established usage, and since they are used almost universally in the literature and are more familiar to those actually working in the field they have been retained throughout this study.

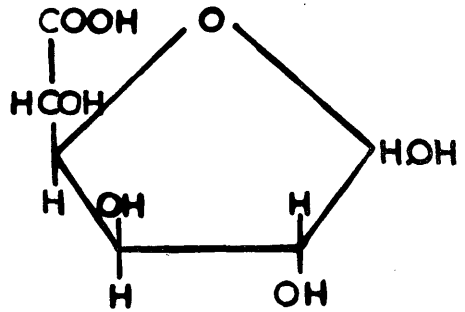
### Structural Chemistry.

The structural chemistry of glucuronic acid is analogous to that of glucose and, as in the case of glucose, it may theoretically exist in both pyran and furan ring forms (Fig. 2 1, I & II)



I

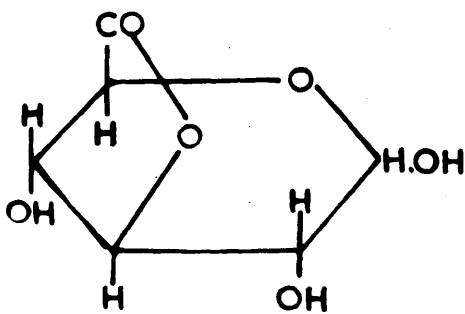
Glucopyruronic Acid



II

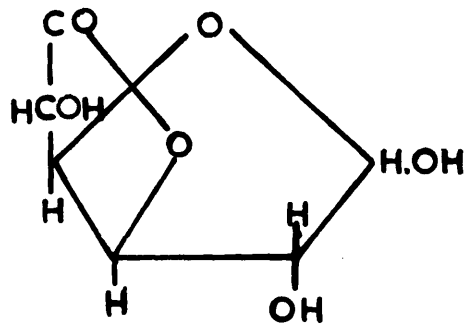
Glucofufururonic Acid

Figure 2.1



III

Glucopyruronolactone



IV

Glucofufuronolactone

Figure 2.2

It has a marked tendency to form a gamma lactone or internal ether, called glucurone, which may also occur in pyran (III) and furan (IV) ring forms (fig. 22) Methylation studies by Pryde and Williams (1931) suggested that the normally prepared crystalline material was glucopyruronolactone but subsequent studies by Reeves (1940 and Smith (1944) have suggested a furan structure (Fig. 22, III & IV)

As in the case of glucose glucuronic acid undergoes mutarotation and hence it consists of both  $\alpha$ - and  $\beta$  - anomers but it is interesting that in biological materials only the  $\beta$ - form has been found to occur.

#### General reactions.

The presence of both aldehyde and carboxyl groups in the molecule accounts for the considerable reactivity of glucuronic acid and this, in turn, accounts for many of the difficulties in its isolation.

Reactions of the aldehyde (hemiacetal, glycosidic) group are exactly analogous to those of the same group in glucose. Consequently glucuronic acid is a reducing substance of the same order as glucose (Kertesz, 1935) and oxidation with bromine converts it readily to glucosaccharic acid.

A number of crystalline derivatives have been described for use in its identification (Thierfelder, 1887; Neuberg, 1900; Mayer, 1900; Giemsa, 1900, 1904) but in general these have proved to be rather unsatisfactory (Naidus, 1903; Goldschmidt and Zerner, 1912). Its identification as the dibenziminazole derivative of saccharic acid (Lohman, Dimler, Moore and Link, 1942) would appear to be much more useful.

Glucuronic acid will condense with a number of materials to form coloured pigments for use in its identification and determination/

determination. Of these the most important are the naphthoresorcinol reaction (Tollens, 1908) and the carbazole reaction, (Dische, 1947) These were the subject of a special study and will be considered later in this section.

The carboxyl group gives glucuronic acid the properties of an organic acid. Ester linkages form very readily in the usual manner while salts are formed and are utilised for the isolation of glucuronic acid and glucuronides. The salts most commonly used for this purpose are the basic lead salts, barium salts and salts formed with the alkaloids brucine and cinchonine. In considering the selection of a salt for the isolation of glucuronic acid the observations of Ehrlich and Guttman (1934) should be considered. These authors found that hot basic lead acetate and also strontium hydroxide but not barium hydroxide acted on galacturonic acid to produce a very strongly reducing yellow salt which eventually gave no orcinol reaction. The same phenomenon was observed in the case of glucuronic acid with calcium hydroxide and barium hydroxide.

Physical constants for glucuronic acid and its derivatives are given in table 2.1

#### Action of acids.

One of the most characteristic reactions of the hexuronic acids is their decomposition by strong mineral acid to/

Table 2.1  
Physical constants of glucuronic acid derivatives.

	M.P. °C	in water	Authority
Glucuronic acid	156 165 (corr)	11.73/36.26 (24°)	Ehrlich and Rehorst (1925) Weinmann (1929)
Glucurone	175-8 180 (corr) 177-8 176-8	16.05/36 (24°) 19.1 (20°) 19.2 (20°) 18.55 (23°)	Goebel and Babers (1933) Fischer and Piloty (1891) Ehrlich and Rehorst (1925) Goebel and Babers (1933) Pryde and Williams (1933) Stacey (1939)
Na salt		20 (20°)	Ehrlich and Rehorst (1929)
K salt		-0.56/22.51 (20°)	"
NH <sub>4</sub> salt		-2.78/22.47 (2.5°)	"
Ba Salt		-4.05/23.17 (20°) 17.45 (20.5°)	" (also Schmiedeberg and Mayer, 1879)
Brucine salt	156-7	-15.08 (20°)	Neuberg (1900)
Dibenziminazole derivative of saccharic acid	238		Lohman, Dimler, Moore and Link (1942)

to form furfural and carbon dioxide. The use of this reaction to determine glucuronic acid has been mentioned earlier and will again be referred to. Link and Niemann (1930) investigated the reaction and found that it could occur quite efficiently in acid concentrations of less than 5%. To a large extent the difficulty of preparing and isolating glucuronic acid is due to this reaction. The yield of furfural is only about 45% of theoretical and this indicates that degradation probably does not proceed via a pentose, as pentoses usually yield nearly theoretical amounts of furfural in the same circumstances.

#### Behaviour in alkali.

In weak alkali the lactone ring of glucuroné is hydrolysed and a salt of glucuronic acid is formed. Sodium bicarbonate or barium carbonate are commonly used for this purpose.

As with most simple carbohydrates strong alkali destroys glucuronic acid in time, most of the end-products being unknown.

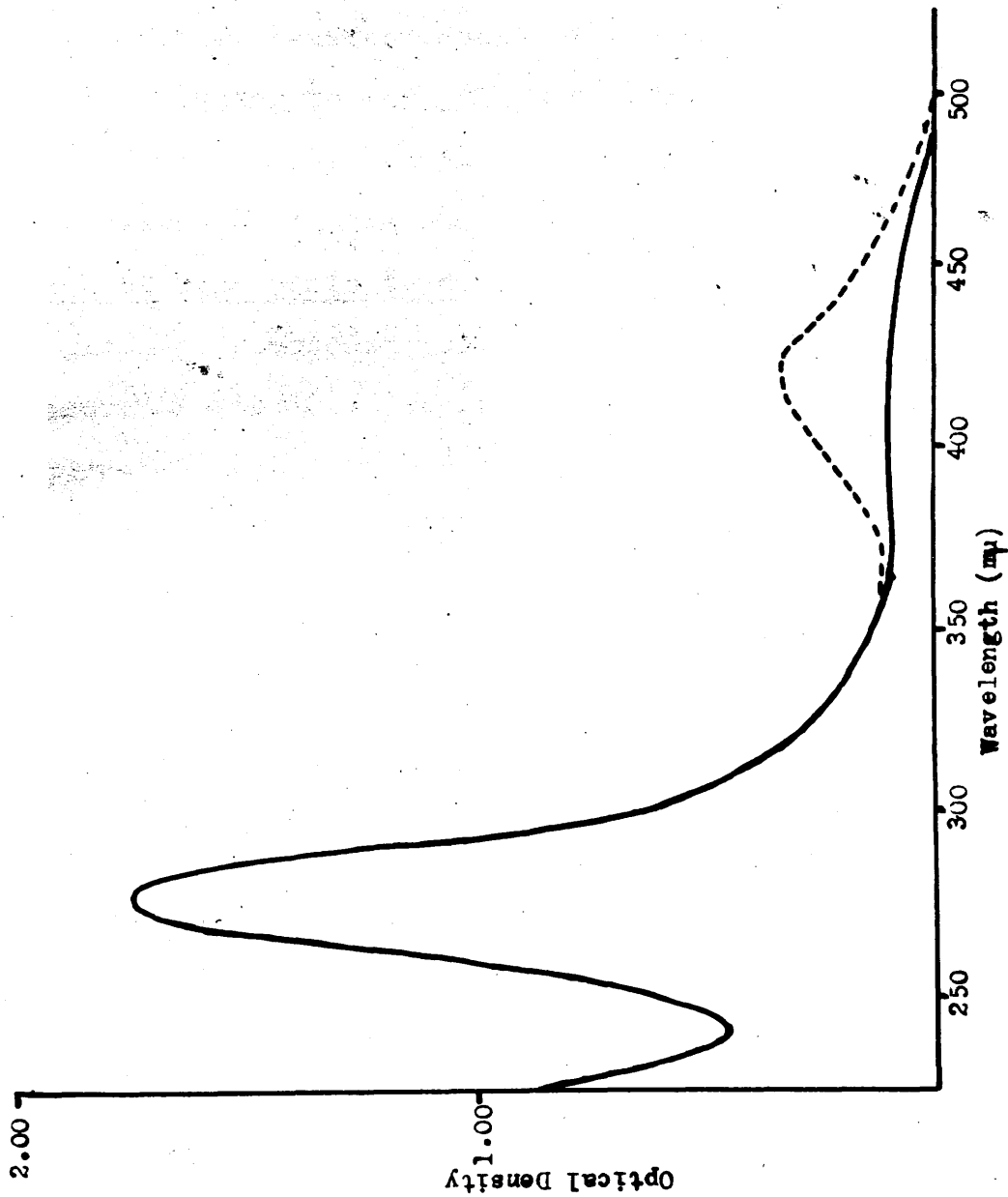
#### Experimental.

In view of the phenomenon reported by Ehrlich and Guttman (1934) and referred to above and similar phenomena occurring with methylglucuronide described by Owen, Peat and Jones (1941) and discussed below the effect of strong alkali on glucurone was investigated.

Crystalline glucurone dissolved in N NaOH gave an intense yellow colour which faded more-or-less rapidly.

Its/





**Figure 2.3** Absorption Spectrum of Glucurone Dissolved in N NaOH.

Its absorption spectrum (fig. 2.3) showed two peaks of maximum absorption at 270  $\mu$  and 420  $\mu$ . respectively. The peak at 270  $\mu$ . remained constant whereas the peak at 420  $\mu$ . corresponding to the yellow colour, faded. Glucurone solutions treated in this way were capable of reducing Fehling's Solution on standing in the cold.

#### Preparation of Glucuronic acid.

Originally glucuronic acid was produced from euxanthic acid or urochloralic acid for preparative purposes (Thierfelder, 1887; Kostanecki, 1887; Mayer, 1900) but the manufacture of purree became illegal and chloral fell out of favour as a sedative. It is now generally prepared synthetically from bornylglucuronide (Quick, 1927; Crismer, 1940; Murer and Crandall, 1940) or from menthylglucuronide (Neuberg and Lacksmann, 1910; Kiliani, 1926; Ehrlich and Rehorst, 1925; Williams, 1940). Both these glucuronides are very readily obtained from urine, bornylglucuronide in the form of its zinc salt and menthylglucuronide as its ammonium salt. The glucuronide is hydrolysed by acid and the aglycone can be removed simultaneously and very conveniently by steam distillation (Swartz and Miller, 1933). The great advantages of these methods are that the material can be purified before hydrolysis, the glucuronides are readily hydrolysed and the aglycones are readily removed. Consequently loss of glucuronic acid/

acid due to decarboxylation and difficulties in isolation due to impurities are avoided. The disadvantage is the trouble involved in feeding animals and collecting a large stock of material. Another type of isolation from biological material has been described by Weinmann (1929), using gum arabic as the source.

Chemical syntheses fall into two classes. In the first group glucuronic acid is obtained by reducing the terminal carboxyl group of saccharic acid. In the second group the primary alcohol group of glucose is oxidised to a carboxyl group. The original synthesis of Fischer and Piloty (1891) was of the first type. A considerable number of syntheses has been developed (Jolles, 1910; Killiani, 1921; Bergmann and Wolff, 1923; Smolenski 1924; Zervas and Sessler, 1933; Stacey, 1939; Leutgoeb and Heinrich, 1939; Smith, Stacey and Wilson, 1944). In the majority of cases the yields have not justified the labour involved while in many cases no glucuronic acid was produced.

Killiani's synthesis involved the direct oxidation of glucose by nitric acid rich in nitrous acid. Recently it was demonstrated by Yackel and Kenyon (1942) Unruh and Kenyon, (1942) and by Maurer and Drefahl (1942) that nitrogen dioxide had the property of oxidising the primary alcoholic/

alcoholic groupings of sugars specifically when the aldehyde groups were blocked. The former authors prepared a polyglucuronic acid from cellulose by this method and the latter authors claimed to have prepared methylglucuronide. It has been suggested that the method might be useful for the preparation of glucuronic acid and the following experiments were performed in order to investigate this possibility.

#### Experimental.

As a preliminary experiment cellulose (cotton wool) was oxidised by nitrogen peroxide gas by the cyclic method of Yackel and Kenyon. (apparatus modified).

About 50 g. **dry**, finely shredded cotton wool were placed in a large aspirator. 120 g. freshly distilled  $\text{NO}_2$  were placed in a flask with 60 g.  $\text{P}_2\text{O}_5$  and the liquid was volatilised by a 15 watt lamp placed underneath as a heater. The gas was led into the aspirator by the bottom opening and the spent gases from the top were led back into a reflux condenser where unused nitrogen peroxide was recondensed and dropped back into the generating flask. After 66 hours treatment the material was removed and washed free of nitrogen peroxide. This material had the same properties as that described by Yackel and Kenyon i.e. it retained its fibre structure, dissolved fairly readily in weak alkali and was insoluble/

insoluble in acid but gave off  $\text{CO}_2$  when treated with strong acid. As might have been expected this material proved very refractory to acid hydrolysis and the possibility of obtaining glucuronic acid from it seemed very slight. It was considered that oxidised starch might prove less refractory to hydrolysis and experiments on the following lines were performed. On this occasion the method was based on some later findings reported by McGee, Fowler, Taylor, Unruh and Kenyon (1947)

Materials. Starch used in the experiment was dried for two hours in an oven at  $110^\circ$  before use.

Carbon tetrachloride was shaken with strong caustic soda, washed exhaustively with distilled water, dried over calcium chloride and redistilled.

Nitrogen dioxide was fractionally distilled, the first fractions, rich in nitrogen trioxide, and the last fractions, rich in nitric acid, being discarded. It was obtained as a white solid.

Reaction. In a large beaker were placed 50 g. starch, 100g carbon tetrachloride, 40 g. nitrogen dioxide and 3 ml. pure nitric acid (Analar, fresh). This was placed in a desiccator containing 100 g.  $\text{P}_2\text{O}_5$  and fitted with a reflux condenser. Care was taken to exclude moisture from the apparatus and the reaction was left to proceed for 72 hours.

The/

The material obtained retained the appearance of starch but was readily soluble in alkali, giving a yellow colour.

Hydrolysis. To 10 g. of this material in a 100 ml. pyrex flask were added 75 ml. 5 N H<sub>2</sub>SO<sub>4</sub>. Heat was applied directly until the material formed a solution and hydrolysis was then continued in a boiling water -bath until the optical rotation remained constant (less than two hours). 2g. good animal charcoal were added five minutes before removing the hydrolysate and filtering. Treatment with charcoal was repeated as required until a clear golden solution was obtained. The bulk of the sulphuric acid was removed by adding carefully rather less than the calculated amount of barium hydroxide solution. Barium sulphate was removed by centrifugation, and neutralisation completed by treatment with barium carbonate. After pH 5 had been reached the insoluble material was again centrifuged off and discarded. The solution was concentrated to a small volume by vacuum distillation and freeze-dried. The solid material left was then extracted with warm alcohol and warm glacial acetic acid successively and these extracts were again concentrated.

The syrup obtained gave a strong naphthoresorcinol reaction but could not be made to crystallise.

### Glucuronides.

Glucuronides are compounds in which a variety of substances, known collectively as aglycones or 'aglucones' (Fishman), are linked to glucuronic acid through its glycosidic group, with the mutual loss of a molecule of water. In the case of ether glucuronides the aglycone is either a phenol or an alcohol and in the case of ester glucuronides the aglycone is a carboxylic acid. Methylation studies (Pryde and Williams, 1933) indicate that glucuronic acid has a pyran ring structure in bornylglucuronide. Glucuronides are, of course, organic acids and occur naturally either in the free state or as their sodium or ammonium salts.

### Properties.

The glucuronides as a class have few common properties other than their behaviour as weak acids. Ether glucuronides can be distinguished from ester glucuronides by their resistance to alkaline hydrolysis, ester glucuronides in contrast being very readily hydrolysed by weak alkali. Resistance to acid hydrolysis varies from glucuronide to glucuronide, depending on the nature of the aglycone grouping. It follows that whereas most glucuronides may be isolated by precipitation as basic lead salts the isolation of ester glucuronides presents more difficulty since they tend to be destroyed in such circumstances. Most glucuronides in the form of the free acid are more-or-less soluble in organic solvents whereas their /

their salts are, as a rule, highly soluble in water but insoluble in organic solvents. The nature and size of the aglycone grouping affects these properties considerably, however, sodium pregnanediol glucuronidate, for instance, being quite soluble in butanol and rather insoluble in water. All the naturally occurring glucuronides so far tried are hydrolysed by the enzyme  $\beta$ -glucuronidase.

#### Preparation.

Glucuronides are usually prepared biosynthetically. The following method for the preparation of p-chlorphenylglucuronide is illustrative of the general type of preparation. The phenylglucuronide and o-aminophenylglucuronide used in this study were also prepared by this technique. In this particular instance better results were obtained by this method than by the extraction procedure used by Spencer & Williams (1950).

To each of a number of rabbits one gram. p-chlorphenol in 50 ml. water was fed by stomach-tube. Urine passed in the next 18 hours was pooled and strained through gauze. A saturated solution of normal lead acetate was added with stirring until precipitation was just complete, the precipitate being removed by centrifugation and discarded. To the supernatant ammonia was added to raise the pH to 10 and then/



then a saturated solution of basic lead acetate was added until maximum precipitation was obtained. This precipitate was centrifuged down and the supernatant discarded. It received three washings with water containing a trace of ammonia. (This latter prevented the formation of colloidal solutions in the later washings). The precipitate was stirred up with a little water to form a creamy mixture and gassed with H<sub>2</sub>S until the supernatant remained clear. Lead sulphide was filtered off, washed twice with small amounts of water and H<sub>2</sub>S removed from the solution by a current of air. The solution was taken to a very small volume by vacuum distillation, the glucuronide allowed to crystallise, collected on a Buchner Funnel and dried in vacuo. Recrystallisation was performed from hot water or acetone-ethyl acetate. (m.p. 150°C ),

For a number of glucuronides more specialised methods are employed and it is necessary to consult original papers. A very complete bibliography on the subject is contained in 'Detoxication Mechanisms' by Williams (1947).

### Chemical Syntheses.

The general type of synthesis employed for glucuronides is the same as that employed for glucosides i.e. condensation of/

of the aglycone with the acetyl halide of glucuronic acid or glucurone in the presence of a suitable catalyst. This method was originally employed by Neuberg and Niemann (1905 a) for phenylgluronide and euxanthic acid. The preparation of intermediates was investigated by Goebel and Babers (1933; 1933; 1934; 1935; 1935a) and the method has been used for steroid glucuronides by Shapiro (1939) and for pregnanediol glucuronide by Hubner, Overman and Link (1944).

The Fischer synthesis ( treatment with an anhydrous solution of an acid in an alcohol) has also been employed (Owen, Peat and Jones (1941). In analogy with glucose when glucuronic acid is refluxed with methanolic hydrochloric acid, methyl  $\beta$ -D-glucopyruronolactone is produced whereas in the cold methyl- D - glucofururonolactone is formed. The interesting behaviour of this latter compound will be discussed (Section 5). It is also interesting to note that the  $\beta$ -anomers were formed almost exclusively in these experiments whereas when methylglucoside is formed in the same way the  $\alpha$ -anomers invariably predominate. Mucopolysaccharides (Polyuronides).

Our detailed knowledge in this field is still somewhat limited but considering the difficulty of the subject a great deal has been established. The structure of all these compounds seems to be based on repeating units of aldobiuronic acids.//

Acids. Aldobiuronic acids, strictly speaking, are defined as disaccharides in which one of the sugar components is a uronic acid, linked by a glycosidic linkage to a hexose or pentose. In the strict terms of this definition chondrosin, at least, is not an aldobiuronic acid since Levene and LaForge (1914) showed that oxidation with bromine gave rise to saccharic acid as one of the end-products after hydrolysis and thus the hemiacetal group of glucuronic acid must be free. This probably applies to others of the group but the generic term is still employed. The non-uronic acid moiety of these aldobiuronides is usually an amino-sugar, in the case of chondrosin aminogalactose and in the case of the aldobiuronic acid from mucin and hyaluronic acid aminoglucose. Two or more of these aldobiuronides are usually linked together and the molecule may, in addition, contain sulphuric acid residues (heparin, chondroitin sulphuric acid and mucoitin sulphuric acid). Mucopolysaccharides are fairly readily hydrolysed by acid to the aldobiuronic stage but these latter are characterised by extreme resistance to acid hydrolysis and in order to split them Levene (1925) found sodium amalgam necessary.

### Mucoproteins.

Since Eichwald (1886) demonstrated that some proteins were capable of reducing Fehling's solution after hydrolysis a considerable amount of work has been done on this group of proteins, /

proteins, but our knowledge is still meagre. It has been established that in many cases the carbohydrate moiety is chondroitin sulphuric acid or mucic acid sulphuric acid and that, as a rule, these substances are readily hydrolysed from the parent protein by dilute alkali, an observation which suggests that the linkage between the two may be a simple ester linkage.

#### The Chemistry of Minute Amounts of Glucuronic Acid.

Whilst many of the methods outlined above are excellent where large quantities of material are to be isolated and analysed it is necessary in biochemical investigations to be able to isolate, identify and estimate amounts of glucuronic acid between 5 and 100  $\mu$ g. where it may be present in a concentration of only 0.0005% in a solution containing a great excess of other substances. The inadequacy of existing micromethods has constantly handicapped our efforts to elucidate the biochemical mechanisms in which glucuronic acid is involved. In an attempt to improve the position the following studies were undertaken.

Ultramicroestimation of glucuronic acid.

Methods based on the reducing properties of glucuronic acid (Goebel and Babers, 1933; Quick, 1924; Fishman, 1938) and the cerimetric methods of Fishman (1939), Levvy (1946) and Mills (1948) are not sufficiently specific for this purpose although they have their place in the assay of glucuronidase activity (Section 4.) The same limitation applies to methods based on the formation of furfural (Günther, De Chalmot and Tollens, 1892; Lefèvre and Tollens, 1907; C. Tollens; 1909; Haendel, 1930; Fürth and Peschke, 1936; Tanabe, 1938). Methods based on the liberation of carbon dioxide by strong acids (Tollens and Lefèvre, 1907; Sauer, 1930; Buston, 1932; Voss and Pfirschke, 1937; Norman, 1939; Whistler, Martin and Harris, 1940; Freudenberg, Gudjons and Dumpert, 1941; McCready, Swenson and Maclay, 1946) require at least a few milligrams of material and, while indispensable for the analysis of mucopolysaccharides are inconvenient for routine glucuronic acid determinations.

Two reactions offer a greater degree of specificity and sensitivity. They are 1) The carbazole reaction (Dische, 1927; 1947) 2) the naphthoresorcinol reaction (Tollens, 1908).

Although the carbazole reaction is in many ways satisfactory it has a few serious limitations. In the first place correction factors are necessary for different glucuronic acid compounds since the colour intensity is not always proportional to glucuronic acid content. Secondly, the fact that colorimetric readings are taken on solutions containing 85% sulphuric acid must limit its application as a routine procedure. Finally, biological materials frequently contain unidentified substances which interfere with the reaction.

Since the first two objections do not apply to the nephthoresorcinol reaction it was considered that it merited further study with a view to increasing its tolerance and specificity. Previous attempts to put the reaction on a quantitative basis have been those of C. Tollens (1908), Ogata and Yamanouchi (1929, 1930, Maughan, Evelyn and Browne (1938), Florkin (1937), Kapp (1940), Mozolowski(1940), Deichmann (1943), Hanson, Mills and Williams (1944), Jarrige (1947), Fuchs and Traumer-Adelpoller (1947) and Bisset, Brooksbank and Haslewood (1948), Jarrige (1950) has recently reviewed some of these methods.

#### Experimental.

##### Nature of the Reactions and properties of the Pigment.

The reaction is performed by heating a solution containing glucuronic acid together with naphthoresorcinol in the/

the presence of a strong acid. A pigment is formed which may be extracted into certain organic solvents for colorimetric determination. Guerrero and Williams (1948) described some of the properties of the pigment and concluded that these were in accord with the theory that it was a direct condensation product rather than a furfural derivative. In this investigation some of their results were confirmed and other observations were made.

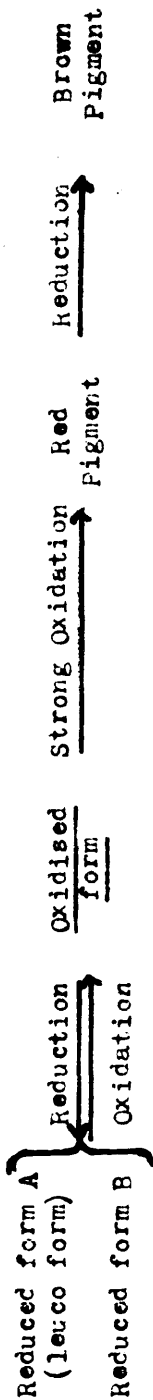
The pigment was found to be sensitive to alterations of oxidation-reduction potential and pH as summarised in the scheme given in fig. 2.4

It was found that only the acid, oxidised pigment gave a satisfactory quantitative reaction for glucuronic acid. It is soluble in the common ethers and esters, giving a typical purple colour and in the lower alcohols, giving a blue colour. The actual colour produced may be related to the hydrogenion concentration in the solvent as will be discussed later. Reduced from 'A' (leuco-form of Guerrero and Williams) is produced by the action of a reducing agent on the formed pigment. The material which has been designated 'reduced form B' is only formed in the course of the reaction in the presence of certain reducing agents (e.g. glucose, formaldehyde). Its formation can be inhibited by the presence of an oxidising agent and it can be oxidised directly (e.g. by chloramine-T) to give the/

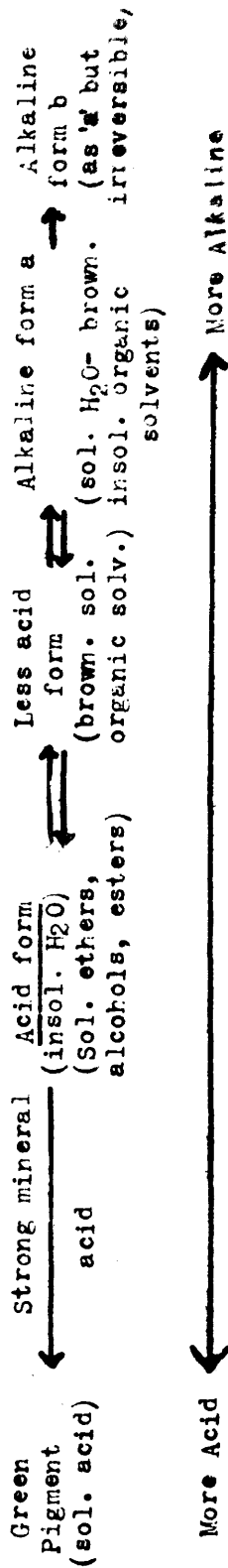
Figure 2.4

Properties of Pigment

Effects of oxidising and reducing agents



Effects of alterations in pH





the oxidised pigment. It is not soluble in ether or ethyl acetate but is soluble in the lower alcohols, to which it imparts a blue colour and a greenish fluorescence. Although it is unsuitable for the determination of glucuronic acid it is important in connection with interfering substances, under which heading it will receive further consideration.

The behaviour of the pigments in organic solvents is important. Besides the uronic acids many other substances form pigments with naphthoresorcinol (Mandel and Neuberg, 1900; Van der Haar, 1918; White and Green, 1932). The lower alcohols, as employed by Hanson et al (1944) extract most of these pigments and also the reduced form 'B'. Moreover, the colours formed are not stable but tend to alter both quantitatively and qualitatively on standing, a red-brown tint becoming more apparent, particularly if the pigment has first been extracted with ether and redissolved in the alcohol. It appeared not unlikely that this change was due to alteration towards an alkaline form of the pigment (see figure 2.4) and indeed, the addition of the correct concentration of acid to the alcohol was found to stabilise the colour.

Ether and ethyl acetate are rather more critical solvents and neither dissolves the reduced pigment 'B' nor some of the other pigments. In these solvents, without the addition of extra/

extra acid, uronic acid pigments give a purple colour whilst those derived from the true sugars give a yellow-brown colour. The addition of acid brings up the blue element and suppresses the yellow element. Now, it had been found that the pigments formed with true sugars tended to alter towards their less acid forms at rather higher acid concentrations than the uronic acid pigments and it seemed plausible that the concentration of hydrogen ions extracted from the reaction mixture by these solvents was such that the uronic acid pigments still remained predominantly in their acid forms whilst the true sugar pigments were predominantly in their less acid state. Whether this be the true explanation or not the nett result was a gain in specificity with these solvents.

Neuberg and Saneyoshi (1911) claimed that only the pigment formed from uronic acids was soluble in benzene and this would appear to be very nearly true in all cases. The pigments from hexoses and pentoses are certainly almost completely insoluble in benzene. The uronic acid pigment itself is not very soluble in benzene and is rather difficult to extract. It was found to be even less soluble in toluene and almost completely insoluble in carbon tetrachloride. Chloroform, on the other, hand, was found to dissolve the uronic acid pigment much more readily, whilst retaining the specificity possessed by benzene.

### Interfering Substances.

Interference in reactions of this type may be considered under two headings, negative interference implying inhibition of the reaction and positive interference implying the formation of an excess of end-products of the reaction, (in this case other pigments).

Negative interference may be caused by a variety of substances. Maughan et al (1938) referred to the effect of ethanol in this respect and I have observed that practically all the lower alcohols are capable of inhibition to some degree. Formaldehyde was also found to have an exceedingly powerful inhibiting action, less than a microgram being found quite effective, and possibly traces of the lower aldehydes are responsible for the interference caused by a number of organic materials. The lower ketones were not found to interfere with the reaction. Some metallic ions may also inhibit colour development but their main disadvantage was found to be a tendency to give rise to opacity in the extracting solvents so that higher readings may actually be obtained. The effects of ethanol and formaldehyde are shown in table 2.2.

Oxidising agents, if present in excess, were also found to inhibit the reaction while, if present in considerable excess, a red pigment was formed. (Figure 2.4) High concentration/

concentrations of protein constituted another source of this type of interference (see later)

In most cases it is possible to avoid the presence of these substances in the reaction mixture. A more serious form of negative interference, since it is not so readily avoided, is that due to reducing sugars and to excess of glucuronic acid itself. In these cases it was observed that, coincident with the failure to develop ether-soluble pigment, there was formed a water and ether-soluble blue pigment, soluble in alcohol and apparently identical with reduced form 'B'. As one would expect if this hypothesis were valid the addition of an oxidising agent to the reaction mixture was found to abolish the effect to a considerable extent. (Figure 2.5).

The other type of interference, is mainly due to pigment formation by sugars and allied substances. It will be more convenient to discuss it from the practical point of view later.

#### Optimum conditions for the reaction.

#### Choice and concentration of acid.

Hydrochloric acid is usually employed in the reaction and, in agreement with Hanson et al., the optimum concentration was found to be 3 ml. conc. HCl in 7 ml. total reagents.

Neuberg and Kobel (1931) demonstrated that the reaction/

Table 2.2

Effect of ethanol and formaldehyde on the naphthoresorcinol reaction. (40 micrograms of methylglucuronide estimated by the sulphuric acid - ethyl acetate modification with additions as shown. Final volume of reaction mixture in all cases - 7 ml.)

Addition	Colorimeter Reading
0	0.355
Ethanol 0.1 ml.	0.340
0.2 ml.	0.290
0.3 ml.	0.245
0.4 ml.	0.210
Formaldehyde 2 $\mu$ g approx.	0.005

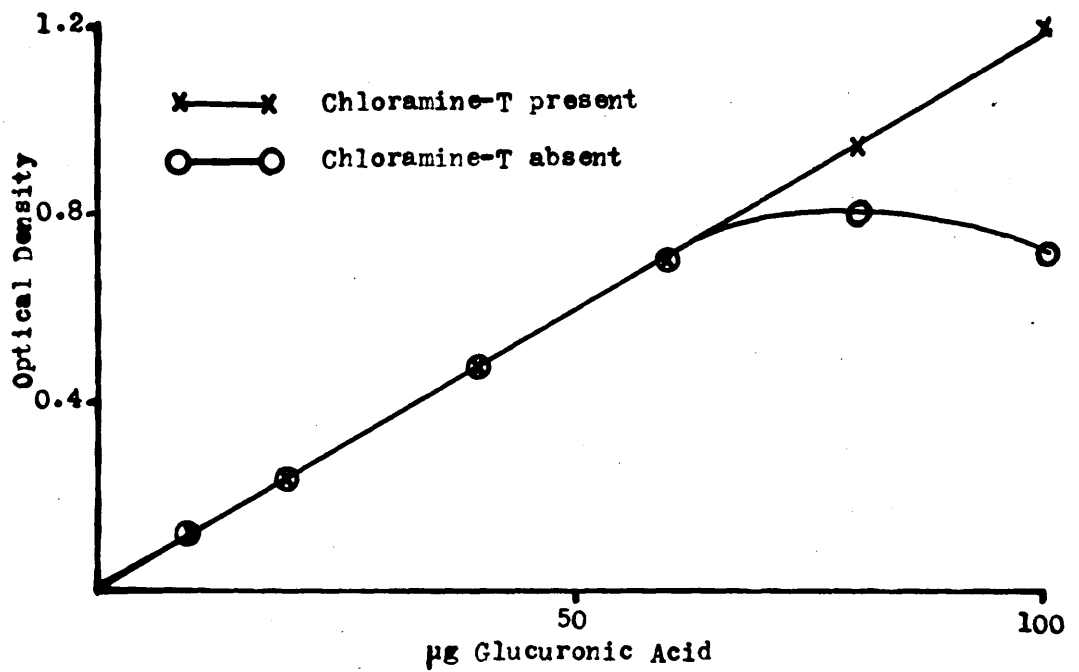


Figure 2.5

Calibration Curves with and without the Addition of an Oxidising Agent to the Reaction Mixture.

reaction could be carried out with sulphuric acid and, although they reported no quantitative experiments, they claimed that it increased the specificity of the reaction. Examination of this point (Table 2.7) established that in some cases sulphuric acid did give improved specificity whilst in other cases the opposite was true. However, sulphuric acid was found to have a great advantage over hydrochloric acid in that it gave a low, stable and reliable blank reading. Whereas with hydrochloric acid the appearance of opacity in the extracting solvent, particularly in low readings and blank tubes, has been a persistent, though rarely mentioned, source of inaccuracy I have not experienced it when employing the reaction using sulphuric acid in the concentrations described. It is interesting also to observe that the blank values apparently did not increase with increasing concentrations of naphthoresorcinol in this case (Table 2.3) and thus a higher concentration of naphthoresorcinol could be used without increasing the blank readings to an unmanageable extent.

These factors combine to increase the dependability of the reaction and I would therefore suggest that sulphuric acid should replace hydrochloric acid. The optimum concentration was found to be 1.25 ml conc. sulphuric acid in 7 ml. of reaction mixture (i.e. 17.85%  $H_2SO_4$ ). The upper limit of concentration cannot be exceeded due to charring. (Table 2.4).

Table 2.3

Naphthoresorcinol concentration on blank reading when reaction is performed with sulphuric acid.  
(Pigment in ethyl acetate)

Naphthoresorcinol Final concentration	Colorimeter reading
% 0.036 0.054 0.071 0.089 0.107 0.125 0.143	0.065 0.067 0.060 0.065 0.060 0.050 0.050



Table 2.4

Optimal concentration of sulphuric acid.  
(2 ml. of glucurone solution (approx. 40 micrograms) + 2 ml. naphthoresorcinol reagent + conc. sulphuric acid as shown + water to 7 ml. Pigment extracted with ethyl acetate).

Sulphuric acid	Colorimeter reading	Remarks
ml. 0.5 1.0 1.25 1.5 1.75 2.0	0.20 0.510 0.535 0.40 0.410 0.405	Charring " "

Preparation and concentration of naphthoresorcinol reagent.

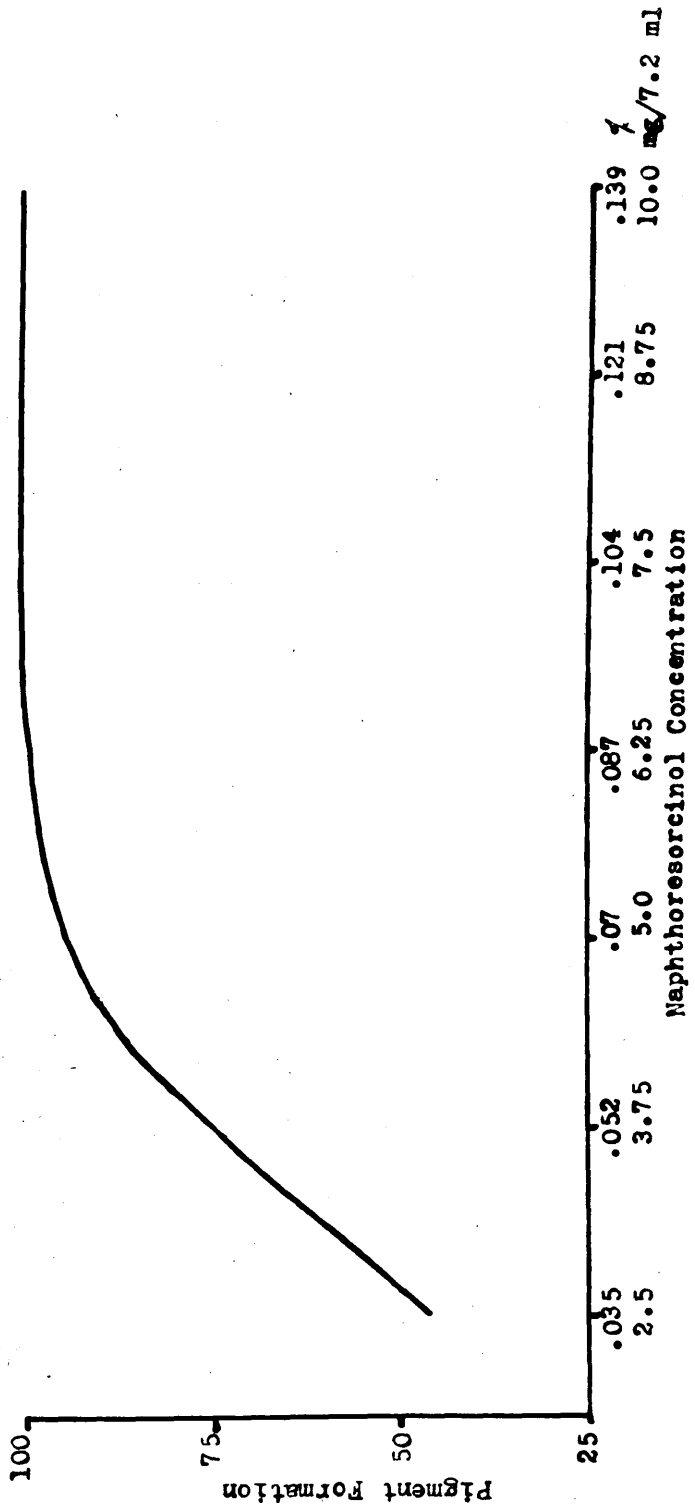
There has been considerable disagreement over the preparation of the naphthorescinol reagent, which is generally considered to be highly unstable. The optimum conditions for stability were investigated and are clearly indicated by the figures in table 2.5. It was found that if naphthoresorcinol was made up in acid solution containing a small amount of a reducing agent it would remain stable in a dark bottle for some weeks. It was nevertheless considered advisable to store it in the refrigerator when not in use.

The optimum concentration of naphthoresorcinol for use in the reaction was sought. In agreement with Hanson et al. (1944) it was found that pigment formation in relation to naphthoresorcinol concentration increased rapidly to a concentration of about 0.07% (2ml. 0.25% naphthoresorcinol in 7 ml.) and then gradually approached a maximum value at a naphthoresorcinol concentration of 0.1%, thereafter remaining constant within the concentration used. (Figure 2.6) Hanson et al. (1944) decided to use the former concentration since blank values became too high with greater concentrations. Since, as has been mentioned, this did not occur using sulphuric acid in place of hydrochloric acid a higher concentration of naphthoresorcinol became //

Table 2.5

Conditions for stability of naphthoresorcinol solution.  
 (After remaining in contact with the additions shown for three days at room temp., four samples of naphthoresorcinol solution were used for the estimation of approximately 50 micrograms of glucurone.)

Addition to naphthoresorcinol	Colorimeter Readings		Difference
	Water blank	Glucurone soln.	
Alkali (KOH)	0.080	0.080	0
Acid (H <sub>2</sub> SO <sub>4</sub> )	0.160	0.880	0.72
Oxidising agent (Chloramine-T)	0.230	0.940	0.71
Reducing agent (NaHSO <sub>3</sub> )	0.115	0.825	0.71



**Figure 2.6** Effect of Naphthoresorcinol Concentration on Pigment Formation.

became permissible. A higher concentration is desirable for the following reasons. Since the curve of pigment formation in relation to naphthoresorcinol concentration has a much smaller slope at 0.1% there is likely to be a greater degree of reproducibility between different batches of naphthoresorcinol, which may vary in purity. Also minor errors in weighing or pipetting reagents are likely to have a negligible effect on results. At this level sensitivity is, moreover, greater and there is possibly also a slight increase in tolerance. For these reasons I chose a final concentration of 0.1% which displayed the above advantages while still remaining economical. Such a concentration corresponds to 2 ml. of a 0.35% solution in a total of 7 ml. of reaction mixture.

#### Choice and concentration of an oxidising agent.

As mentioned previously, the addition of an oxidising agent was found to increase the tolerance of the reaction considerably, although excessive oxidation inhibited colour development. The presence of metallic ions was found undesirable since they caused opacity and tended to reduce the sensitivity of the reaction; therefore such substances as ferricyanide were rejected. Two effective systems were found. Where hydrochloric acid was used in the reaction, benzoquinone, added/

added as 1% (w/v) to the acid before mixing with the other reagents proved both convenient and effective provided no other substances were present with which benzoquinone, itself a chromogen, would react to form a pigment soluble in the extracting solvent. However, since this limited its application and, moreover, sulphuric acid had been found more satisfactory than hydrochloric acid this system was rejected. For general purposes chloramine-T was found to be the most useful oxidising agent and the optimal addition to 7 ml. of other reagents was found to be 0.2 ml. of a 1% (w/v) (table 2.6) In certain circumstances higher concentrations of chloramine-T are necessary (Estimation of glucuronides in blood, part 3).

#### Time of Heating.

This point has been thoroughly investigated by other workers (Kapp, 1940,) Hanson et al, (1944). Two hours heating in a vigorously boiling (Mills, 1946) water-bath is considered suitable for routine tests but in the case of certain difficulty hydrolysible glucuronic acid-containing materials the time may have to be extended to four and a half hours or even more in order to achieve complete colour development.

#### Choice of an extracting solvent.

Ethers are reliable extracting solvents. Ether for this purpose must be freed of peroxides (Maughan et al 1938). For colorimetric/

Table 2.6

Optimal addition of chloramine-T solution to reaction mixture.  
(20 micrograms glucuronic acid in 2 ml. water + 3 ml. conc. HCl  
+ 2 ml. naphthoresorcinol reagent + 1% (w/v) chloramine-T solution  
as shown. Pigment extracted with ethyl acetate).

Chloramine-T added.	Colorimeter Reading
ml.	
0	0.276
0.10	0.275
0.15	0.275
0.20	0.275
0.25	0.263
0.30	0.252
0.35	0.241

colorimetric work, however, the common ethers are inconveniently volatile while purification of the higher ethers may be hazardous. For these reasons Hanson et al (1944) rejected ether and employed amyl alcohol. However, as pointed out earlier, alcohols are rather uncritical solvents. More reliable results can be obtained by extracting the pigment with ether, evaporating to dryness and redissolving in alcohol to which has been added acid (0.5 - 1% v/v HCl) in the cases of ethanol and propanol. (Fig. 2.7)

For routine purposes, where the amounts of substances causing positive interference were insignificant the most satisfactory solvent was found to be ethyl acetate. The pigment is highly and very readily soluble in this solvent and is extracted almost 100% by a single extraction with the minimum of mixing. The latter point minimises the possibility of error from incomplete solution of the pigment. In ethyl acetate the absorption spectrum and intensity of the colour remain constant for hours after the first few minutes, no addition being necessary (fig. 2.7) The colour formed is intense and gives the reaction a high degree of sensitivity.

For determination in the presence of materials liable to give rise to positive interference, on the other hand, there was little doubt about the superiority of chloroform (see table 2.8) Sensitivity with this solvent is just as great as with ethyl/



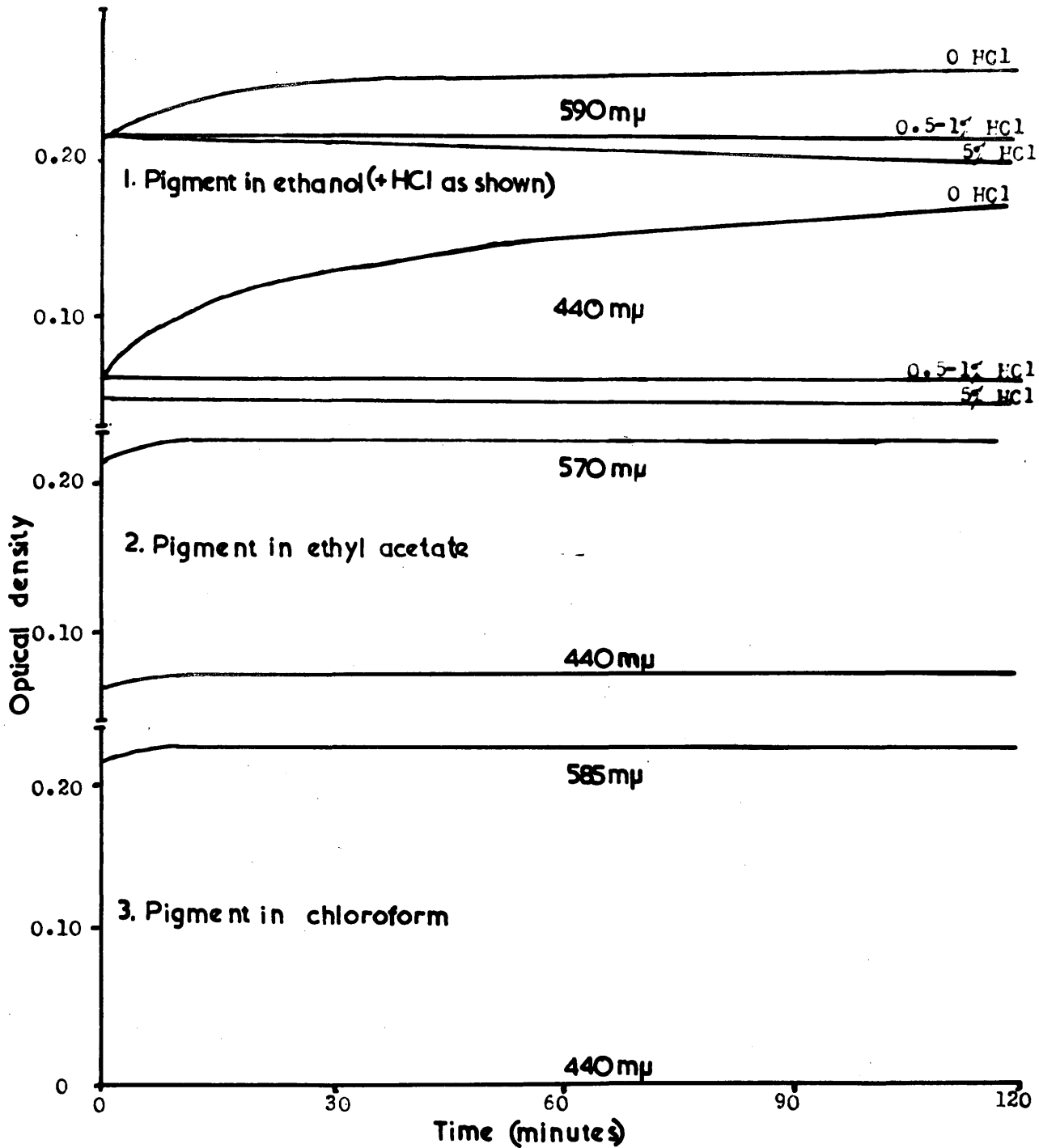
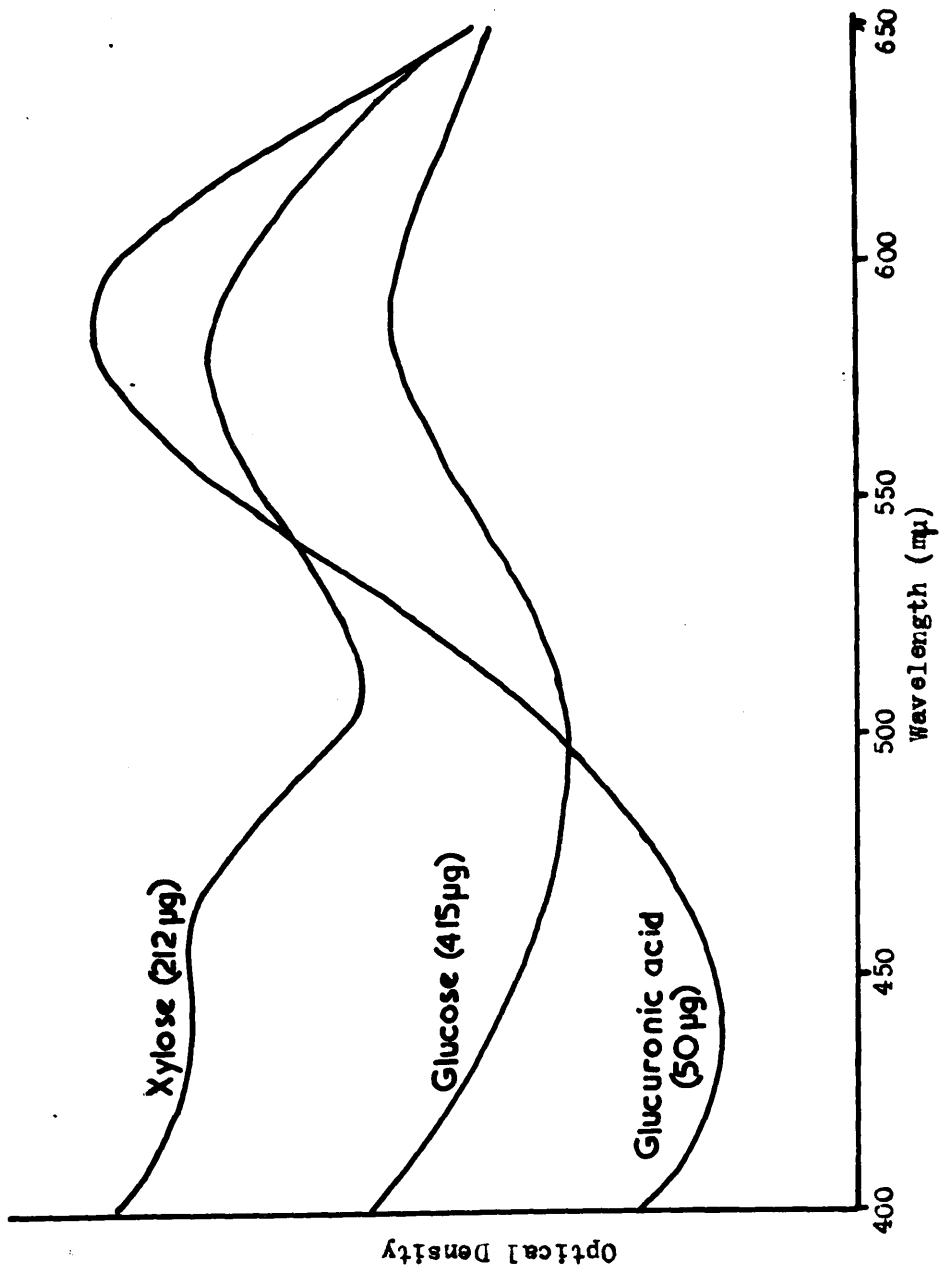


Figure 2.7 Stability of Pigment in Different Solvents.

ethyl acetate but, owing to the fact that the pigments formed with true sugars are almost insoluble in it, specificity is vastly increased. The two disadvantages of using chloroform are that the pigment is not so readily soluble and the emulsion is slow to clear. These two disadvantages are readily overcome though the technique then requires a little more care and time. To overcome the former it was only found necessary to ensure that shaking with the solvent was carried out very thoroughly while the latter problem was solved either by centrifuging at a medium speed for a few minutes or by transferring the solvent layer to a tube containing a little anhydrous sodium sulphate (which did not adsorb the pigment significantly). The absorption spectrum of the pigment dissolved in chloroform is also stable after a few minutes (Fig. 2.7) Commercial chloroform contains about 1% of ethanol. It was found that this concentration of alcohol did not affect extraction of the pigments significantly and therefore it was used for routine purposes.

The amount of solvent to be used in extracting the pigment depends entirely on the type of colorimeter to be employed. Absorption spectra of different pigments in the various solvents are shown in Figs. 2.8, 2.9, & 2.10. Density of the solutions should be determined in acid alcohol at 590  $m\mu$  in ethyl acetate at 570  $m\mu$  in chloroform at 585  $m\mu$  /



**Figure 2.8** Absorption Spectra of Pigments in Acid Ethanol.

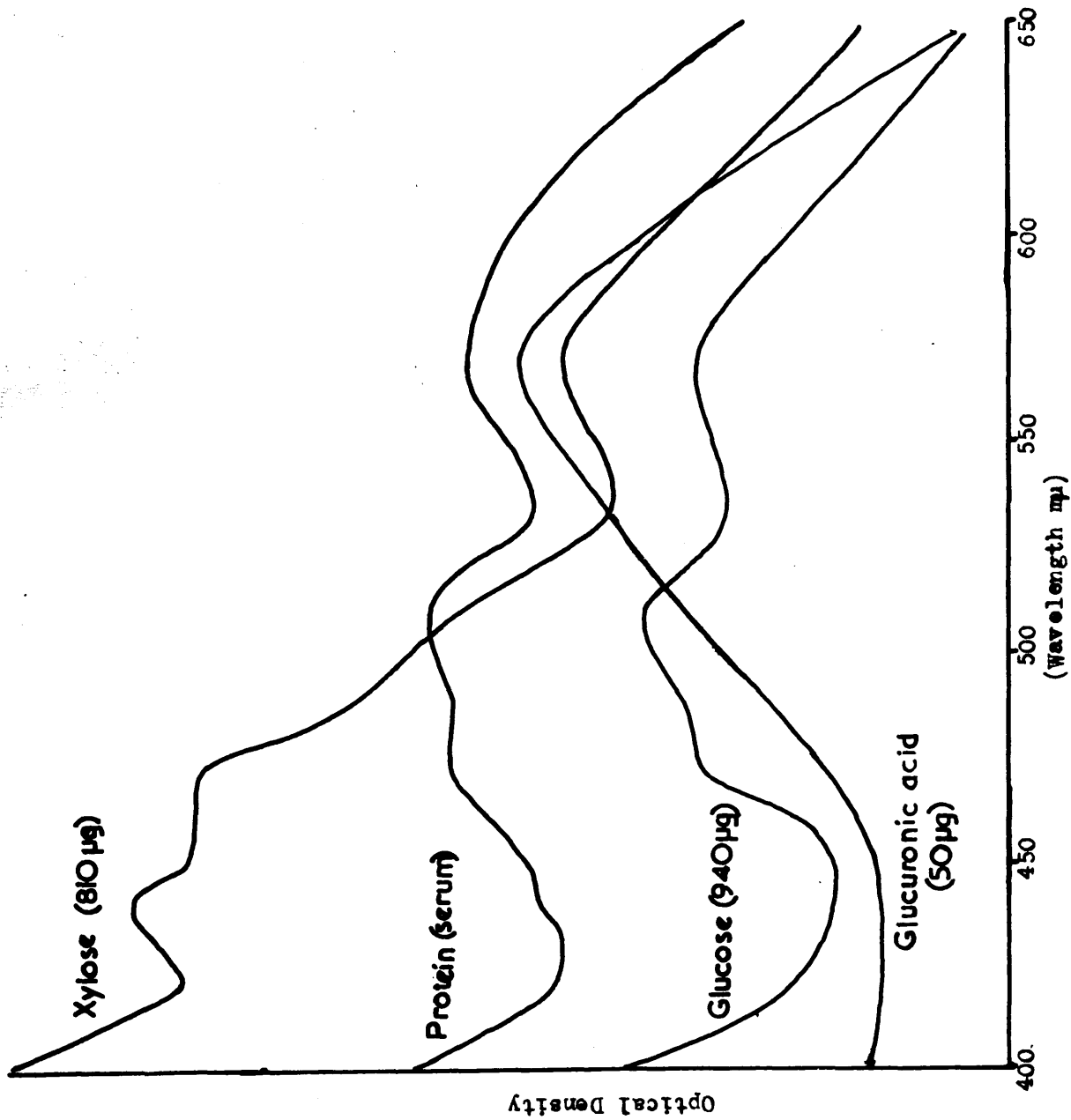
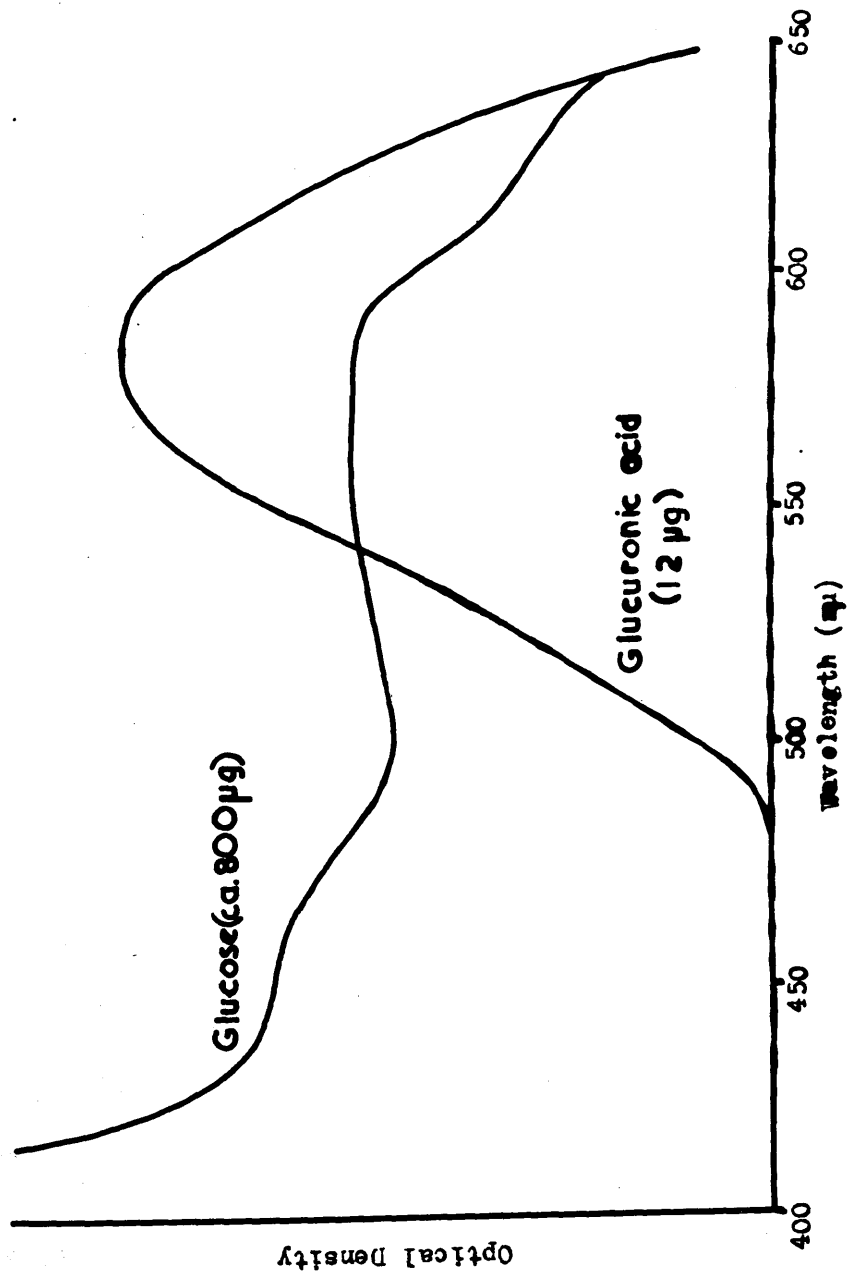


Figure 2.9 Absorption Spectra of Pigments in Ethyl Acetate.



**Figure 2.10** Absorption Spectra of Pigments in Chloroform.

585  $m\mu$  and in benzene at 555  $m\mu$  (Ilford filter yellow 606 for all except benzene, for which the yellow-green 605 filter should be used).

Method of estimation.

Re-agents.

- 1) Naphthoresorcinol, 0.35 g. dissolved with gentle heat in 100 ml. of 0.01 N  $H_2SO_4$  containing 0.01 ml. of a 41% solution of sodium bisulphite (S.G. 1.34) and filtered if necessary. Stored in a dark bottle in the refrigerator.
- 2) 15 N sulphuric acid (Analar)
- 3) 1% w/v solution of chloramine-T
- 4) (a) Ethyl acetate. (b) Chloroform B.P. (McFarlane).

Procedure. 2 ml. of a solution containing not more than 200  $\mu g$  of glucuronic acid (preferably 5-50  $\mu g$ ) 3 ml. 15 N sulphuric acid, 2 ml. of the naphthoresorcinol reagent and 0.2 ml. of the chloramine-T solution are mixed intimately, immediately after the addition of chloramine-T, in 6" x  $\frac{5}{8}$ " Pyrex boiling tubes, conveniently fitted with ground glass stoppers (Quickfit and Quartz), and placed in a vigorously boiling water-bath for two hours (the ground-glass stoppers being replaced by glass bubbles). After cooling the pigment is extracted either with a) a suitable volume of ethyl acetate (usually 8 ml.) or b) a suitable volume of chloroform. Where procedure b) is employed the solvent must be cleared either by/

by transferring to test-tubes containing about 0.5 g. anhydrous sodium sulphate or by transferring to centrifuge tubes and spinning for a few minutes at medium speed. The density of the coloured solutions is estimated in a suitable colorimeter, using the wavelengths or filters listed above.

A reagent blank, prepared by replacing the glucuronic acid solution by 2 ml. of distilled water, should be carried through the entire procedure and used for zeroing the colorimeter rather than a solvent blank. It is advisable to carry out determinations in duplicate and blank readings in triplicate in order to recognise errors due to extraneous factors.

#### Modifications.

Most of these have already been implied. Different quantities may be employed provided the proportions are kept the same and, in particular, 1.25 ml. conc.  $H_2SO_4$  may be used, permitting up to 3.75 ml. of a dilute solution of glucuronic acid to be estimated without increasing the other quantities. If desired benzoquinone and hydrochloric acid may be used in place of sulphuric acid and chloramine-T. Though more convenient this procedure has disadvantages which have already been mentioned.

#### Sources of error.

Apart from the usual sources of inaccuracy in quantitative determinations the following were found important.

If/

If the solutions were not mixed thoroughly, immediately after the addition of chlorimine-T, the high local concentration of the latter was sometimes sufficient to oxidise some naphthoresorcinol and give rise to the red pigment.

As emphasised by Mills (1946) it was found essential that the water-bath should be boiling vigorously throughout the entire course of the reaction in order to obtain reproducible results.

The design of the water-bath was found of importance since, in the course of two hours vigorous boiling, it was observed that water from the bath was liable to spurt on to the glass bubbles and thence into the reaction mixture.

It was found that reduced pigment 'B' when formed, could be re-oxidised by shaking with chloramine-T but that this procedure gave rise to very erratic results and it cannot be recommended. If an excess of reducing material is present reduced pigment 'B' will, of course, be formed and the readings obtained will be low. It can usually be recognised by the presence of a greenish tinge in the reaction mixture and is readily recognised at the solvent-water interference with ether and ethyl acetate. All such determination should be rejected and repeated **with** more dilute solutions.



Sensitivity and accuracy.

With most colorimeters (Hilger Spekker absorptionmeter, Unicam SP 500 and Beckmann (Model DU ) photoelectric quartz spectrophotometers and Unicam (SP350) diffraction grating spectrophotometer), from 5-100 microgrammes of glucuronic acid may be determined with an accuracy of  $\pm 1 \mu\text{g}$  whilst up to  $200 \mu\text{g}$  may be estimated with rather less accuracy since the calibration curve is no longer linear. In the presence of interfering substances the upper limit of tolerance is reduced to an extent depending on the amount of interfering material present and there is probably also a slight loss of accuracy though in many experiments values within the limits given above were achieved.

Specificity.

The high degree of specificity of the reaction is best appreciated from a study of tables 2.7 & 2.8.

A number of substances were found to react with naphthoresorcinol to produce pigments soluble in ethyl acetate but the sensitivity for glucuronic acid was very much greater than for any other substance tested while the absorption spectra were quite distinctive (Fig. 2.9).

When chloroform was used as the solvent, specificity for glucuronic acid was almost complete (Other uronic acids probably produce the same pigment but were not available in a sufficiently pure state for quantitative experiments).

Of/

Table 2.7  
Naphthoresorcinol reaction of various substances with  
different techniques

Substance in solution	Amount micrograms	Colorimeter Reading in ethyl acetate			
		HCl	HCl+C-T	H <sub>2</sub> SO <sub>4</sub>	H <sub>2</sub> SO <sub>4</sub> +C-T
Water		.075	.075	.050	.050
		.13	.125	.060	.060
		.055	.088	.046	.052
Glucurone	27.17	.276	.325	.302	.330
		.305	.315	.280	.320
Pyruvic acid	879	.325	.302	.331	.320
Xylose	99.6	.105	.185	.110	.216
	199.2	.195	.150	.165	.090
Arabinose	100.35	.095	.107	.085	.258
Ascorbic acid	99.9	.135	.155	.100	.090
Glucose	100.9	.060	.115	.100	.115
	201.8				.085
	403.6				.183
Glycogen	206.8	.148	.157	.070	.085
	413.6				.175
Glucosamine HCl	199.8				.015
Gluconic acid	ca. 500	.100	.155	.076	.090
Mucic acid	499.4	.040	.045	.075	.060
Saccharic acid	1,016	0	.025	.025	.010
Serum 1/25		.24	.38	.18	.34
Serum (dial.) 1/25		.15	.30	.172	.29
Serum 1/200 (dial.)				.074	.080

The substance to be estimated was dissolved in 2 ml. water to which was added 3 ml. of conc. HCl or 15 N H<sub>2</sub>SO<sub>4</sub>, and 2 ml. of naphthoresorcinol solution. To this 0.2 ml. of 1% chloramine-T solution (C-T) was added in the cases indicated.

Table 2.8

Specificity with different solvents

The figures in the table represent IR values

IR (Dische) - ratio of the reaction intensity of a compound to that of glucuronic acid

Substance tested	Pigment dissolved in		
	Acid-ethanol	Ethyl acetate	Chloroform
Glucurone	1.00	1.00	1.00
Pyruvic acid	-	0.019	0.011
Xylose	0.20	0.076	0.00
Arabinose	-	0.075	0.00
Ascorbic acid	-	0.097	0.094
Glucose	0.075	0.035	0.0043
Glycogen	0.075	0.035	0.0043
Glucosamine	-	0.004	0.00
Gluconic acid	-	0.015	0.00
Mucic acid	-	0.010	-
Saccharic acid	-	0.00083	-

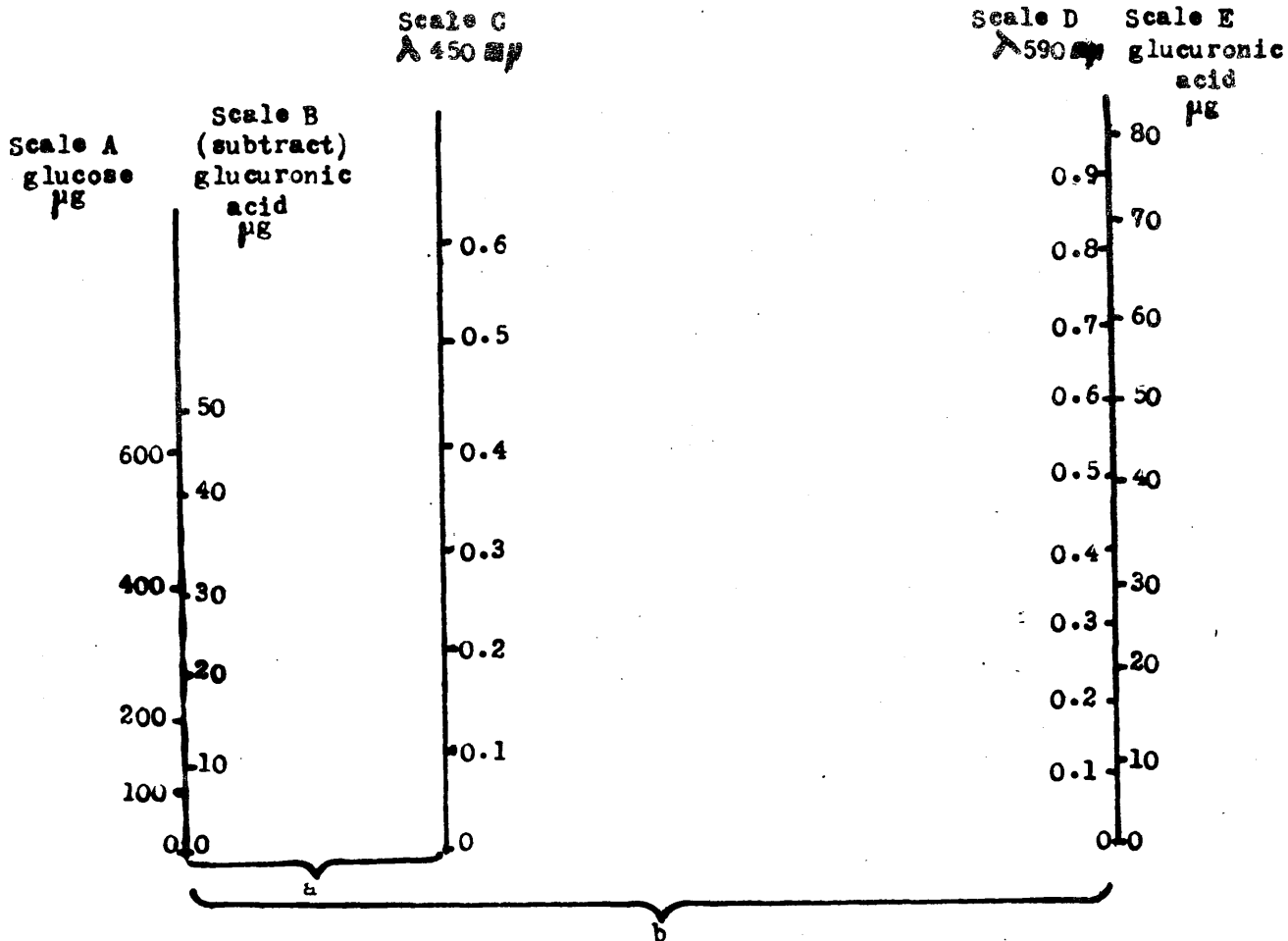
Of all the materials likely to be present, only ascorbic acid and pyruvic acid gave rise to coloured solutions and the intensity of these was such that it seems highly unlikely that any normal biological fluid would contain them in sufficient excess to interfere significantly with the reaction. Large amounts of glucose also gave rise to a slight amount of positive interference (Section 3) but a three - or fourfold excess gave no detectable absorption.

Determination in the presence of interfering substances.

Substances causing negative interference have already been discussed and it has been pointed out that in most cases it is only necessary to be aware of the possibility of interference to circumvent it, while in the case of reducing sugars the addition of an oxidising agent to the reaction mixture has been shown to solve the problem. By this latter method interference by sugars was reduced to positive interference alone and, as has also been shown, positive interference can be accounted for by using a critical solvent, such as chloroform. Two cases still remain to be considered, the case in which the interfering substance is present in such excess that the amount of oxidising agent is insufficient to deal with it, and the case where a pigment soluble in chloroform is formed to an extent sufficient to interfere with the reaction.

Both//

Both problems are solved if the interfering material can be separated from the glucuronic acid to be measured but this solution is not always conveniently available. In the case where a reducing sugar appears in great excess (e.g. in blood) it will be shown that the effect can be counteracted by increasing the concentration of chloramine-T (Section 3.). In the second case two solutions are available. If the interfering material can be determined separately it is, of course, possible to make an allowance for it. This criterion is not frequently fulfilled and use has to be made of the other solution which depends on the fact that the absorption spectra of such substances and glucuronic acid are quite distinct in the cases investigated so far. If the absorption spectrum of the interfering material can be determined along with the absorption spectrum of glucuronic acid it becomes possible, by taking readings at two different wavelengths and solving a simple simultaneous equation, to determine the relative amounts of the two substances present in a mixture. Interpretation of the results is simplified by constructing a nomogram of the type illustrated in Fig. 2.11 which was designed for the simultaneous determination of glucose and glucuronic acid, the pigments being dissolved in acid-ethanol. With the other solvents such a subterfuge is rarely necessary but the solution is available should the circumstances occur.



**Figure 2.11** Nomogram for simultaneous determination of glucose and glucuronic acid.

To read. Join colorimeter readings at 590  $m\mu$  and 450  $m\mu$  with a ruler. Intercept on scale A gives glucose value. Subtract reading at intercept on scale B from reading at intercept on scale E to obtain glucuronic acid value.

Construction.  $\frac{a}{b} = \frac{\text{Glucuronic acid rdg. @ } \lambda 450}{\text{do. @ } \lambda 590} \times \frac{\text{Glucose rdg. @ } \lambda 590}{\text{do. @ } \lambda 450}$

Scale D = colorimeter readings corresponding to amounts of glucuronic acid at the same levels on scale E.

Scale B = scale E

Scale A/scale E =  $1/I_R$  (glucose/glucuronic acid.  $\lambda$  590)

Scale C = colorimeter readings at 450 corresponding to the amounts of glucose at the same levels on scale A.

Some remarks may be appended on interference by protein. Some anomalous results were obtained on investigating this problem. At low concentrations of protein interference could be overcome by increasing the concentration of naphthoresorcinol in the reaction mixture, whereas at high protein concentrations the presence of more naphthoresorcinol increased interference (Table 2.9)

Obviously the mechanism underlying such a phenomenon must be complicated. The matter is of little practical importance since it is usual to remove protein before conducting a determination of glucuronic acid and the common deproteinising agents have not been found to interfere with the reaction.

Within the limitations mentioned this modified form of the naphthoresorcinol reaction has been found very satisfactory in actual practice and would seem to exceed all previous modifications in sensitivity, specificity, tolerance and accuracy for the determination of glucuronic acid in the free state and in the common glucuronides.

The significance of this study will be discussed in Section 5.

Table 2.9

Interference by protein

Conc. of protein	Conc. of naphthoresorcinol reagent		
	0.25%	0.5%	1%
0	0.38	0.40	0.40
Serum 1/200	0.34	0.40	-
Serum 1/20	0.16	0.06	0.00

The figures represent the colorimeter reading due to the addition of 50 micrograms of menthylglucuronide to a standard reaction mixture modified as indicated.



The isolation of minute amounts of glucuronic acid compounds

The two general methods for the isolation of glucuronides from urine, viz. precipitation as insoluble salts and extraction into organic solvents are, unfortunately, of limited application as general methods when the glucuronide concentrations are very low. Precipitation methods are quite useless in this respect for although a minute degree of solubility may be negligible when dealing with concentrations even as low as 0.01% it usually becomes very significant when concentrations fall below that value and since one may be dealing with concentrations as low as 0.0025% e.g. in tissue slice experiments, the method obviously has no practical application as a quantitative procedure in such cases. Moreover, where basic salts are used ester glucuronides are destroyed partially or completely. Bisset, Brooksbank and Haslewood (1948) attempted to entrain glucuronides on barium phosphate but here again the results became too inaccurate at the low concentrations occurring naturally and they had to employ a preliminary concentration before their method could be applied. Orientating experiments which I performed in an attempt to entrain glucuronides on lead phosphate yielded no better results and the method was not considered promising.

One/

One of the original general procedures for the extraction of glucuronides was by means of ether-alcohol mixtures (Neuberg and Schewket 1912) and for individual glucuronides extraction procedures such as this are very satisfactory. However, since the partition coefficients of glucuronides between organic and aqueous solvents vary widely such methods cannot be said to have general applications. It was found that the majority of glucuronides tried could be extracted efficiently from acid solution by means of n-butanol and this procedure almost satisfies the criteria of a general method. However, butanol is tedious to concentrate in large quantities and many other substances are extracted by it. Ether, which is much more convenient, unfortunately extracts only a few glucuronides. As a useful compromise ethyl acetate was found useful in many cases. (table 2.10) Not all glucuronides are extracted by it (notably o-aminophenyl-glucuronide) but most of the common glucuronides are more-or-less easily extracted while some may actually be extracted 90% or more in a single extraction. The method was found generally useful in tissue slice experiments and in other cases where the glucuronide to be extracted was known to be readily soluble in ethyl acetate./

Table 2.10

Recoveries of glucuronides by ethyl acetate extraction.

Material added	Amount /ug	% Recovered	
		1	2
Glucurone	20	0	0
o-aminophenyl glucuronide	50	2	0
Menthyl glucuronide	50	87	90
Phenolphthalein glucuronide	50	100	98
Pregnanediol glucuronide	50	100	100

acetate. If determination of glucuronic acid by means of the naphthoresorcinol test is to follow extraction in these cases it is, of course, necessary to remove all trace of the solvent. This may be achieved by evaporating the bulk of the ethyl acetate by means of a current of warm air and then drying in vacuo over NaOH and H<sub>2</sub>SO<sub>4</sub>.

#### Ion-exchange methods.

Since almost the only common property of all the glucuronides is their behaviour as organic acids the possibility of employing ion-exchange resins for their isolation suggested itself. In selecting a resin for this purpose it was necessary to select one, not only with appropriate groups for taking up these weak acids but also, since it was intended to perform a naphthoresorcinol reaction on the effluent, able to be purified to a degree such as would prevent it from interfering with the reaction. For the latter reason Deacidite F (Permutit Co.Ltd) had to be rejected although it apparently had a more suitable structure than Deacidite E (Permutit Co. Ltd.) which was found to hold some promise. The methods eventually evolved different in some/

some respects from orthodox methods of using these resins and this may have been due to the fact that orthodox methods rarely aim at more than approximately quantitative recoveries from solution of 0.1% strength whereas the aim of this investigation was to obtain very nearly completely quantitative recoveries from solutions of concentrations as low as 0.0005%

#### EXPERIMENTAL

For experimental purposes a batch-exchange technique was employed

Preparation of material. About four times as much resin as was finally required was ground up finely. (For this purpose a mechanical method was found necessary). This material was suspended in distilled water and allowed to settle for thirty seconds, the sediment being rejected. The remaining suspension was centrifuged at 3,000 revolutions/minute for ten minutes and the supernatant suspension discarded. It was then washed, by resuspending and recentrifuging, with warm alcohol, ether, alcohol and finally water. Thereafter it was subjected to about twenty cycles, each consisting of suspending in 2N HCl, centrifuging for ten minutes and discarding/

discarding the supernatant, and repeating the process with water, 2 N NaOH and water. After the supernatant had remained clear for two cycles subsequent cycles were performed on a Buchner funnel with suction, whereby the process was rendered less tedious. Finally the resin was treated with the appropriate charging solution and washed until the effluent had the same pH as the washing water. An approximately equal volume of distilled water was added and the 50% suspension was ready for use.

To a given volume of the solution from which ions were to be removed was added a suitable volume of this suspension and they were shaken together mechanically for a suitable time. Separation of the resin from the supernatant was achieved by centrifuging for ten minutes. To the resin, containing the required ions, was then added a suitable volume of the eluting solvent and these were shaken together for a suitable time. The suspension was once more separated by centrifugation and the supernatant removed for analysis, with great care not to include any resin. A second centrifugation was usually necessary to ensure this.

After preparing a new batch of resin it was found necessary to perform recovery experiments by adding a known amount/

amount of glucuronide to a sample of eluting fluid which had been treated by shaking together with the resin. If in the control experiment, using water in place of glucuronide solution, a large blank reading was obtained, or, if in the recovery experiment one hundred per cent recovery was not obtained the resin was again "cycled" as above, ten times. On this occasion the various treatments were carried out on a Buchner funnel. Treatment was repeated if necessary until no interference occurred due to the resin itself. Thereafter it was possible to recover the resin after use.

In the first instance the displacement method used by Partridge and Brimley (1949) for amino-acids was tried. The resins available at the time were Deacidite E and Deacidite F (Permutit Co.Ltd.). Deacidite F is described as a strongly basic resin with a high capacity for anions and appeared to have the properties desired but unfortunately it was not found possible to remove interfering materials from this resin to such an extent as to allow determination of glucuronic acid by the naphthoresorcinol method on the eluted material. This may have been due to a slight degree of solubility of the resin itself in alkaline solution or to the continuous release of a trace of formaldehyde or similar material./

material. Deacidite E is described as a resin of medium basicity, having a fairly high capacity for all but the weakest of anions. It was found possible to purify this resin to the required extent.

The resin was charged by treating with 2N NaOH and washing with distilled water until the effluent had the same pH as the washing water. A 50% suspension was prepared as above, to 1 ml of a solution containing 50 $\mu$ g of menthylglucuronide, in a centrifuge tube, was added 1 ml of the suspension and the mixture was shaken mechanically for 30 minutes. It was then centrifuged for ten minutes and the supernatant retained for assay. To the precipitate was added 1 ml of 2N HCl and this mixture was again shaken and centrifuged, the supernatant being retained for assay. The method was found to be very unsatisfactory at these concentrations, very little of the glucuronide being taken up by the resin and practically none of that taken up being displaced by HCl. These findings suggested that, by this method and at these concentrations, such glucuronide as had been taken up by the resin had adhered to it by simple van der Waal's forces.

The suggestion was made by Weiss (1950) that ion-exchange resins could be used in a novel manner by employing this/



this phenomenon of simple adsorption. He suggested that acids could be made to adhere to a cation-exchange resin by employing conditions in which both acids and resin were unionised i.e, in strongly acid conditions in most cases. By altering conditions so that both the resin and the acid became ionised the anions could be expelled quantitatively since they both possessed the same type of free electrostatic charge.

In a trial of this method the resin used was Zeokarb 215(Permutit Co.Ltd), a sulphonated resin with a high capacity for cations. A 50% suspension was prepared as before, the resin being employed in the hydrogen state i.e. by treating it finally with 2N HCl. On this occasion it only received one washing with distilled water and therefore contained a considerable excess of hydrogen ions. This procedure was found as satisfactory as washing with water till the effluent was neutral and then treating with acid in the course of the reaction, the object of the excess hydrogen ions, of course, being to ensure that both acid and resin would remain unionised. Times and quantities used in the experiment were determined experimentally (table 2.11).

To/

Table 2.11

Optimum conditions for Zeokarb 215 technique

Amount of suspension Added (ml)	Time shaken together		% of menthylglucuronide not adsorbed	Eluted
	Adsorption	Elution		
0.25	30 min	30 min	15	-
0.50	30 "	30 "	10	-
0.75	30 "	30 "	10	-
1.00	30 "	30 "	10	-
0.50	10 "	30 "	50	-
0.50	20 "	30 "	10	-
0.50	30 "	30 "	10	-
0.50	40 "	30 "	10	-
0.50	20 "	10 "	-	86
0.50	20 "	20 "	-	90
0.50	20 "	30 "	-	90
0.50	20 "	40 "	-	90

Menthylglucuronide originally present in 1 ml. of water - 50 micrograms

To 1 ml of a solution containing  $50\mu\text{C}$  of menthylglucuronide were added 0.5 ml of suspension. These were shaken together for ten minutes and the supernatant removed for determination of glucuronic acid remaining. Elution was achieved by treating with 2 ml of  $2\text{N}$  NaOH and shaking for 20 minutes. It will be seen from the table that almost all the material was taken up by the resin and eluted.

These results were very promising and although recoveries were only about 90%, the percentage recovery remained constant over a wide range of concentrations 'fig. 2-12'. Possibly the menthylglucuronide used in the experiments had undergone a little hydrolysis but, of course, there are many other possible sources of loss in the method. Unfortunately, when interfering substances were added to the solutions it soon became apparent that the capacity of the resin was limited by this method and though interesting, it offered little promise as a practical technique.

In order to obtain high capacity it seemed that an anion-exchange resin would prove necessary, but that in order to ensure that it would take up anions from a very dilute solution a different technique would be required.

Now, since menthylglucuronide had been made to adhere firmly to a cation-exchange resin, apparently by simple adsorption/

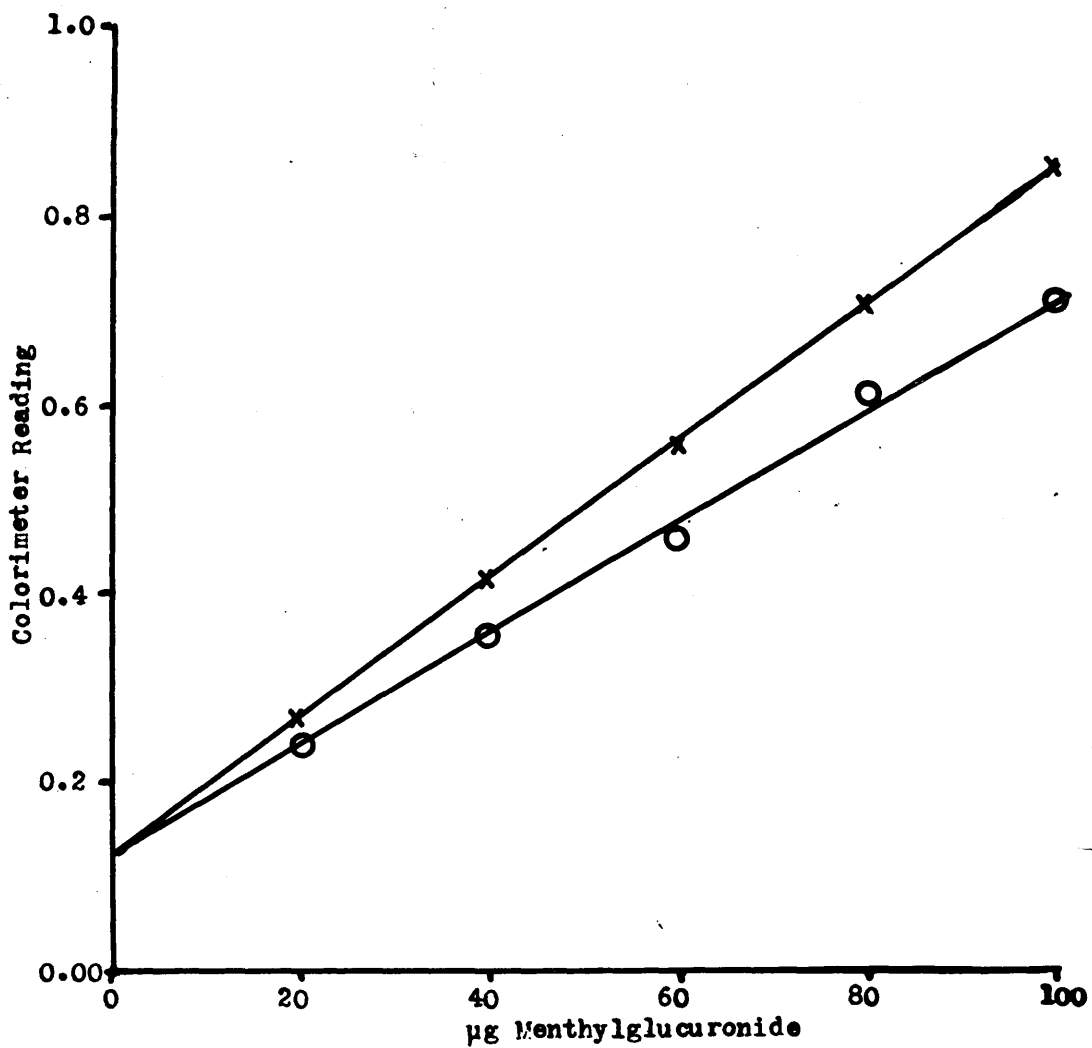


Figure 2.12 Recovery of Menthylglucuronide by "Zeokarb 215"

x — x Control Determinations  
o — o Recovery Determinations  
(Naphthoresorcinol Method)

adsorption forces alone, it seemed likely that, in similar circumstances, it should have an even greater tendency to adhere to an anion-exchange resin where free electrostatic charges would be of an opposite nature. However, in theory, anions of stronger acids should tend to replace it. Since, in the case of glucuronides we are dealing with much larger molecules than the smaller ions usually employed in such studies it seemed possible that behaviour might differ due to the greater effect of van der Waal's forces. In preliminary experiments it was, in fact, found that shaking a glucuronide solution, a Deacidite E suspension and a little sulphuric acid together resulted in the glucuronides being taken up almost completely by the resin. The effect is rather difficult to explain though it may be that the combination of van der Waal's forces and electrostatic forces in the case of a molecule of large size are sufficient to overcome the displacing effect of stronger acids.

Having solved the problem of making the glucuronide adhere to the resin there still remained the problem of eluting it. Alkali itself was not completely effective and it seemed likely that simple adsorptive forces might also be involved so that the use of an organic solvent might improve recoveries./

recoveries. A solution of ammonia in ethanol (10% v/v of 0.88 ammonia) achieved the required result.

Method. The resin (Deacidite E) was prepared as before. 1 ml of the 50% suspension was added to 1 ml of a solution containing a glucuronide and 0.2 ml of 2 N H<sub>2</sub>SO<sub>4</sub> were added. After centrifuging and discarding the supernatant 1 ml of ammoniacal ethanol was added. These were shaken together for 15 minutes, the eluate removed and the process repeated. The two eluates were combined and recentrifuged to remove the last possible traces of resin. The supernatant was transferred to a Pyrex tube and placed in a vacuum desiccator over KOH and H<sub>2</sub>SO<sub>4</sub>. After evaporation 2 ml of water were added and the material was assayed for glucuronic acid by the naphthoresorcinol reaction. Recoveries achieved from a Krebs-Ringer-Phosphate saline containing 0.1% of glucose are shown in table 2.12.

### Use of columns

The knowledge obtained from these experiments was now applied to column chromatography, the eventual object being the isolation of glucuronides from urine and similar biological fluids./

Table 2.12

Recoveries of glucuronides from a  
Krebs-Ringer-Phosphate saline containing 100 mg %  
glucose by the Deacidite E method.

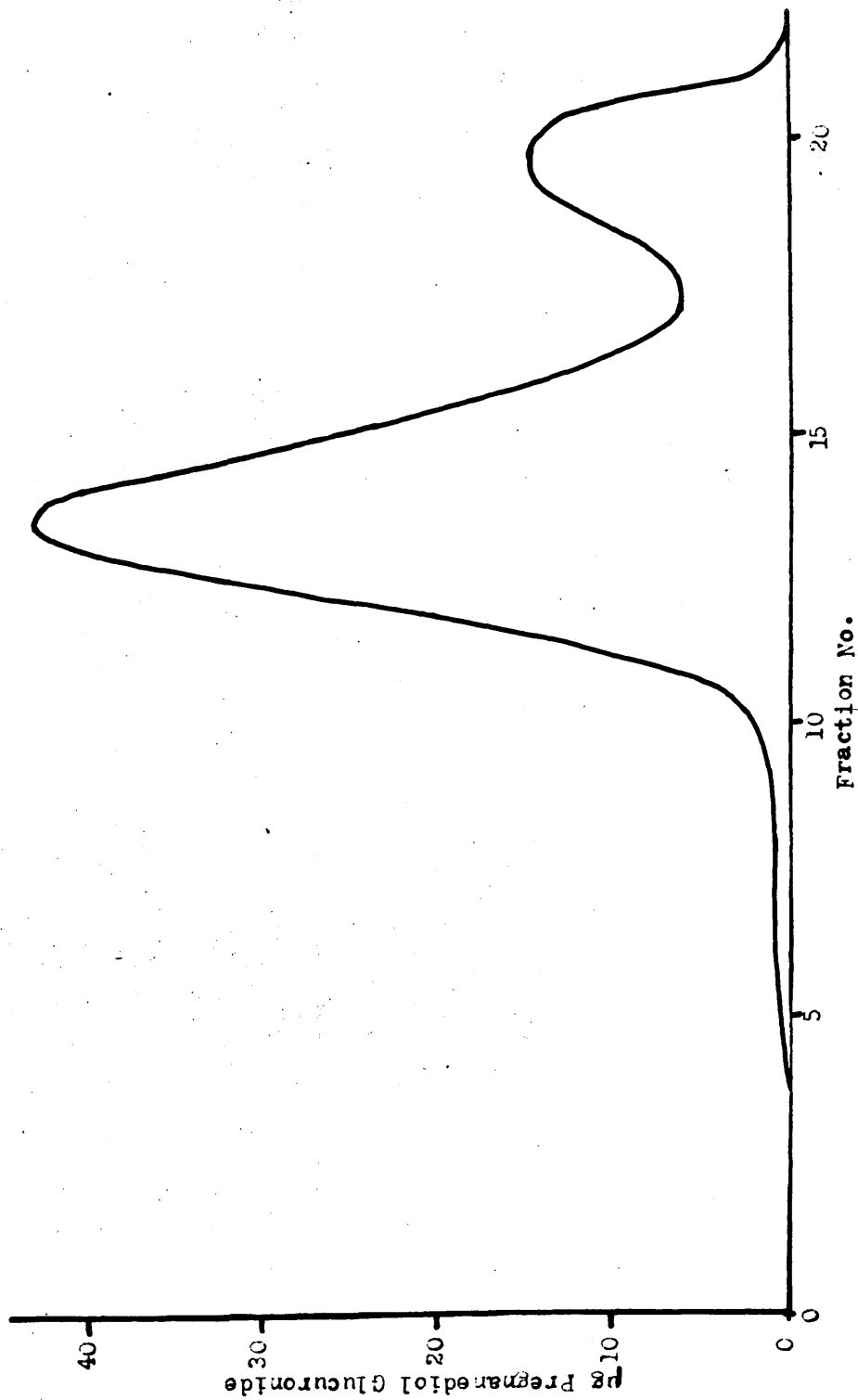
Substance added	Amount /ug/l ml	Recovery %	
		1	2
Glucurone	20	0	0
Menthylglucuronide	50	75	80
Phenolphthalein glucuronide	50	80	85
Pregnanediol glucuronide	50	98	100

fluids.

Preparation of Material. 7g Deacidite E were ground to a fairly fine granular state by means of a coffee mill and packed in a column 1 cm in diameter and about 15 cm high. It was subjected to cycles of acid and alkali as described for the former technique, with frequent back-washings to remove 'fines'; until the effluent at no part of the cycle interfered with the naphthoresorcinol reaction.

Recovery experiment. In this experiment Deacidite E was finally treated with NaOH and washed till the effluent had the same pH as distilled water. Through it was passed in the course of 18 hours 50 ml of a solution of pregnanediol glucuronide which had been acidified by the addition of 5 ml 2N H<sub>2</sub>SO<sub>4</sub>. The total amount of pregnanediol glucuronide present was 200 micrograms. (concentration of 0.000007 mmol/ml). No glucuronic acid was detected in the effluent. Elution was then commenced with 10% ammoniacal ethanol, the effluent being collected in 1 ml fractions in a fraction collector. The result of the analysis of these fractions is shown in fig 2.13. The total amount of glucuronic acid was accounted for and had been concentrated well within 10 ml. The second peak may be an/





**Figure 2.13** Recovery of Pregnenediol Glucuronide by Anion-exchange Resin.  
Analysis of Effluent.

an artefact but it should be pointed out that pregnanediol glucuronide as prepared by the method of Venning (1938) contains about 10% of pregnane-3- $\alpha$ -20-one probably as a glucuronide (Marrian and Gough, 1946) and this may represent a chromatographic separation of the two.

Capacity experiment. On this occasion the resin was treated differently, being charged with 2N HCl instead of with NaOH as before. Through this were passed two litres of a solution containing a total of 2 g, of menthylglucuronide (concentration approximately 0.0025 mmol/ml.) In the first 1500 ml of effluent no glucuronic acid was detected but in the last 500 ml were estimated 20 mg. of menthylglucuronide. Elution was carried out as before, the effluent being collected by means of a fraction collector. In this case ammonium chloride crystallised out in the column and in the effluent. The fractions, which contained glucuronic acid in fractions 10 to 25, i.e. in 15 ml were pooled and taken to dryness. Menthylglucuronide was recovered by extracting with acidified ethanol and estimated by weighing after again taking to dryness. 1.7 g. (85% of the original charge) was recovered. Thus by this technique the resin was found to have a capacity of approximately 0.8 mmol. menthylglucuronide per gram. For such/

such a large anion this is quite a large capacity.

In consideration of these very favourable results the method promised to be directly applicable to the isolation of glucuronides from urine. It was not expected that  $\text{Cl}^-$  ions nor urea would interfere while the recoveries on the micro-scale from Krebs-Ringer-Phosphate saline suggested that  $\text{PO}_4^{=}$  and  $\text{SO}_4^{=}$  ions would also give rise to no interference. These hopes have not yet been realised although the two techniques described and another in which cations were removed by treatment with a cation - exchange resin beforehand have been investigated. In the presence of urine the capacity of the resin for glucuronides seems to be small and they soon appear in the effluent. It would seem that they are either displaced by other anions or by some other substances which adsorb more strongly or upset the adsorption forces in the column.

Although as yet inapplicable to untreated urine the method in either of the above forms should prove useful in purifying already relatively pure solutions of glucuronides.

The separation and identification of small amounts of glucuronides.

On occasions it may be necessary to separate and identify the separate glucuronides present in a few milligrams of material prepared from natural sources, or to prove the presence of a glucuronide in a very dilute solution. It will rarely be possible to apply the ordinary methods of organic chemistry to such a problem. The method of partition chromatography suggests itself as at least one solution.

The first aim was the development of a method suitable for the positive development of very small amounts of glucuronic acid. Since glucuronic acid forms its lactone rather readily in acid solutions while in alkaline solutions the lactone ring is hydrolysed and a salt formed it was considered that this property might be exploited by employing both acid and basic solvents. A suitable acid solvent was found to be ethyl-acetate-acetic acid-water (3-1-3) as recommended by Jermyn and Isherwood (1949) while a suitable basic solvent was found, after experiment, to be sec-butanol-ammonia-water (4-1-4). In each case the solvent was prepared by mixing the components in these proportions, the solvent phase being utilised and the aqueous phase rejected.

The separation of glucuronides posed additional problems since/

since they are all of rather similar structure in that they consist of carboxylic acids with large organic side-groups. Generally speaking the free acids are fairly soluble in the more polar organic solvents and insoluble in non-polar solvents. On the other hand salts of most of the phenolic glucuronides are virtually insoluble in all organic solvents although some of the salts of the steroid glucuronides and terpene glucuronides have varying degrees of solubility in the lower alcohols. Most ester glucuronides are hydrolysed readily in alkaline solvents and some of the ether glucuronides hydrolyse fairly readily in acid solution. In consideration of these points it was considered that once more the possession of both acid and basic solvents would be of advantage.

Sec-butanol-ammonia-water again proved to be a suitable alkaline solvent and good separations, with RF values between 0.25 and 0.8, were obtained with a number of glucuronides. Sometimes a tendency to streaking was observed and, since it seemed likely that this might be due to the separation of phases on the paper due to the presence of the glucuronides, a modification was introduced, which simply consisted of adding 1 ml of sec-butanol to each 100 ml of the solvent.

The/

The latter then being unsaturated there occurred greater tolerance in the presence of other substances and this device produced more compact spots. In this solvent ester glucuronides could readily be recognised by the long tail of glucuronic acid they left in their wake due to hydrolysis.

After some experiment useful acid solvents were found to be those of the ethanol-benzene-acetic acid-water type. The amount of Benzene that could be included was limited by the fact that where it was present in high concentrations the rate of flow was too rapid for equilibration to take place and streaking resulted. A mixture which was found useful was n-butanol-benzene-acetic acid-water in the proportions 3-2-1-3, the solvent phase of the mixture being used as before. This solvent also had a disadvantage, since with weak acid in low concentrations ionisation, and therefore distribution between phases, is related to the concentration of the acid and thus some variation of rate of flow occurs. In order to overcome this problem 1 ml conc. HCl. was added to the mixture before separation of the phases. The presence of the hydrochloric acid in the solvent stabilises ionisation, and therefore partition coefficients, and consequently leads to better reproducibility./

reproducibility. This type of solvent was not so satisfactory as the basic solvent and the various factors were found to be more difficult to control due to the complicated phase system but, with care, it gave quite useful results.

Neither of these solvents provided very satisfactory separations of fast-flowing glucuronides and several other similar solvents were tried. The most satisfactory were sec-butanol-KOH (9 parts sec-butanol-1 part 40% KOH) and pentanol-ammonia-water (4-1-4). In the latter solvent many of the slower glucuronides did not move at all but the more rapidly flowing ones were quite satisfactorily separated in most cases.

#### Methods: Filter-paper chromatography

For all filter-paper chromatograms Whatman no. 1 paper was used, being first washed with acetic acid and then water and dried.

The solution to be analysed was freed from any interfering ions. This was found to be particularly important in acid solvents where the only permissible cation was found to be the hydrogen ion. Also in the basic solvents results/

results were much more reproducible if the cation in the solvent was the only one present. Desalting was simply performed in the case of glucuronides by extracting them from acid solution with ethyl acetate or butanol and concentrating the extract to a suitable volume. In the case of glucuronic acid itself the problem was much greater. The most satisfactory procedure evolved for dealing with this case and also with glucuronides not extracted by the above procedure was as follows. The solution to be tested was neutralised and a little formalin added. It was vacuum distilled to a small volume at 35-40°C and finally freeze-dried. The dried material was extracted with a fairly large volume of ethanol containing 1% of glacial acetic acid. Extraction was carried out at about 40°C and adequate time given for glucuronic acid to be dissolved. The alcoholic solution was taken to dryness by vacuum distillation and then by drying in a vacuum desiccator over KOH and H<sub>2</sub>SO<sub>4</sub>. It was re-extracted with anhydrous ethanol and concentrated immediately to a small volume and applied to the paper.

The following were the reasons for the above steps. It was necessary to neutralise the solution since, on concentration excess of strong alkali or acid destroys glucuronic acid.

Formalin/



Formalin or some other reducing material was added to prevent oxidation to saccharic acid due to the concentration of oxidising materials which might have been present. The temperature was kept low to prevent side-reactions destroying the minute amounts of glucuronic acid present.

The material thus prepared was applied to the paper by means of a micropipette with simultaneous evaporation of the solvent by means of a current of warm (not hot) air. The smaller the spots applied the neater the end results were found to be and 5 mm was the maximum diameter permitted. It was found necessary to take care to equilibrate the atmosphere in the tank with the solvent to be used before adding solvent to the trough. This was particularly important in the case of the highly volatile acid solvents containing benzene. It was also found advisable to protect the tank from wide temperature variations in the course of the experiment.

All the chromatograms described in this section were run by the technique of descending chromatography.

#### Development

Glucuronic acid and reducing sugars were detected by spraying the paper with ammoniacal silver nitrate or aniline hydrogen oxalate (Partridge 1949) and heating for 5 - 10 minutes in the oven at 110°C.

These/

These methods were not applicable to the glucuronides in which the reducing group, on which these tests depend, is blocked. Three methods were developed for the localisation of glucuronides on paper.

1) Many glucuronides could be revealed by ultraviolet light, some e.g. stilboestrol glucuronide, being fluorescent, and others quenching the fluorescence of the filter paper.

2) Glucuronides could be identified as free anions by spraying the paper with a suitable indicator. For this purpose a 0.1% solution of bromocresol green in 50% alcohol was found satisfactory. All ammonia or acetic acid was driven from the paper before spraying by placing it in an oven at  $110^{\circ}$  for about thirty minutes after drying in air. Anions were revealed as yellow spots on a green background while cations, if present, showed up as blue spots. Contrast could be improved temporarily by exposing carefully to ammonia vapour.

The interesting observation was also made that brown spots frequently appeared at the site of glucuronides on paper treated this way after it had been left in a drawer for some months. It was found that the same effect could be/

be produced, though not so effectively, in fresh chromatograms by heating very strongly before an electric fire.

3) Hydrolysis of the glucuronides and identification of glucuronic acid by a suitable method seemed an obvious solution. Hydrolysis with acid rendered the paper brittle and hydrolysis by glucuronidase suggested itself as a better solution. Provided a pure enzyme preparation was used one would expect a high degree of specificity. Spleen glucuronidase (part 4) was used in these experiment but possibly bacterial glucuronidase (Buehler, Katzman and Doisy, 1949; Smith and Mills 1950) would also be useful. It was found advisable to use a powerful preparation, containing at least 5,000 phenolphthalein units to the ml (substrate concentrations probably being very low). In the first instance the paper having been dried and freed of solvents was simply sprayed with a buffered solution of glucuronidase, incubated in a moist atmosphere for a suitable time and sprayed with ammoniacal silver nitrate. This method was found to give irregular results since diffusion tended to occur due to excessive moisture, or no hydrolysis due to drying. More reproducible results were obtained with the following technique which is easy to manage. The chromatogram was sandwiched between a sheet of/

of filter paper moistened with glucuronidase solution and another similarly moistened with pH 5.2 acetate buffer. These three sheets were, in turn, sandwiched between two sheets of plate-glass and placed in an incubator for an hour or two. After drying, all three sheets were sprayed with ammoniacal silver nitrate. It was found useful to run duplicate chromatograms, one of which was treated with glucuronidase and the other not, both being subsequently sprayed with ammoniacal silver nitrate. It was then considered that it could be reasonably assumed that spots presenting in the hydrolysed chromatogram but not in the unhydrolysed one were due to glucuronides. However, it should be kept in mind that most crude glucuronidase preparations contain large amounts of esterase and frequently contain phosphatase and possibly other enzymes.

The third method has the advantage of specificity but the first two methods are much more convenient.

Two methods for glucosides have recently been described by Buchanan, Decker and Long (1950), based on their degradation by metaperiodate and lead tetra-acetate respectively and these may also be applicable to chromatograms of glucuronides.

Interpretation of paper chromatograms. While it is usually necessary/

necessary to employ reference samples of the pure material suspected in the unknown it is often convenient to employ one readily recognised material as a reference substance for the relative rates of flow of the others. In the case of glucuronides it is suggested that phenolphthalein glucuronide be used for this purpose for the following reasons. 1) It is fairly readily prepared (Di Somma, 1940) (Talalay et al., 1946) and is, in fact, available commercially in America (Sigma Chemical Co. St. Louis, Missouri). 2) It is detected by all the methods described. 3) It has an RF value midway between the very fast and the very slow glucuronides and can therefore be used for reference by both groups. 4) It is readily recognised after hydrolysis by the pink colour which appears on spraying the paper with ammoniacal silver nitrate. RF values referred to phenolphthalein glucuronide could conveniently be called  $R_{\phi}$  values, (RG being already in use for sugars)

Results Contact prints of glucurone run in conjunction with xylose and glucose are shown in fig 2.14, the solvents used being those already described. It will be seen that whereas there appears but one rapidly moving spot in the acid solvent, due to glucurone, there appear two slowly moving spots in the basic solvent due, according to Partridge (1948), to glucurone and glucuronic/

Xylose ○



Glucuronic ○



Glucose ○



ETHYL ACETATE - ACETIC ACID - WATER  
6 HOURS.

Xylose ○



Glucuronic ○



Sec. Butanol - Ammonia - Water  
24 Hours.

Glucose ○



Figure 2-14

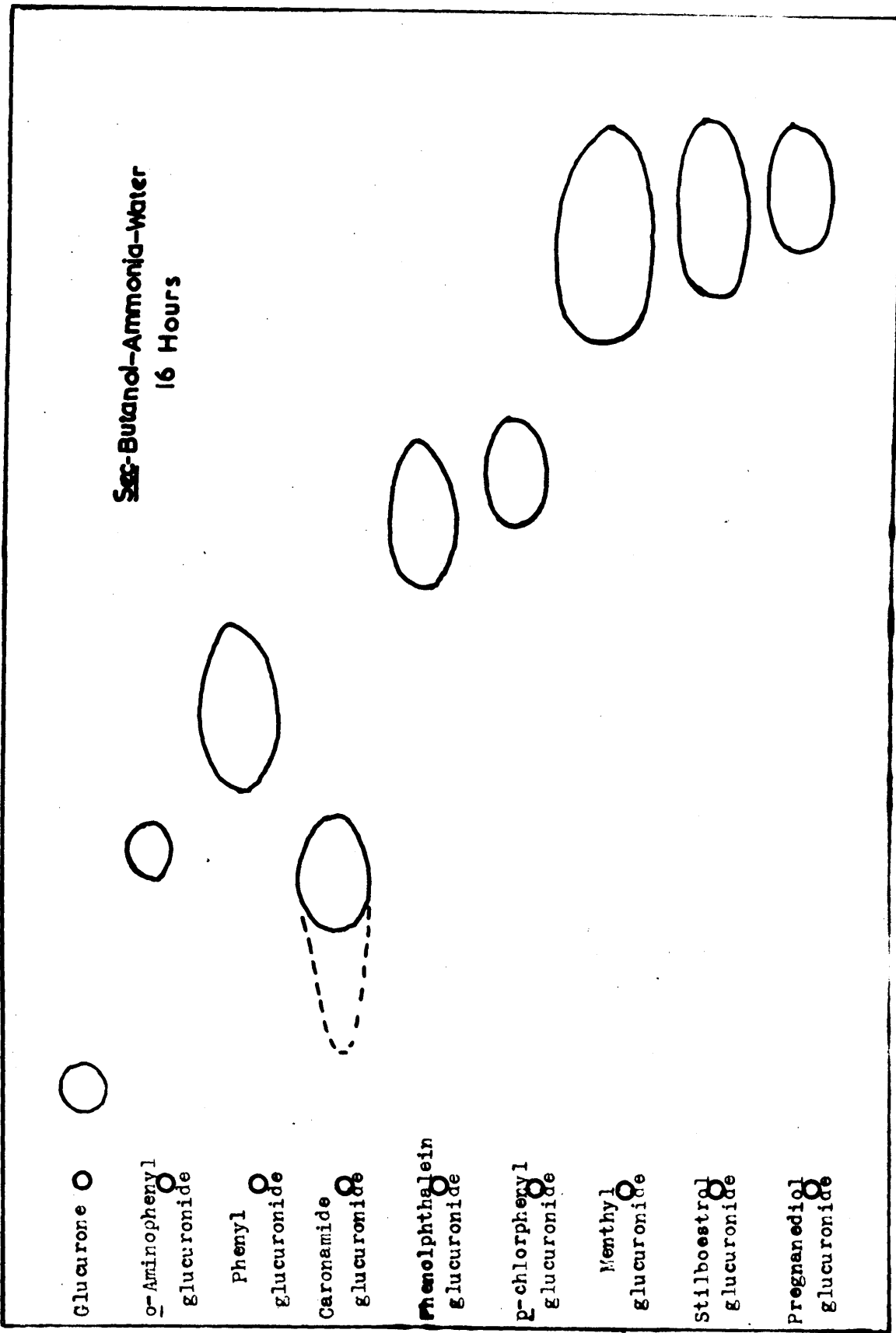
glucuronic acid.

Figs 2.15 and 2.16 are tracings of chromatograms developed by method 3. For comparison figures 2.17 and 2.18 are contact prints of similar chromatograms developed by the indicator method (2) Photographic prints of these chromatograms are difficult to obtain since photographic papers do not distinguish readily between yellow and green. Figure 2.19 is a contact print of the same chromatogram as in fig 2.15 taken in ultraviolet light. It may be seen that while this is very sensitive for many glucuronides it is by no means a general procedure.

'R<sub>0</sub> values' for a number of glucuronides in different solvents are listed in table 2.13.

#### Methods: column chromatography

Whilst discrete spots can be eluted from paper and analysed with a high degree of accuracy in the case of the sugars (Flood, Hirst and Jones 1947) it seemed unlikely that this method would prove applicable to the analysis of unknown mixtures of glucuronides where there might occur several with closely similar mobilities so that the spots would tend to overlap/



**Figure 2.15**



n-Butanol-Benzene-Acetic acid-Water  
5 Hours



Glucurone



o-Aminophenyl  
glucuronide



Phenyl  
glucuronide



Carceramide  
glucuronide



Phenolphthalein  
glucuronide



p-Chlorophenyl  
glucuronide



Menthyl  
glucuronide



Stilboestrol  
glucuronide



Pregnenediol  
glucuronide

Figure 2.16



Glucurone

*o*-Aminophenyl  
glucuronide

Phenyl  
glucuronide

Caronamide  
glucuronide

Phenolphthalein  
glucuronide

*p*-chlorphenyl  
glucuronide

Menthyl  
glucuronide

Stilboestrol  
glucuronide

Pregnanediol  
glucuronide

Figure 2.17



Glucurone

*o*-Aminophenyl  
glucuronide

Caronamide  
glucuronide

Phenyl  
glucuronide

Pregnanediol  
glucuronide

*p*-Chlorophenyl  
glucuronide

Phenolphthalein  
glucuronide

Menthyl  
glucuronide

Stilboestrol  
glucuronide

Figure 2.18

Glucurone

*o*-Aminophenyl  
glucuronide

Phenyl  
glucuronide

Carbamide  
glucuronide

Phenolphthalein  
glucuronide

*R*-chlorophenyl  
glucuronide

Menthyl  
glucuronide

Stilboestrol  
glucuronide

Pregnenediol  
glucuronide



Figure 2.19

Table 2.13

R<sub>0</sub> values for a number of glucuronides in several different chromatographic solvents.

Glucuronide	<u>Sec</u> -Butanol-NH <sub>3</sub> -H <sub>2</sub> O	Pentanol-NH <sub>3</sub> -H <sub>2</sub> O	<u>Sec</u> -Butanol- KOH	Butanol-benzene- acetic a-H <sub>2</sub> O
<u>o</u> -aminophenyl- caronamide- phenyl-	0.32 0.52-0.58 0.72-0.77 1.0-1.1	0.00 0.00 0.27-0.29 1.10-1.16	0.26 0.54 0.54 0.98	0.00 0.91-0.915 0.75-0.80 0.67-0.85
<u>p</u> -chlorphenyl-				
Phenolphthalein-	1.00	1.00	1.00	1.00
menthyl- stilboestrol- pregnanediol-	1.45-1.50 1.45-1.50 1.50	3.70-3.73 6.00-6.20 6.25-6.40	1.48 1.48 1.52	1.15-1.22 1.30-1.41 1.32-1.49

overlap.

Such a problem is best solved by the method of column chromatography as applied to the amino-acids (Elsden and Synge, 1944)

Preparation of columns. Starch columns were prepared by a standard method (Stein and Moore, 1948). 10 g of potato starch were made into a slurry with 20 ml n-butanol and 0.8 ml water. This was poured gently into a 1 cm. diameter chromatography column and allowed to pack under 15 cm. of mercury pressure. It was then washed for 48 hours with the solvent to be employed.

Filter-paper powder columns were prepared by adding 10 g of Solka-floc (Brown & Co. Ltd.) to a suitable volume of acetone to form a slurry and pouring the column in this form. It was then packed under 10 cm. mercury pressure using acetone as the solvent and thereafter washed with 100 ml each of absolute ethanol and 80% ethanol. It was found advisable to boil and cool the solvents to prevent gas bubbles coming out of solution when the solvents mixed. The column thus prepared was then washed through with the solvent to be used for 24-48 hours/

hours. This method of preparation is modified from one used by Dr T.S. Work and recently published by him (Campbell, Work and Mellanby, 1951).

Charging The solvent was driven just into the top of the column and the charge, in not more than 0.5 ml, placed on the surface with great care not to disturb it. The charge in turn was driven just into the top of the column, solvent was added carefully and pressure applied to obtain a convenient rate of flow. The effluent was collected in fractions of equal size, usually 0.5 or 1 ml, by means of a fraction-collector ( See appendix - Apparatus).

Analysis of fractions.

Analysis was carried out by means of glucuronic acid determinations on each fraction. In order to prepare the tubes it was first necessary to drive off all traces of solvent as mentioned previously. This was readily achieved by leaving them in an evacuated desiccator over NaOH and H<sub>2</sub>SO<sub>4</sub> for a suitable period of time. 16-24 hours was sufficient in the case of most solvents but n-butanol required several days for complete removal. The following modification of the naphthoresorcinol reaction was devised for convenience, economy/

economy and speed.

The reagent was prepared by mixing 3 parts 15 N sulphuric acid, 2 parts naphthoresorcinol reagent and two parts 0.1% chloramine-T solution in that order, immediately before carrying out the estimations. To each tube, from which the solvent had been evaporated as above, were added three ml of this reagent from a burette or from a dispenser. The tubes were placed in a vigorously boiling water-bath for 2 hours, cooled, the pigment extracted with 8 ml of ethyl acetate and assayed colorimetrically. Ethyl acetate was used for convenience though, of course, if substances other than glucuronic acid likely to give rise to pigments soluble in ethyl acetate had been present it would have been necessary to use chloroform. The use of chloroform can be rendered a little less tedious in a mass experiment of this kind, where slight individual errors are insignificant, by immersing the tubes briefly in hot water and allowing to cool, a procedure which usually succeeds in separating any emulsion.

By this technique up to 50  $\mu$ g of glucuronic per tube may be determined with a high degree of accuracy but when the amounts exceed this inaccuracies are liable to occur. If high/



high concentrations are to be expected tolerance may be doubled by using twice the amount of reagent and so on.

### Interpretation of results

In order to interpret the results the amounts of glucuronic acid were graphed against the number of fractions passed. Where complete separation was not obtained the peaks could be analysed in an approximate manner akin to that used for the analysis of electrophoretic pictures.

### Results

The graphs from experiments with different solvents and with different adsorbents are shown in figs 2.20 and 2.21. With the solvents used separation of the more rapidly flowing glucuronides was not satisfactory and probably better results would be obtained with these by employing the slower solvents which were mentioned earlier. Unfortunately the RF values of the slower glucuronides are so low in these solvents that it would require many weeks for them to appear, using the highest permissible pressure. It may therefore be necessary, in order to achieve satisfactory separation, of all the glucuronides in a mixture, to employ two solvents, changing from the slow to the fast when the rapidly flowing glucuronides have/

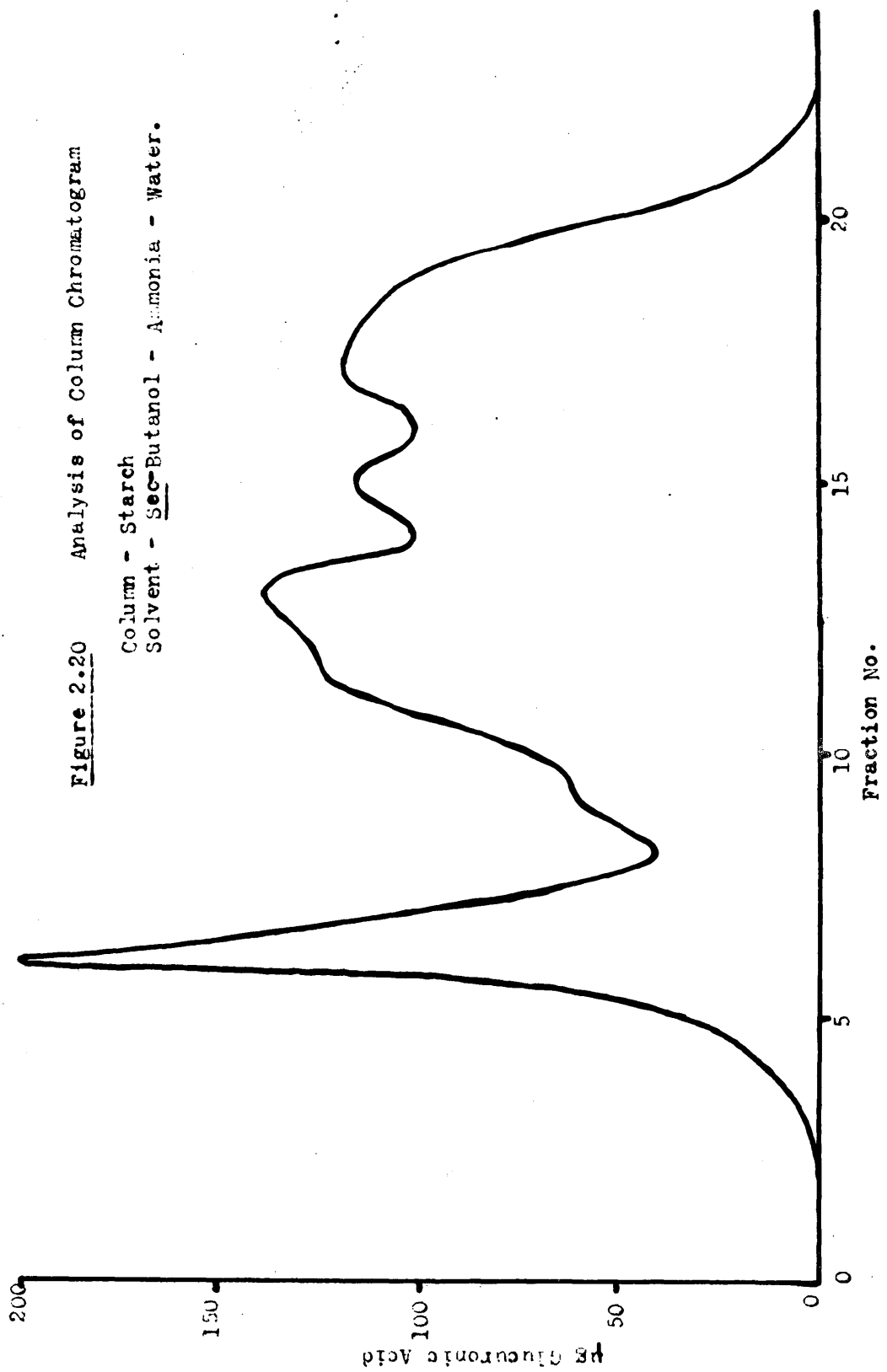
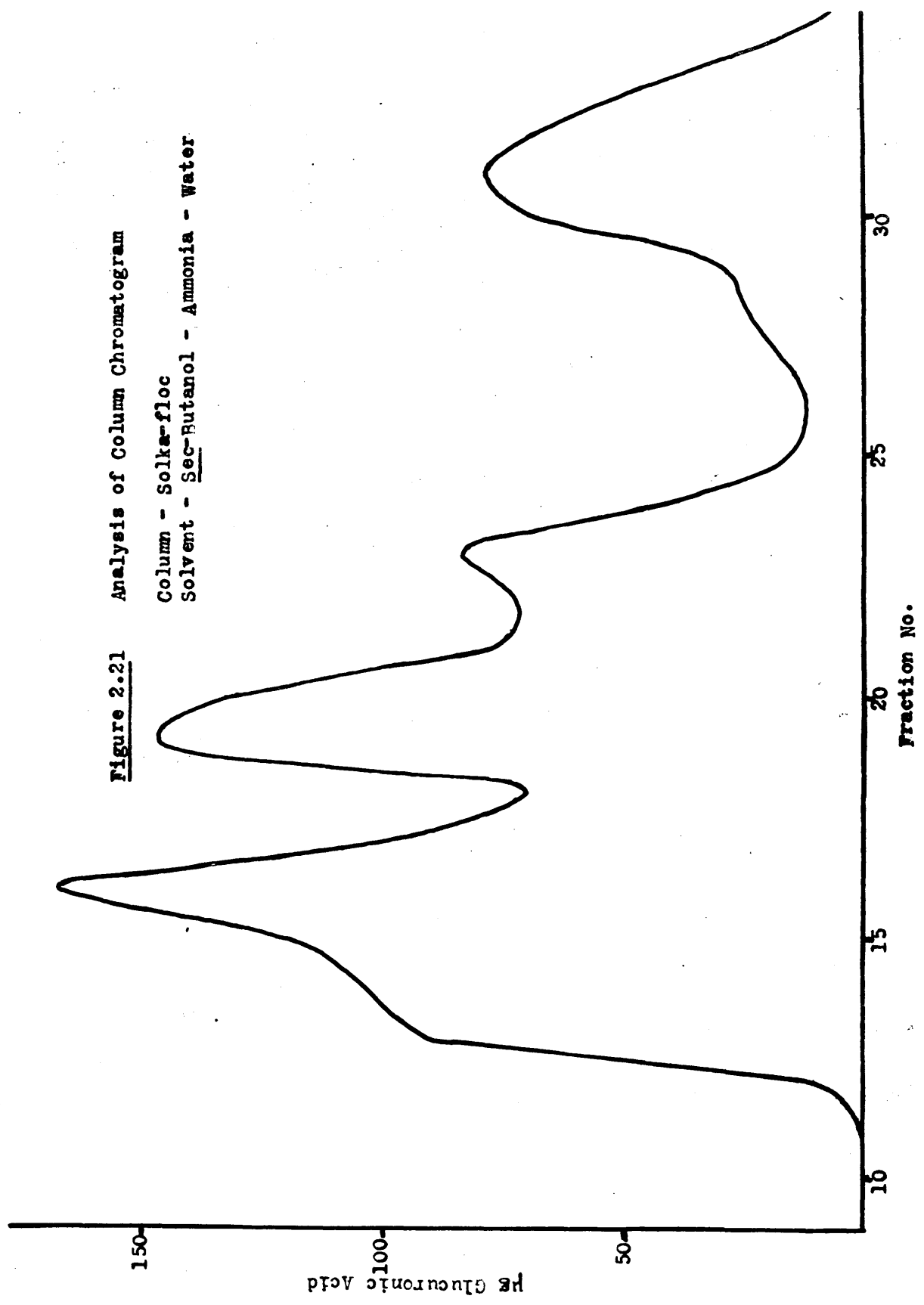


Figure 2.20 Analysis of Column Chromatogram  
Column - Starch  
Solvent - Sec-Butanol - Ammonia - Water.

Figure 2.21 Analysis of Column Chromatogram

Column - Solker-floc  
Solvent - Sec-Butanol - Ammonia - Water



have passed. This type of procedure is likely to involve some difficulty since two different phase mixtures are liable to cause mutual separation when meeting in such circumstances.

SECTION 3.

Occurrence and Synthesis of Glucuronic Acid  
in Animal Tissues.

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Synthesis and Conjugation of Glucuronic Acid.	
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Natural occurrence and synthesis of glucuronic acid  
in animal tissues.

The mucopolysaccharides and mucoproteins form a high percentage of the "ground material" of many tissues, particularly connective tissues. As a component of these, glucuronic acid occurs in fairly large amounts, as mucoitin sulphuric acid in all mucin, as chondroitin sulphuric acid in all cartilage, as hyaluronic acid in most connective tissues and also in a few other materials such as heparin. Our knowledge of the origin and synthesis of these materials is by no means in proportion to the importance one feels justified in ascribing to the evidence of such widespread distribution.

This is almost certainly due to the very great chemical difficulties involved in the study of the subject and, to date, such investigations as have been conducted on the synthesis of glucuronic acid-containing materials have been confined almost entirely to the chemically simpler glucuronides. The experimental work now reported constituted a pilot investigation into the present position in this field.

Occurrence of glucuronides.

Before investigating the synthesis of these materials it was considered advisable to investigate their natural occurrence.

X  
The/

The urine is the most readily accessible source of glucuronides and contains relatively few substances likely to interfere with their isolation. It is therefore not surprising that glucuronides were originally described in the urine and that attention has remained almost entirely centred on the glucuronic acid present there. As has already been mentioned, Thierfelder (1886) originally tried to determine them by laevorotation and reducing material released by hydrolysis. Such methods are greatly lacking in specificity and are of little value. C. Tollens (1909) was probably the first to apply a rational method to the study of the glucuronic acid excretion in urine and he employed the furfural distillation method of Günther et al., (1892) and also attempted to apply the naphthoresorcinol method of B. Tollens (1908). Values obtained in normal urine by this method were 500 mg/day (C. Tollens, 1909) later modified to 300-400 mg/day (C. Tollens and Stern, 1910). Thereafter a large number of investigators, particularly in France, attempted to apply the naphthoresorcinol test directly to urine as a test of liver function. Limitations of the method were pointed out by Schmid (1922) and Brulé, Garban and Amer (1925). Bernier (1912) made one of the first rational attempts to remove interfering/

interfering substances by employing mercury salts to remove indoxyl which he reported as interfering. Roger (1916) also attempted to improve the reliability of the test by precipitating glucuronides as the basic lead salts before applying the reaction. The test fell into disrepute for many years but later Sauer (1930) applied the decarboxylation method to the ether extract of urine. However, not only was such a method tedious but it only estimated the fraction of glucuronides soluble in ether. Hence his values for human urine (220-280 mg./day) are almost certainly low. Florkin and Crismer (1939) pointed out, quite rightly, that the absorption spectrum of the pigment in the extracting solvents normally used was not identical with the spectrum from solutions of pure glucuronides and they attempted to circumvent the effects of interfering substances by removing indoxyl by the method of Bernier (1912) and treating the urine with copper-lime. Since their technique possibly would not estimate some glucuronides precipitable as mercury salts and since ester glucuronides would be destroyed when copper-lime was employed their estimates of 50-200 mg./day are also almost certainly low. Ogata and Yamanouchi/



Yamanouchi (1930) had earlier attempted a non-specific method for removing interfering materials by subjecting the urine to a preliminary hydrolysis. However, one would expect such a procedure to destroy some glucuronides (Link and Niemann, 1930) and that this is so can be demonstrated readily. Salt (1935) attempted to remove interfering materials by lead acetate precipitation and then to precipitate the glucuronides with basic lead acetate before determination by the naphthoresorcinol reaction. He stated that his method could not be applied quantitatively but Deichmann (1943) nevertheless applied virtually the same technique to a quantitative determination of glucuronic acid and also subjected the urine to a preliminary hydrolysis. The sources of error in his method are very numerous. Among many others, some glucuronides may be precipitated by normal lead acetate, not all are precipitated by basic lead acetate and very few are precipitated completely, acid hydrolysis destroys some glucuronic acid, lead ions in high concentrations may interfere with the naphthoresorcinol reaction. Therefore Deichmann and Thomas's (1943) figures of 65-239 mg/day are almost certainly a considerable underestimate. The methods employed by Maughan, Evelyn and Browne (1938) and Wagreich, Kamin and Harrow (1940) were more direct applications of the naphthoresorcinol/

naphthoresorcinol method and their normal estimates of 375-456 mg/day in the former and 350-650 mg/day in the latter case were probably subject to errors in both directions. Owing to the uncritical nature of the extracting solvent used, Hanson, Mills and Williams (1944) undoubtedly overestimated the total glucuronic acid (the authors did not claim otherwise) and their figures of 1 g/day are probably rather high.

Owing to the high degree of tolerance achieved by the modification of the naphthoresorcinol reaction described in section 2 it seemed likely that it might satisfy the requirements of a direct reaction for estimating the glucuronic acid in urine. The method was applied directly to urine diluted 1/40, the pigment being extracted with and assayed in chloroform. The absorption spectrum of the resulting coloured solutions was identical with that obtained by similar treatment of a pure solution of menthylglucuronide. Recovery experiments indicated that there was little interference (table 3.1).

In all human urine so far tested there would seem to be little material which interferes with the reaction and a few determinations have indicated a daily excretion of about 0.5g. glucuronic acid per day.

Some/

Table 3.1

Recovery of glucuronic  
acid added to human urine.

Amount added to 2 ml urine diluted 1/75	Amount recovered
$\mu\text{g}$	$\mu\text{g}$
5	5.2
10	10.4
20	19.7

Some results which have been obtained with chinchilla rabbits are shown in table 3.2. Unfortunately evidence has been obtained that there exists in rabbit urine a powerful interfering substance. It may be mentioned that a useful test for the presence of materials causing negative interference was found to consist of performing a number of assays on a number of different dilutions of urine. If the readings graphed against the concentrations of urine did not give a straight line up to near the usual limit of tolerance the presence of an interfering substance was proved.

As has already been mentioned, the majority of investigators in the past have been preoccupied with the presence of glucuronides in the urine although in the early part of the century Bial and Hüber (1902) had claimed to have demonstrated it in the faeces. The reactions employed by Bial et al (1902) Mayer, and van Leersum (1902) were unsatisfactory but the observation of Abel and Rowntree (1909) that conjugated phenolphthalein, extractible by ethyl acetate, could be found in the urine of dogs, coupled with Di Somma's (1940) finding that only about 45% of administered phenolphthalein could be recovered from the urine of rabbits provided further suggestive evidence./

Table 3.2

Daily excretion of glucuronic acid in chinchilla rabbits. (See remarks in text. Many more figures were rejected as unreliable, and the reliability of these is also in doubt, but they probably represent fairly closely the daily glucuronic acid excretion).  
 Results expressed as milligrams of glucuronic acid per day.

Sex	Male		Female		Pregnant Female	
	1	2	3	4	5	6
Rabbit No.						
Day	117	38.5	89	38.5	144	253
	110	96.5	95	100	175	163
	-	-	116	72	112	114

evidence. Hasegawa (1943), investigating the sugars in bile, claimed to have found 1.96 mg/ml of glucuronic acid in the bile. The Japanese journal in which this report appeared was inaccessible to the present author who was therefore unable to confirm whether this estimate was arrived at after the removal of mucin. It seems important to establish whether significant amounts of glucuronic acid can be excreted as glucuronides in the bile since proof of this would invalidate many of the kinetic studies and percentage conjugation figures obtained from investigations confined to urine.

A number of ox- and guinea-pig bile specimens, freed of protein by treatment with trichloroacetic acid and sulphuric acid were tested and found to give a positive reaction with naphthoresorcinol, the pigment being soluble in chloroform to give an absorption curve identical with that produced by treating a pure solution of menthylglucuronide similarly. In one specimen of fresh ox-bile figures corresponding to a concentration of about 100 mg. glucuronic acid/litre were obtained.

It appeared highly likely from an early date that glucuronides must appear in the blood. P. Mayer (1901) claimed to have demonstrated their presence while Lépine and Boulud/

Boulud (1902) attempted to determine the amounts present by optical rotation. Orskov (1931) and Masamune (1933) attempted to determine the plasma glucuronide content by applying the Hagedorn-Jensen method to hydrolysed ether extracts. Masamune estimated 18-70 mg/ litre in guinea-pigs. The furfural distillation method was tried on the basic lead acetate precipitate from normal serum by Haendel (1930). Stepp (1919), Diebschlag (1920) and Stepp and Diebschlag (1921) were probably the first to try to apply the naphthoresorcinol method to blood but decided that there were too many interfering substances for it to be of any value. However, Mazolowski (1940) and Ratish and Bullowa (1943) applied the method of Maughan, Evelyn and Browne more-or-less directly to blood while Deichmann and Dierker (1946) applied Deichmann's modification. One is at a loss to explain the apparently excellent recoveries obtained by Ratish and Bullowa in particular, for the many observations on the effect of interfering substances from the time of Mandel and Neuberg make it obvious that the high concentrations of glucose would alone invalidate the test as usually applied. Probably on a sounder basis was the method of Florkin et al (1940, 1942)

Blood/

Blood was freed of protein by ultrafiltration and glucose destroyed by copper-lime before applying the test. These authors estimated that human serum contained less than 3 mg. per 100 ml, of glucuronic acid. Their method, of course, only estimated the ether glucuronides.

Since, in the method described in section 2 no appreciable positive interference was obtained due to glucose and negative interference due to relatively small amounts of glucose could be overcome by the addition of chloramine-T it seemed feasible that the principle could be extended to higher concentrations of glucose by increasing the amount of chloramine-T.

The following principle was employed. The unknown solution was standardised to contain a given amount of reducing material, estimated as glucose. Sufficient chloramine-T, determined experimentally, to neutralise this reducing material was included in the reaction mixture and the test was then carried out as before. A convenient amount of reducing material, chosen arbitrarily, was the equivalent of 500  $\mu$ g of glucose (equal to 1 ml of blood containing 50 mg/100 ml)..

Experimentally the optimum amount of chloramine-T to deal with this amount of glucose was determined (table 3.3) and found/



Table 3.3

Effect of chloramine-T concentration on the naphthoresorcinol reaction in the presence of large amounts of glucose. Figures represent colorimeter readings.

Contents of test solution	Reaction mixture containing 1% chloramine-T				
	0.5 ml	0.6 ml	0.7 ml	0.8 ml	0.9 ml
500 $\mu$ g glucose	0.085	0.100	0.105	0.122	✕
" plus 15 $\mu$ g glucuronic acid	0.120	0.207	0.265	0.321	✕
Difference (glucuronic acid)	0.035	0.107	0.160	0.190	-

✕ - pigment production inhibited by excessive oxidation.

found to be 0.75 - 0.8 ml of a 1% solution.

As one might have expected the sensitivity of the test was reduced to some extent by this procedure, probably due to some glucuronic acid being oxidised to saccharic acid before it could combine with naphthoresorcinol. The obvious method of dealing with this reduced sensitivity was by increasing the concentration of naphthoresorcinol and as shown in table 3.4 this was to some degree successful. Although full colour production had not been reached a final concentration of 0.15% gave fairly high sensitivity and still remained fairly economical. A further increase in sensitivity was achieved by using a smaller amount of solvent to extract the pigment and by using a reagent blank with glucose solution in place of a solvent blank to zero the colorimeter.

### Reagents

10% W/V sodium tungstate solution. 4/3 N sulphuric acid solution. Folin and Wu blood sugar reagents.

Concentrated sulphuric acid (Analar).

Naphthoresorcinol reagent. 1.4 g. naphthoresorcinol dissolved in 100 ml of 0.01 N H<sub>2</sub>SO<sub>4</sub> with the addition of 0.025 ml saturated sodium bisulphite solution. 4% (W/V chloramine - T. Standard glucose solution, 50 mg/100 ml.

### Procedure/

Table 3.4

Effect of increasing naphthoresorcinol concentration in the presence of 500 micrograms of glucose neutralised by the inclusion of 0.75 ml 1% chloramine-T solution in the reaction mixture.

Contents of test solution	Reaction mixture naphthoresorcinol conc.		
	0.10%	0.114%	0.128%
500 $\mu$ g glucose	0.138	0.148	0.148
" plus 15 $\mu$ g glucuronic acid	0.275	0.301	0.302
Difference (glucuronic acid)	0.137	0.153	0.154
			0.143%
			0.125
			0.312
			0.187

### Procedure

A sample of at least 1 ml of serum was employed. A 1/10 protein-free blood filtrate was prepared by the method of Folin (Hawk and Bergeim, 1937) and the reducing material in the blood filtrate was determined by the original method of Folin and Wu (1920) using 2 ml of the filtrate for this purpose. The method was slightly modified for use with a photoelectric colorimeter (see section 4). Based on the value obtained a volume of protein-free filtrate containing the equivalent of 500  $\mu$ g of glucose was pipetted into a 6"x  $\frac{5}{8}$ " Pyrex boiling tube and sufficient water added to make the volume up to 7 ml. 1.8 ml of concentrated sulphuric acid, followed by 1 ml of the naphthoresorcinol reagent, were added. 0.2 ml chloramine-T solution was then added with great care to ensure accuracy and the whole was intimately mixed immediately. With each batch of estimations a control tube containing 1 ml of the standard glucose solution in place of the blood filtrate was prepared.

The tubes were placed in a vigorously boiling water-bath for two hours and, after cooling, the pigment was extracted with 5 ml chloroform. This was cleared by centrifugation and the/

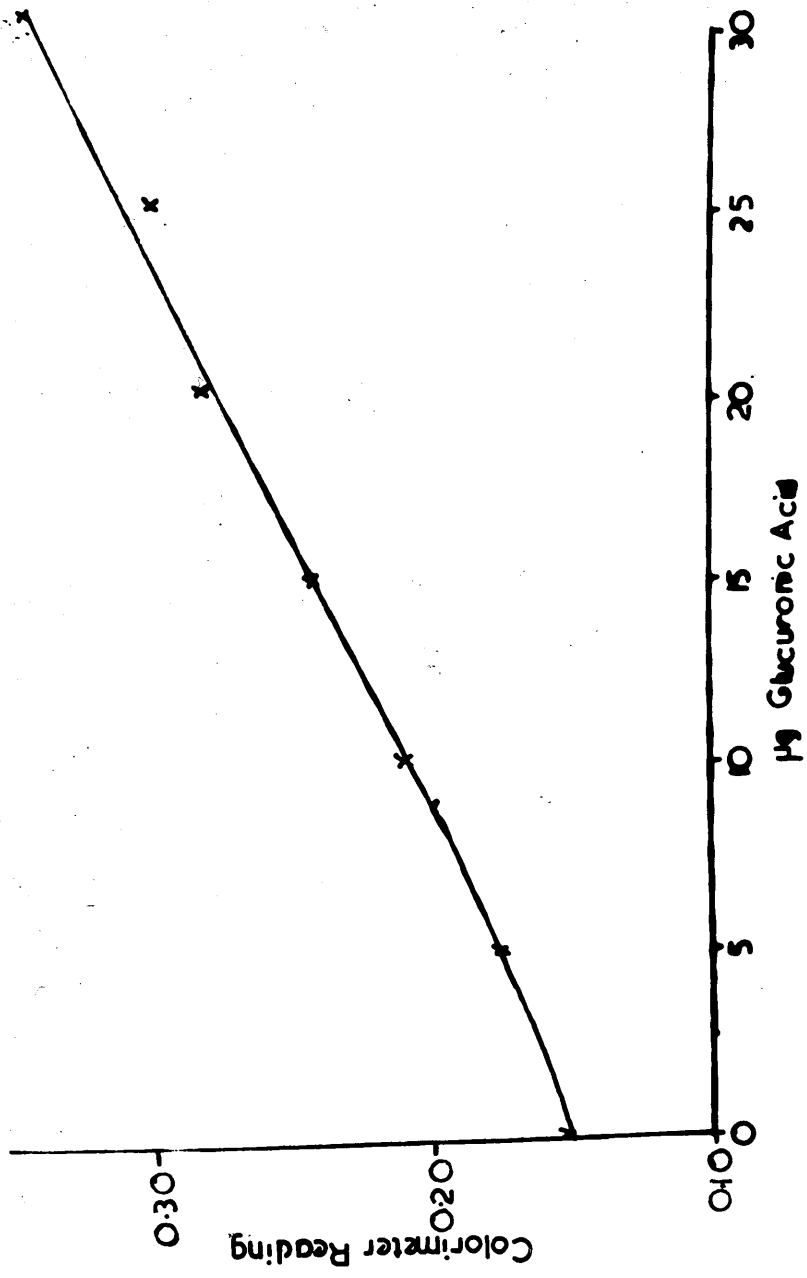
the optical density determined at 580  $m\mu$ , using the glucose control to zero the instrument. Remarks applying to the general method in section 2 also apply to this modification.

### Results

A calibration curve in the presence of 500  $\mu$ g of glucose is shown in fig. 3.1. It will be seen that the curve tends to be concave upwards and this would suggest that the concentration of the oxidising agent was probably a little too high for the range tested, resulting in some inhibition of the colour development at lower levels.

### Recovery of menthylglucuronide added to serum.

Table 3.5 indicates some of the recoveries obtained from normal serum by this method. The results of determinations on a few normal sera are also given.



**Figure 3.1.** Calibration Curve for the Determination of Glucuronic Acid in the Presence of 500 µg Glucose

Table 3.5

Recovery of glucuronic acid added to rabbit serum  
and values found for normal human serum

Serum sample	glucuronic acid estimated mg%	glucuronic acid added mg%	Extra glucuronic acid recovered mg%
Rabbit	2	1.5 1.5	1.4 1.5
Human " " " "	1.7 0.46 1.57		

Synthesis and conjugation of glucuronic acid

Previous investigations have been detailed in the introduction and it will be remembered that a considerable measure of disagreement existed on many points. This orientating investigation was designed to cover the main observations made by previous authors with a view to verifying them or otherwise.

Experimental

Two techniques were employed, the method based on the diazotisation of *o*-aminophenylglucuronide employed by Storey in his investigations and an extraction method similar to those employed by Lipschitz and Bueding (1939) and Crépy (1947).

The diazotisation technique (Levy and Storey, 1949) was used more-or-less in the original form described by these authors. One modification was considered desirable. The originators of the method found that the pink colour was developed optimally at a pH of 2.25 and they employed a mixture of molar phosphate at pH 2.25 and molar trichloroacetic acid at pH 2.25 as a protein precipitant and in order to ensure this pH. This mixture has a rather poor buffering action and it was suspected that, in some cases where strong buffering/



buffering substances, e.g. sodium pyruvate, were present, some of the variability of the results was due to slight pH shifts. This proved to be the case in some instances and it was found more satisfactory to employ a mixture of equal parts of molar glycine-HCl buffer and molar trichloroacetic acid, both at pH 2.25, for this purpose. (see table 3.10). Probably the best and most convenient material would be a glycine-trichloroacetic acid buffer.

The second method employed the extraction of the glucuronides by a suitable solvent and direct determination of glucuronic acid in the extract by the naphthoresorcinol method. Method. 300 mg l-menthol were dissolved in 1 litre of distilled water by leaving in contact in an airtight vessel for several days. Using this stock solution in suitable proportions with water the Krebs-Ringer stock solutions described by Umbreit, Burris and Stauffer (1945) were prepared, to contain suitable concentrations of menthol as substrate for conjugation with glucuronic acid. Control salines were prepared in the same way using distilled water in place of the menthol stock solution. In all cases the stock salines were made up to 4/3 the strength finally required in the course/

course of the experiments. 1.5 ml, saline was added to each flask and the volume made up to 2 ml by the additions described in the tables or by distilled water.

The animals were stunned by a blow on the head and bled to death, guinea-pigs first being anaesthetised with ether. The liver was immediately dissected out and placed on crushed ice. A suitable quantity of slices was cut by means of a microtome (Stadie and Riggs, 1944) which permitted the cutting of large, even slices in a short time. As they were cut the slices were stored in ice-cold saline. Two slices were then quickly dried of excess water on filterpaper, weighed rapidly on a torsion balance and transferred to the prepared flasks (25 ml flasks, Hysil). A suitable gas-phase was passed into the flasks for thirty seconds, the flasks stoppered and placed in the bath at  $37.25^{\circ}\text{C}$ . agitation being commenced immediately. In a few minutes the stoppers were released momentarily and then retightened. At the end of the experiment the slices were removed from the flasks, with care to remove as little saline as possible and 2 ml of 2 N sulphuric acid was added to each. A specimen of the liver was weighed, dried at  $110^{\circ}$  to constant weight, and reweighed to provide a wet Weight/

weight/dry weight factor. By this means most results were referred to the approximate dry weight.

Extraction. To the acidified contents of each flask were added 5 ml ethyl acetate and they were shaken mechanically in a microflask shaker (Baird and Tatlock) for one minute before transferring the contents to test-tubes from which the solvent layers were transferred to centrifuge tubes. To each flask was again added 5 ml of ethyl acetate, with which it was rinsed, the ethyl acetate transferred to the appropriate test-tube and again shaken with the aqueous layer. After adding the ethyl acetate layers to the previous extracts they were centrifuged to separate any emulsions that had formed and these were again transferred to Pyrex boiling tubes and evaporated in a current of air in a warm bath. The last traces of volatile material were removed by placing the tubes in an evacuated desiccator over potassium hydroxide and fresh concentrated sulphuric acid for at least twenty-four hours.

Determination of glucuronic acid. To each tube were added 2 ml of distilled water and the glucuronic acid was determined by the routine method (section 2) using chloroform as the extracting solvent for the pigment.

Modifications. In experiments where glucose or glucurone were added/

Table 3.6

Bestruction of glucuronic acid and glucose by alkali.

Treatment. Cold NaOH - to 1 ml. of test mixture was added 1 ml. of N NaOH and this was left at room temperature for 30 minutes before applying the naphthoresorcinal reaction. Hot NaOH - to 1 ml. of test mixture was added 1 ml. of N NaOH and this was placed in a boiling water-bath for two minutes and cooled before estimating.

(Figures represent colorimeter readings).

Test mixture	Untreated	Cold NaOH	Hot NaOH
Water	0.014	0.015	0.015
(A) Glucose 0.1%	‡	‡	0.015
(B) Glucurone 0.0020%	0.720	0.710	0.015
(C) Menthylglucuronide 0.0025%	0.640	0.650	0.620
(A plus C)	‡	‡	0.650

‡ - "reduced form B" formed due to excess of reducing material

added to the salines, instead of adding 2 ml of distilled water to the evaporated contents of the tubes 2 ml of N NaOH solution were added and they were placed in a boiling water-bath for five minutes. This method effectively destroyed any glucose or glucuronic acid which might have been carried through to this stage. (table 3.6).

In experiments where pyruvic acid was added the method employed by Lipschitz and Bueding was used to prevent passage of pyruvic acid into the solvent i.e. a large amount of sodium bisulphite was added to the aqueous acidified mixture before extracting. Some pyruvic acid obviously did pass to the ethyl acetate but the amount was reduced to proportions which would not be expected to interfere significantly with the reaction. It may be observed that some passage of pyruvic acid into the solvent would be expected as the pyruvic acid-bisulphite compound is readily hydrolysed by acid.

#### Synthetic experiments employing rats.

The rats employed in these experiments were albino rats and the method used was the method of Levvy and Storey (1949).

Evidence of synthesis was obtained readily in rat liver. The effect of the concentration of O-aminophenyl in the saline is shown in table 3.7. The optimum concentration in these experiments/

Table 3.7

Effect of o-aminophenol concentration on glucuronic acid formation in rat liver

<u>o</u> -aminophenol concentration (%)	glucuronic acid formed mg/g dry tissue/hr.	average
0.00125	0.278 0.251 0.254	0.261
0.0025	0.328 0.415 0.443	0.395
0.005	0.450 0.474 0.425	0.437
0.01	0.288 0.610 0.425	0.441

experiments was rather higher than that described for mouse liver by Storey (1950) but as demonstrated in this and later experiments the degree of conjugation was about the same.

Synthesis was inhibited by KCN and by fluoride (cf Hemingway, Pryde and Williams, 1934; Lipschitz and Bueding, 1939) as shown in table 3.8.

Synthesis was not obtained in homogenates with the addition of cytochrome c and/or ATP but some synthesis was obtained on adding a boiled liver extract (Storey, private communication; Dutton and Storey, 1951). Barium-soluble ethanol insoluble and barium-insoluble fractions of the acid-extractible phosphorus from rabbit liver already contained a large amount of diazotisable material and no satisfactory conclusion could be reached at this stage regarding the effect of adding these to liver homogenates. Inhibition of glucuronidase activity by the addition of saccharate to a brei also had no effect in producing synthesis. (Table 3.9)

The substitution of a bicarbonate saline for phosphate saline produced a considerable increase in glucuronide production (Storey, 1950) but the addition of pyruvate to the medium produced no significant increase (in contrast to the findings of Lipschitz and Bueding but in agreement with the findings/

Table 3.8

Inhibition of glucuronic acid synthesis by cyanide and fluoride

Saline containing <u>o</u> -aminophenol and	Concentration	glucuronic acid mg/g dry tissue/hr.
-		0.472
KCN	0.002 <u>M</u>	0.00
NaF	0.01 <u>M</u>	0.00



Table 3.9

Effect of various factors on synthesis in homogenates.

10% homogenate containing o-NH <sub>2</sub> -phenol and	Glucuronic acid formed mg/g dry tissue/hr.
Cytochrome C	0.00
ATP	0.00
Cytochrome C plus ATP	0.00
Saccharate 0.04 M	0.00
20% boiled liver extract	0.117
	0.162
	0.080

findings of Storey (1950) and Crépy (1947) (table 3.10).

This table also illustrates that there was apparently some inhibition by pyruvate when Levvy and Storey's phosphate-TCA system was used but that such inhibition obviously did not occur when glycine-HCl-TCA was used.

Insulin was not found to stimulate synthesis (table 3.11)

Attempts to demonstrate synthesis of other glucuronides in the rat by employing the extraction technique were successful in the case of p-chlorphenol but repeatedly unsuccessful in the case of menthol. This latter finding is in agreement with the finding of Crépy (1947) but in disagreement with the finding of Lipschitz and Bueding 1939 who claimed to have demonstrated the conjugation of menthol in this animal. In attempts to confirm their findings their own method of ether extraction was followed closely but only on one or two occasions was there any suggestion at all of the appearance of extra glucuronic acid in the extracts. On the other hand almost invariably there appeared some material reacting with naphthoresorcinol in the typical fashion of glucuronic acid, whether menthol was added or not. (table 3.12)

Experiments on guinea-pigs.

In/

Table 3.10

Effect of bicarbonate and pyruvate on synthesis

Expt	Saline	Addition	Conc.	Glucuronic acid formed mg/g dry tissue/hr.	Technique
1	Phosphate	---	---	0.163	Phosphate-TCA
	" Bicarbonate	Pyruvate	0.02 M	0.138	
	"	Pyruvate	0.02 M	0.408 0.267	
2	Phosphate	---	---	0.266	Glycine-HCl - TCA
	" Bicarbonate	Pyruvate	0.02 M	0.266	
	"	Pyruvate	0.02 M	0.440 0.376	

Table 3.11

Effect of insulin on glucuronide synthesis

Phosphate saline containing $\alpha$ -NH <sub>2</sub> -phenol, pyruvate and	Glucuronic acid formed mg/g dry tissue/hr.	
		Av.
No insulin	0.31	0.300
	0.331	
	0.26	
Insulin (0.1 unit/ml)	0.28	0.319
	0.321	
	0.357	

Table 3.12

Conjugation of p-chlorophenol and menthol in rat-liver.

Saline containing	Glucuronic acid formed mg/g dry tissue/hr.	Technique
p-chlorophenol	0.0025%	Ethyl acetate extraction.
	0.005 %	
	0.0075%	
menthol	0.007 %	
	0.014 %	
	0.021 %	
no addition	0.231	Ether extraction (Lipschitz and Bueding 1939)
	0.182	
menthol	0.190	
	0.242	

In marked contrast to rats the synthesis of menthylglucuronide was readily demonstrated with guinea-pig liver slices. The optimum concentration was found to be in the same region as that found by Lipschitz and Bueding (1939) about 0.018% (table 3.13).

Synthesis in different organs was investigated. It was readily demonstrated in liver, was present to a much smaller degree in kidney and absent in spleen. There was a suggestion of synthesis in intestinal mucosa (sliced from the posterior muscle layer by the microtome). These results are presented in table 3.14.

By this technique also it was verified that synthesis was greater in bicarbonate saline than in phosphate saline.

Experiments on the addition of substances which have been claimed to increase glucuronic acid production led to some interesting, if somewhat confusing, results. (table 3.16) In the first experiment the results showed similarities with some of those of Lipschitz and Bueding (1939) in that there was practically no synthesis in the absence of additions other than the aglycone itself. When not only pyruvate but also glucose and glucurone were added a considerable degree of conjugation occurred. In the second experiment, however/

Table 3.13

Effect of menthol concentration on glucuronic acid synthesis  
in guinea-pig liver

Menthol conc. %	Glucuronic acid formed mg/g dry tissue/hr.
0.007	0.397
0.014	0.443
0.021	0.503

Table 3.14

Ability of different organs to form conjugated glucuronides  
(Menthol conjugation in guinea-pig)

Organ	Glucuronic acid formed mg/g dry tissue/hr.
Liver	0.51
Kidney	0.108
Intestine	0.070 (?)
Spleen	0.00



Table 3.15

Effect of bicarbonate on conjugation of menthylglucuronide.  
(guinea-pig liver)

Saline medium (containing menthol)	Glucuronic acid formed mg/g dry tissue/hr.
Bicarbonate Ringer Phosphate Ringer 0.9% NaCl	0.376 0.134 0.00

however it was found that not only was glucuronic acid present in the flasks which had contained menthol alone but also in the cases where glucuronic acid and glucurone were added there appeared to be quite a considerable amount of glucuronic acid extracted by the ethyl acetate even when menthol was absent. In a third experiment there was no evidence for an increase in conjugation on the addition of pyruvate.

At first sight there would appear to be no explanation for this phenomenon other than a fault in the method but an indication of a possible other reason for the discrepancy was obtained when a paper chromatogram was run on some of the material extracted by ethyl acetate. Not only did a spot appear corresponding fairly closely to a reference spot of pure menthylglucuronide but another spot was also present with an even higher RF value. As the chromatogram was developed with the bromocresol method there was, however no evidence that this material was necessarily a glucuronide at all.

Moreover, it may be noted that in experiment 1 the saline medium contained no magnesium ions. Since it is doubtful whether the saline medium used by Lipschitz and Bueding contained magnesium, the findings may explain the discrepancy between their results and those of later workers as discussed in/

Table 3.16  
 Effect of carbohydrate materials on the synthesis of  
 methylglucuronide in guinea-pig liver slices.

	Saline containing		Glucuronic acid formed mg/g dry tissue/hr.
1.	No Mg <sup>++</sup>	Menthol " & pyruvate (0.02 M) " & glucose (0.02 M) " & glucurone (0.02 M)	0.04 0.332 0.284 0.383
2.	Mg <sup>++</sup> present	No addition Menthol " & Glucose (0.02 M) " & " (0.02 M) " & Glucurone (0.02 M) " & " (0.02 M)	0.00 0.753 0.534 0.631 0.317 0.573
3.	Mg <sup>++</sup> present	No addition Menthol " & Pyruvate (0.02 M) " & " (0.02 M)	0.00 0.633 0.343 0.582

in section 5.

An observation had been reported by DeMeio and Arnolt (1944) to the effect that phenol conjugation was inhibited in liver-slice experiments by iodoacetate but that this inhibition could be reversed by the addition of glucuronic acid to the saline medium. This observation obviously suggested that glucuronic acid, if preformed, could be conjugated with an aglycone in the absence of all enzyme systems inhibited by iodoacetate. The significance of such an observation is so great that it is surprising that no previous investigator has tried to verify the facts by actual determination of glucuronic acid conjugation. The two experiments in table 3.17 convincingly demonstrate that liver slices in which the formation of glucuronic acid has been inhibited by iodoacetate are still capable of conjugating menthylglucuronide when glucurone is added to the medium.

Function of the glucuronide-synthesising system.

A few experiments were carried out with the double object of trying to find if there existed any relationship between the changes reported in the glucuronidase activity of tissues and changes in glucuronide-synthesising power and also of verifying some of the observations of Karunairatnam, Kerr and/

Table 3.17

Reversibility by glucurone of the iodoacetate inhibition of methylglucuronide conjugation.

Expt.	Bicarbonate saline containing		Glucuronic acid formed mg/g dry tissue/hr.
	menthol %	iodoacetate M	
1.	0.014	M	0.441
	0.014	-	0.070
	0.014	0.004	0.765
	-	0.004	0.232
2.	0.014	-	0.483
	0.014	0.004	0.046
	0.014	0.004	0.353
	-	0.004	0.00

and Levvy (1949).

### Hepatectomy.

Increase in glucuronidase activity after hepatectomy has been reported (section 4). In this experiment a large number of rats was subjected to partial hepatectomy, about two-thirds of the liver being removed at operation. A batch of rats was killed daily up to ten days after operation and at the first, second, third, fifth, sixth and eighth days fresh samples of liver were assayed for glucuronide-synthesising power. On the average four rat livers were assayed on each occasion, each assay being carried out in triplicate. Simultaneously, other workers investigated glucuronidase and phosphatase activity and the ribonucleic acid, deoxyribonucleic acid and protein contents of the same livers, (Goodlad, Mills, & Smith (1951). The author is indebted to these workers and to Dr W.C. Hutchinson for permission to reproduce their figures on protein nitrogen, deoxyribonucleic acid and glucuronidase values.

Figure 3.2 shows no significant change in the synthesising ability of this liver and no relationship between this and glucuronidase activity. There was, of course, a considerable scatter of results, characteristic of the method.

Table/

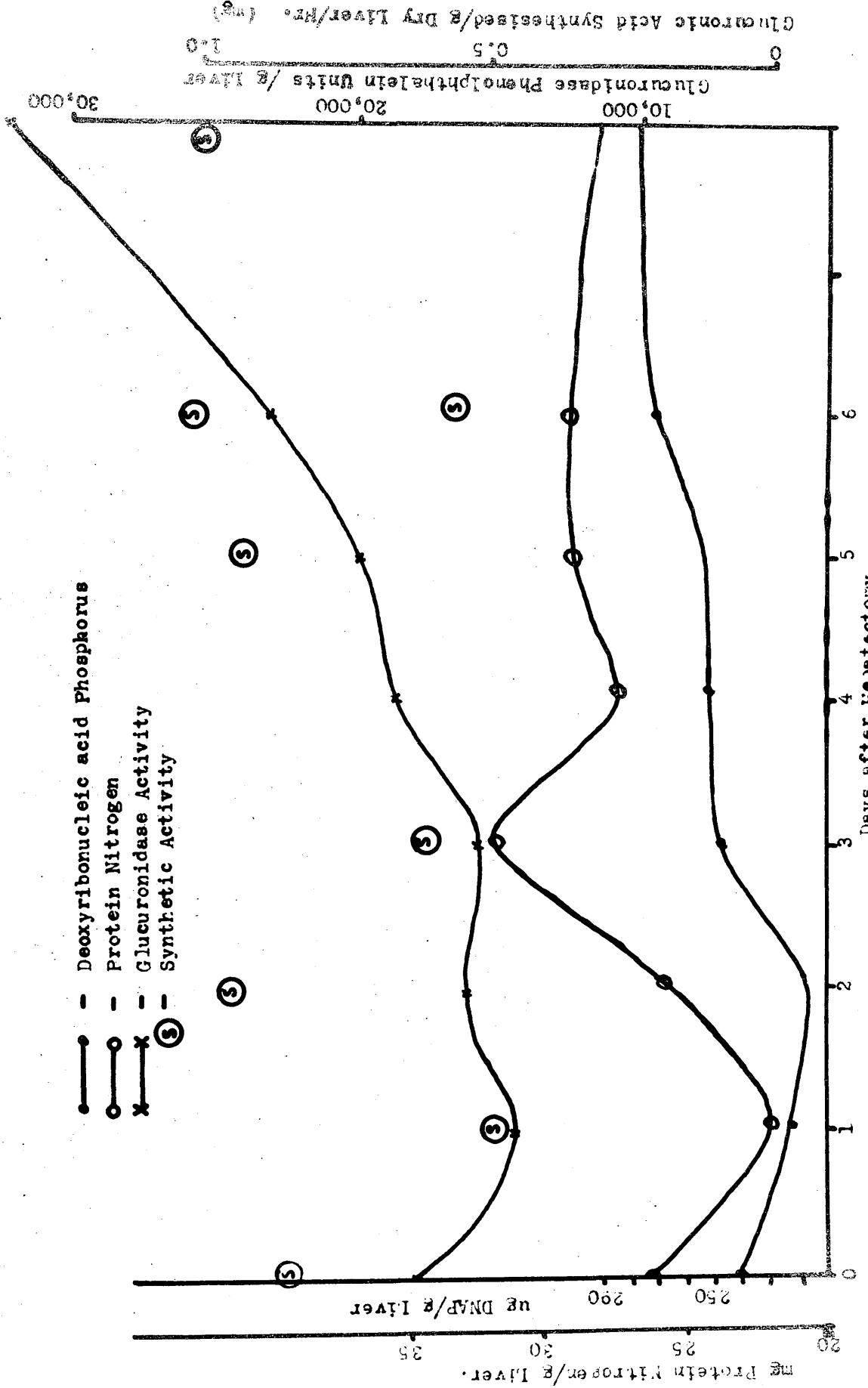


Figure 3.2 Glucuronide Synthesis in Regenerating Liver.

Table 3.18 shows the results of assays on the livers of 3 albino rats which had been fed on a high fat, low choline diet for fifteen days and had fairly markedly fatty livers. Again there is no significant alteration of synthesising power.

In table 3.19 are shown the results of an experiment in which the synthesising ability of a pregnant rat's liver was compared with that of two non-pregnant females of the same age and weight. Again no significant difference is to be observed.



Table 3.18

Glucuronide conjugating power of fatty liver of rat

Animal No.	F 1	F 2	F 3	Control 1	Control 2
Glucuronic $\bar{x}$ (mg/g/hr.)	0.866	0.559	0.425	0.650	0.870

Table 3.19

Glucuronide conjugating power of liver of pregnant rat

Animal	Glucuronic acid formed mg/g dry tissue/hr.
14-day pregnant Female 1 Female 2	0.598 0.700 0.629

S E C T I O N 4.

Degradation of Glucuronic Acid Compounds in the Animal Body.  
B-Glucuronidase.

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DEGRADATION OF GLUCURONIC ACID COMPOUNDS IN THE ANIMAL BODY

$\beta$ -Glucuronidase.

A number of relatively pure enzymes has now been prepared which are capable of attacking most of the naturally occurring compounds containing glucuronic acid. Mucopolysaccharides are attacked by a number of depolymerising enzymes of which the best known is hyaluronidase (Duthie and Chain, 1940), which attacks hyaluronic acid. A chondroitinase is also present in most hyaluronidase preparations and possibly the enzymes are identical. The hydrolysis of the simpler compounds, the glucuronides, is achieved by the enzyme  $\beta$ -glucuronidase which was the subject of the present study. Masamune (1934) was the first to isolate this enzyme in a relatively pure state and he demonstrated that it hydrolysed  $\beta$ -glucuronides with ease but did not attack, glucosides or  $\alpha$ -glucuronides. On the basis of these findings he named the enzyme  $\beta$ -glucuronosidase, now shortened to  $\beta$ -glucuronidase. The enzyme is also frequently referred to simply as glucuronidase and this would seem to be quite adequate since in animal tissues no  $\alpha$ -glucuronides are known to occur and no  $\alpha$ -glucuronidase has been reported. Moreover, although there is no reason to doubt Masamune's observations no independent observer has yet attempted to verify them and until this is done it is possibly a little premature to insist on the full/

full name. In the following pages glucuronidase and  $\beta$ -glucuronidase are regarded as synonymous.

### Methods of Assay

Fishman (1950) divides the methods of assay into three groups, a) reductimetric methods, b) naphthoresorcinol methods, c) aglucuronometric methods.

The naphthoresorcinol method (Florkin et al 1940, 1942) may be dismissed as of only historical interest since it involves a considerable purification of material and separation of the glucuronide and free glucuronic acid, or destruction of free glucuronic acid.

Reductimetric methods were the first used for the determination of glucuronidase activity. Methods were described by Masamune (1933, 1934) and Fishman (1938). Fishman (1939) also developed a cerimetric method and Levvy (1946) introduced a number of improvements, while Mills (1948) introduced the cerimetric method now in general use.

### Experimental

In order to combine the general usefulness of a reductimetric method with the convenience of a colorimetric method the following modification of the Folin and Wu blood sugar/

sugar method was developed.

Assay 1 ml buffer solution was incubated with 0.5 ml enzyme solution and 0.5 ml substrate solution at 37.5°C for a suitable time. The reaction was stopped by adding 0.5 ml 4/5 N sulphuric acid and 0.5 ml 6% sodium tungstate and protein removed by centrifuging.

#### Determination of reducing material

2 ml of the supernatant was pipetted into a 3/4" boiling-tube, 2 ml of Folin and Wu reagent A, added and the tube placed in a boiling water-bath for ten minutes. After cooling 2 ml reagent B, was added with mixing and the colour read after 5 minutes at a wavelength of 670 m $\mu$  (Ilford dark red filter, 608).

Control Tubes In reductimetric assay methods the controls employed are frequently inadequate. For reliable results it is necessary to employ the following controls.

- a) Substrate blank. Enzyme is placed by water.
- b) Tissue blank. Substrate is replaced by water.
- c) Reagent blank. Enzyme and substrate are replaced by water no incubation being required.

The disadvantages of reductimetric methods are non-specificity and the need for many controls. In general they are/

are only useful for assaying rather pure enzyme preparations acting on rather stable substrates. However, they have the advantage of being general methods, applicable to any substrate attacked by glucuronidase and hence they are particularly valuable in kinetic studies where one is generally working with purified enzyme preparations and a variety of substrates.

Two aglucuronometric methods have been developed. The first of these, that of Talalay, Fishman and Huggins (1946) is undoubtedly the most convenient and the most generally useful method for the determination of glucuronidase activity in tissue fluids and extracts, It is based on the colorimetric estimation of the pink colour of phenolphthalein in alkaline solution after the hydrolysis of phenolphthalein glucuronide. Experience with this method has shown that, in removing protein, precautions must be taken to prevent loss of phenolphthalein by adsorption on to the precipitated protein, which takes place very readily in acid solution.

In the second method Kerr, Graham and Levvy (1948) and Mills (1948) applied the King-Armstrong method for phosphatase to the assay of glucuronidase activity, using phenylglucuronide in place of phenyl phosphate. The method (Mills 1948) used in this/

this study was as follows.

1 ml buffer, 0.5 ml phenylglucuronide (0.02M) and 0.5 ml enzyme solution were incubated for 1 hour at 38°C. 2 ml of Folin-Ciocalteu reagent were added and protein centrifuged off. 3 ml of the supernatant were pipetted off and 3 ml, 10% sodium carbonate added. The reaction mixture was then incubated for 40 minutes at 38°C to complete colour development. 2 ml of water were added and the optical density of the blue solution measured in the Hilger Spekker absorptiometer with Ilford dark red filter, no. 608.

Units of activity. These have been chosen arbitrarily and the two units commonly in use are the following.

Phenolphthalein unit. That amount of enzyme which will release one  $\mu$ g. of phenolphthalein from phenolphthalein glucuronide in the course of one hour.

Phenyl unit. That amount of enzyme which will release one  $\mu$ g. of phenol from phenylglucuronide in one hour.

Fishman et al use pH 4.5 for their assays while Levvy and his co-workers use pH 5.2 in citrate buffer.

Preparation of Enzyme Solutions.

The Japanese workers Masamune (1934) and Oshima (1934,1936), were/



were the first to try to prepare purified glucuronidase solutions. Their method consisted of a preliminary autolysis of the tissue followed by alcohol precipitation. After redissolving in water, reprecipitating with alcohol and concentrating, the enzyme was adsorbed on to kaolin and eluted with phosphate. Fishman (1939) had difficulty in repeating this preparation, which is not surprising since glucuronidase is quite readily denatured by alcohol at moderate temperatures. He produced a simple method of preparation which involved an isoelectric precipitation of undesired protein at pH 5 followed by ammonium sulphate precipitation. Graham (1946) investigated Fishman's method further and introduced an acetone precipitation of the enzyme in the early stages as did also Mills (1948) who emphasised in addition the necessity for careful temperature control in the ammonium sulphate fractionation. There has been some doubt recently about the reliability of different methods of preparation and Kerr and Levvy (1951) have very recently reinvestigated factors involved in preparation of these enzyme solutions. They claim to have shown that glucuronidase is distributed between the mitochondria and cytoplasm of the cells and that on incubation with citrate buffer the amount of glucuronidase/

glucuronidase released by the mitochondria is small whereas on incubation with acetate buffer at pH 5 nearly all the glucuronidase activity appears in the supernatant in the course of four hours. The significance of these observations will be discussed elsewhere.

In the course of this investigation two general methods of preparation were employed.

Method 1 For large preparations of glucuronidase solutions for kinetic and preparative studies the method of Mills (1948) was used. The tissue was minced finely and treated with two volumes of acetone which was then filtered off. After a second acetone treatment, acetone was driven off from the powder and this was extracted with water, filtered or centrifuged off and the extract dialysed. Its pH was adjusted to 5 with acetic acid and it was incubated for 4 hours at 37°C and clarified in the Sharples supercentrifuge. Solid ammonium sulphate was added to give a final concentration of 60% and the resulting precipitate was separated by centrifugation, dissolved in water and dialysed.

Method 2 This modification of Fishman's method was used for smaller preparations where a quantitative yield of enzyme was desired.

desired.

The tissue was homogenised with sufficient water to form a 20% homogenate. To each 10 ml of homogenate was added 1 ml of molar acetate buffer of pH 5 and the material was incubated for 4 hours at 37°C. The precipitated material was removed by centrifugation and ammonium sulphate added to the supernatant to a concentration of 60%. After 18 hours in the refrigerator the precipitate was separated by centrifugation, redissolved, dialysed and the volume measured.

On occasions simple tissue homogenates were also employed, phenolphthalein glucuronide being used as the substrate in these cases.

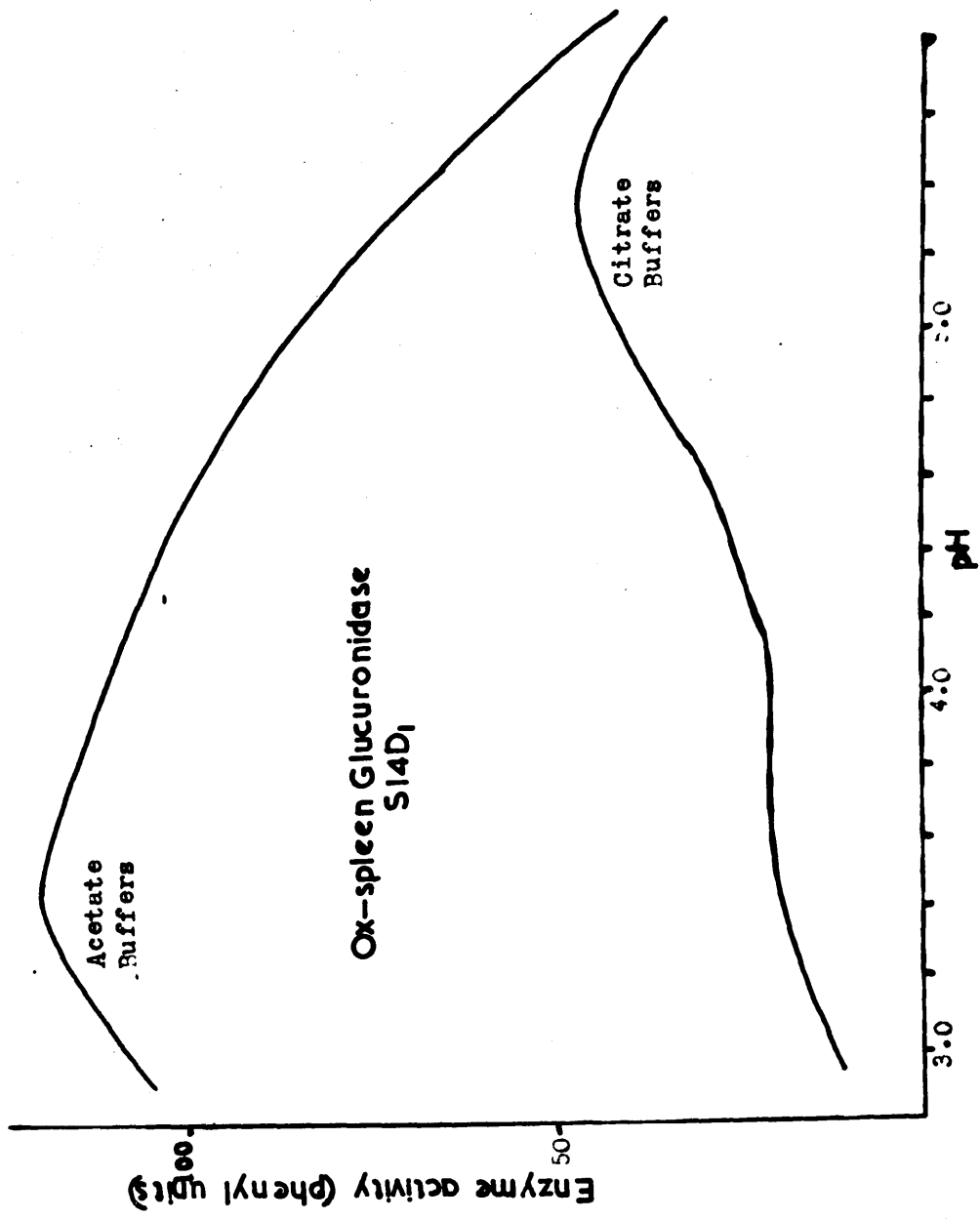
#### Multiplicity of the glucuronidases.

Original kinetic studies indicated pH optima for glucuronidase around pH 5-5.6 (Masamune, Oshima). Fishman (1940) using different substrates, later reported some pH optima in the same range and others around pH 4.4. Subsequent investigators (Mills 1948, Kerr et al, 1948) also reported two different optima, one about 5.2 and the other about 4.5. The reason for these discrepancies was revealed by Mills (1948) who, on the basis of salting-out studies (Falconer and Taylor, 1946) deduced that there were actually two glucuronidases, one with the higher/

higher pH optimum and one with the lower. By ammonium sulphate fractionation he claimed to have separated the two and this was subsequently confirmed by Levvy et al (1948).

Subsequently, in the course of investigating the effect of different buffers on pH optima the two curves shown in fig 4.1 were obtained and these suggested the presence of a further peak of activity at a still lower pH, the enzyme with activity at these levels being inhibited by citrate.

Attempts to separate the three enzymes were made and this proved to be a very difficult task. The methods initially employed were those originally used by Mills in separating glucuronidases 'I' and 'II'. These methods were not successful and, in fact, Mills own findings were not confirmed in attempts lasting over a year, so that there may exist a subtle factor in this separation which we have not yet discovered. In respect to the claims of Mills two points should be mentioned. In the first place the author was privileged to have the opportunity of carrying out pH activity curves on two preparations of Dr Mills which demonstrated two enzymes clearly. Secondly, it was later observed that if enzyme preparations were kept for some time or subjected to harsh methods all enzymatic activity tended to disappear except that at pH 5.2-5.3 and this would tend/



**Figure 4.1** Simultaneous pH Activity Curves Demonstrating:-  
 a) Low optimum pH.  
 b) Inhibition by citrate.

tend to suggest that this enzyme is more stable than the others. Hence it is easy to see that with an original preparation with a peak of activity at pH 4.5 it would be easy to produce the effect of apparent separation into two different enzymatic fractions and, in fact, this was later found to be a source of some confusion and doubt.

Attempts to separate the enzymes having proved only partially successful despite careful control of all conditions attempts were made to prove the existence of three separate proteins and to obtain more critical methods for their separation by other techniques.

In the first instance salting-out curves were constructed, employing the technique, based on the variable solvent solubility test of Falconer and Taylor, originally employed by Mills but employing a lower pH of assay for the enzyme fractions than that originally employed by Mills. As is seen in fig 4.2 these curves apparently indicated the presence of three different phases associated with glucuronidase activity. Now, from these curves one would imagine that, at the lower ammonium sulphate concentrations the phase coming out of solution should consist almost entirely of one enzyme. However, pH activity curves carried out on the precipitates at different ammonium sulphate/

Assay - Phenylglucuronide at pH 4.3 in Acetate

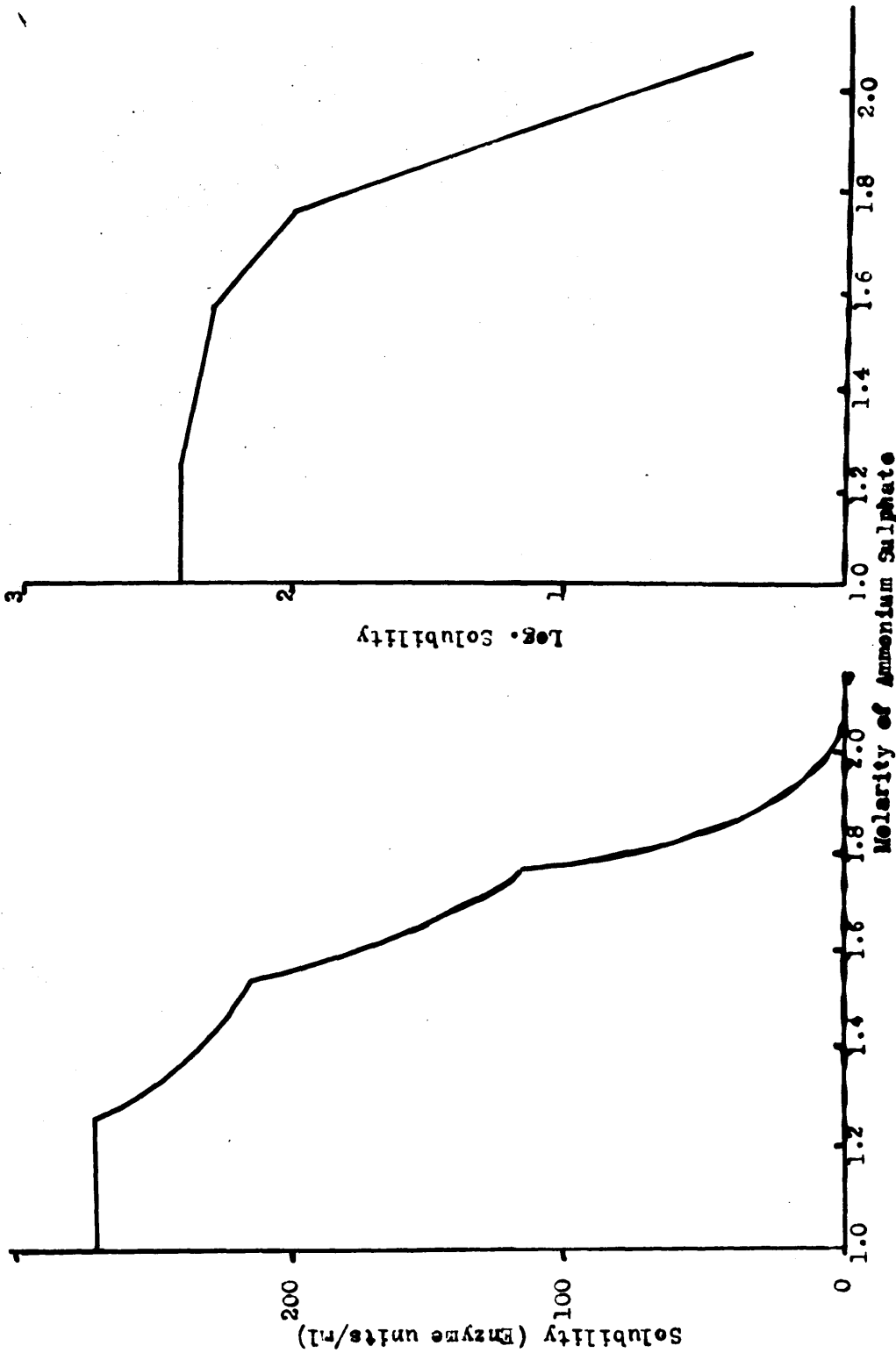


Figure 4.2 Variable Solvent Solubility Test at pH 5.0

sulphate concentrations in the course of the test revealed no significant change whatsoever in their shape. Moreover, it must be admitted that, where more than two phases are involved the variable solvent solubility curve tends to leave too much scope for subjective factors to influence the fitting of points. Against this, however, should be set the fact that the results have proved to be very reproducible, Dr Mills and the author independently achieving virtually the same results with different substrates. It is, of course, possible that the presence of other proteins of similar solubilities might affect the phase charges. Whatever the case may be it would seem that the test itself provides information of limited significance in this case.

Separation of the individual proteins was then tried by a chromatographic technique. The method was based on the concept of a salting-out chromatogram of a type similar to that suggested independently by Tiselius (1948). Of several techniques tried the most successful was as follows.

A spot of the protein solution was placed near the bottom of a 2-inch wide strip of filter-paper. The bottom of the paper was then allowed to touch the surface of some distilled water so that the protein spot was concentrated into a narrow band and pushed at the solvent front for about an inch up the paper. The distilled/



distilled water vessel was then removed and the strip was allowed to dip into a 2M solution of ammonium sulphate in its place. As the ammonium sulphate advanced up the paper in the wake of the water a gradient of ammonium sulphate concentrations was formed which salted out each protein as its critical concentration was reached. The presence of glucuronidase was then revealed by spraying the paper with a buffered solution of phenolphthalein glucuronide and incubating it for some time. On exposure to ammonia a pink spot appeared at the position of the enzyme. A photograph of one of these preparations is shown in fig 4.3. In this preparation the protein was developed by the method of Tursa and Ehenkel (1950) which is employed for demonstrating the situation of protein in the filter-paper electrophoresis technique. It may be seen from this photograph that ferritin and glucuronidase could be readily separated. As a rule no separation of the glucuronidase spot into more than one spot was achieved although on one or two occasions there did occur such an appearance. Since this was never regularly reproducible it may have been due to an artefact.

The method lacked definition and in an attempt to improve definition a column chromatogram on the same principle was employed

A/

Tecumseh, Mo. Cat 5.



Ferritin

A-Glucuronide (pH 4.5)

Figure 4.3

**CONTACT PRINT OF A PROTEIN CHROMATOGRAM OF A SPLEEN  
GLUCURONIDASE PREPARATION.**

In the original the ferritin appeared as a brown stain and glucuronidase was revealed by demonstrating the presence of phenolphthalein after incubation with phenolphthalein glucuronide.

A one-inch diameter starch column was prepared and equilibrated against saturated ammonium sulphate. On top of the column a six-inch space containing saturated ammonium sulphate was left and this was joined by means of a syphon to a reservoir of distilled water situated alongside at the same hydrostatic level. The protein to be analysed was precipitated at the top of the column. Then as the solvent flowed through the column distilled water was drawn through the syphon into the space on top of the column and mixed with the ammonium sulphate solution there, stirring being achieved by means of a current of air. In this way an ammonium sulphate gradient was achieved and, as the solution became more dilute, protein was gradually eluted. The effluent was collected in a fraction collector and the fractions were assayed a) for ferritin by direct colorimetry and b) for glucuronidase by assay with phenolphthalein glucuronide at three different pH levels (3.4, 4.5, 5.2). As may be seen from figure 4.4, some degree of separation of ferritin from glucuronidase again occurred but there was no separation of the glucuronidases. The degree of definition was still inadequate. In order to attempt to improve this a number of different adsorbents (calcium phosphate, diatomaceous earth, filter-paper powder) were tried but the results were not encouraging.

The/.

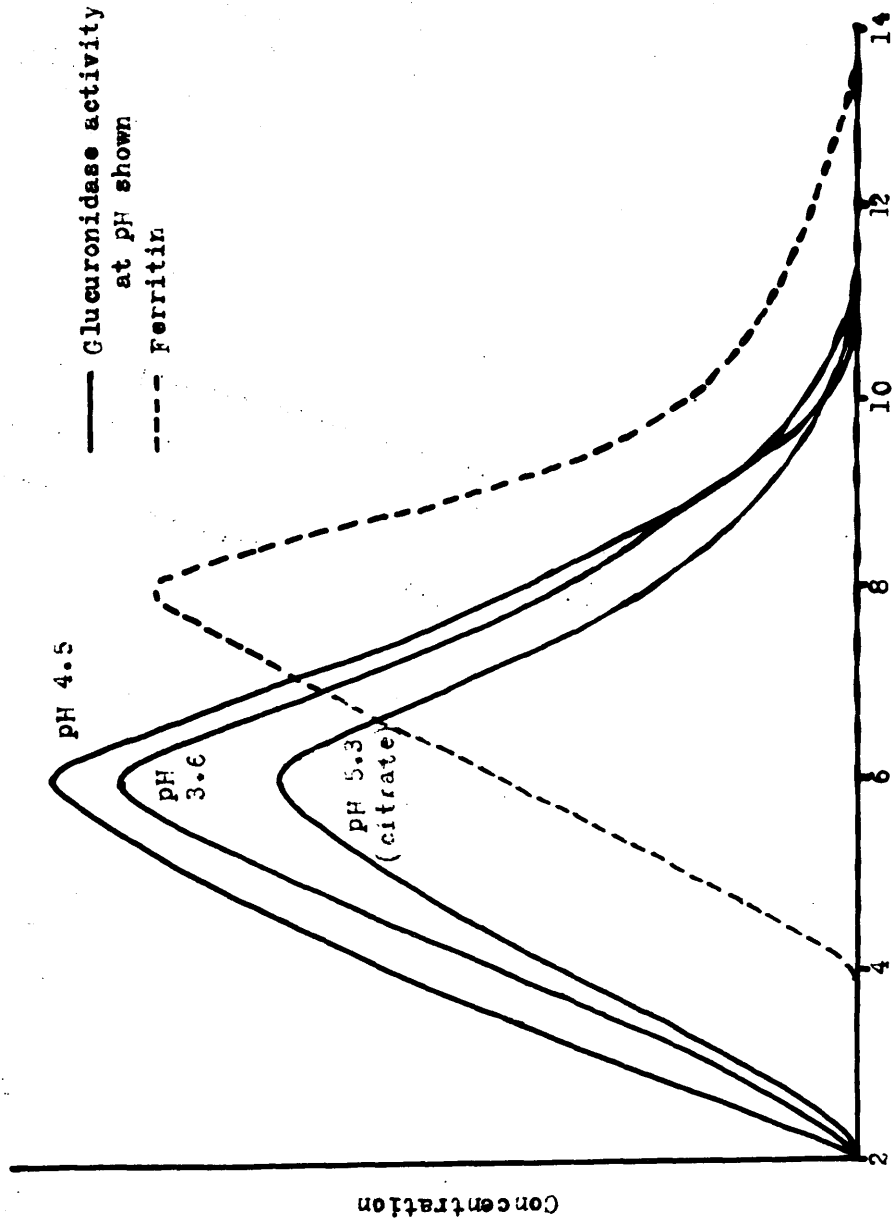


Figure 4.4 Protein Chromatogram of Crude Sp. aer. Glucuronidase.

The possibility of a stepwise elution rather than a continuous elution was now considered and the method of 'Fractional elution' was developed. The adsorbent chosen was Celite 519A and the technique was as follows. To 50 ml of a crude enzyme preparation were added 2 g of Celite and 50 ml of 4M ammonium sulphate solution. This was thoroughly mixed and allowed to equilibrate for thirty minutes. It was then filtered through a Buchner funnel with the minimum amount of suction. The precipitate was stirred up with about 20 ml of 2M ammonium sulphate and again filtered. This process was repeated. The combined filtrates comprised the 2M fraction. The precipitate was then stirred up with 20 ml of 1.8M ammonium sulphate solution, allowed to equilibrate half-an-hour, filtered and washed twice as before. The combined filtrates formed the 1.8 M fraction. This process was repeated with 1.6 M, 1.4 M, 1.2 M, 1.0M, 0.8M ammonium sulphate and water. Solid ammonium sulphate was then added to each fraction to achieve a concentration of 2.4 M (approx. 60%). The precipitate was redissolved in distilled water and made to a standard volume and then assayed. The results of such a fractionation are shown in fig. 4.5 and it will be observed that if the figures for enzymatic activity are considered cumulatively one obtains a picture/

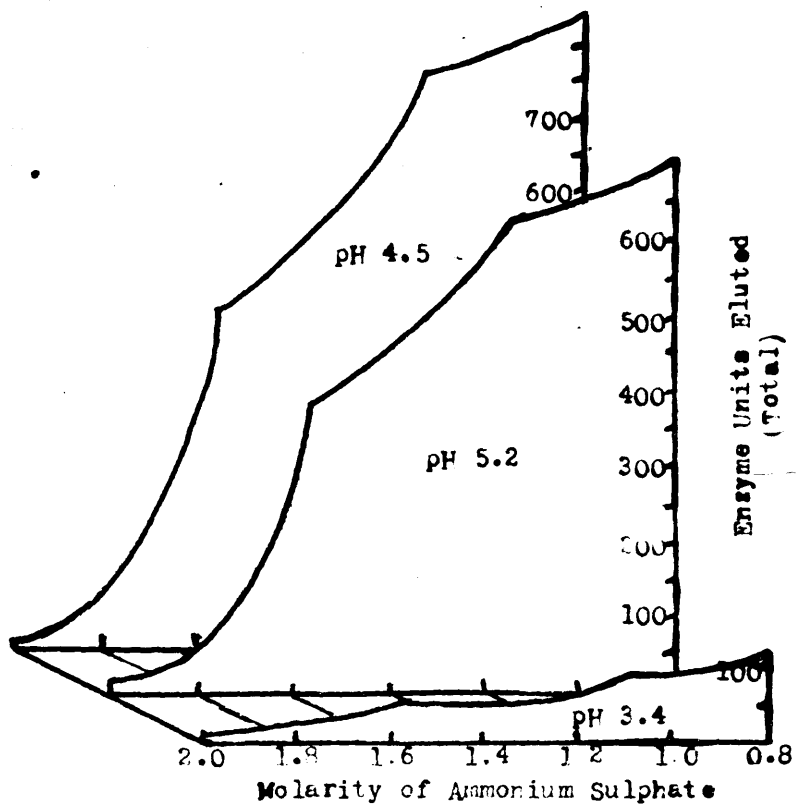


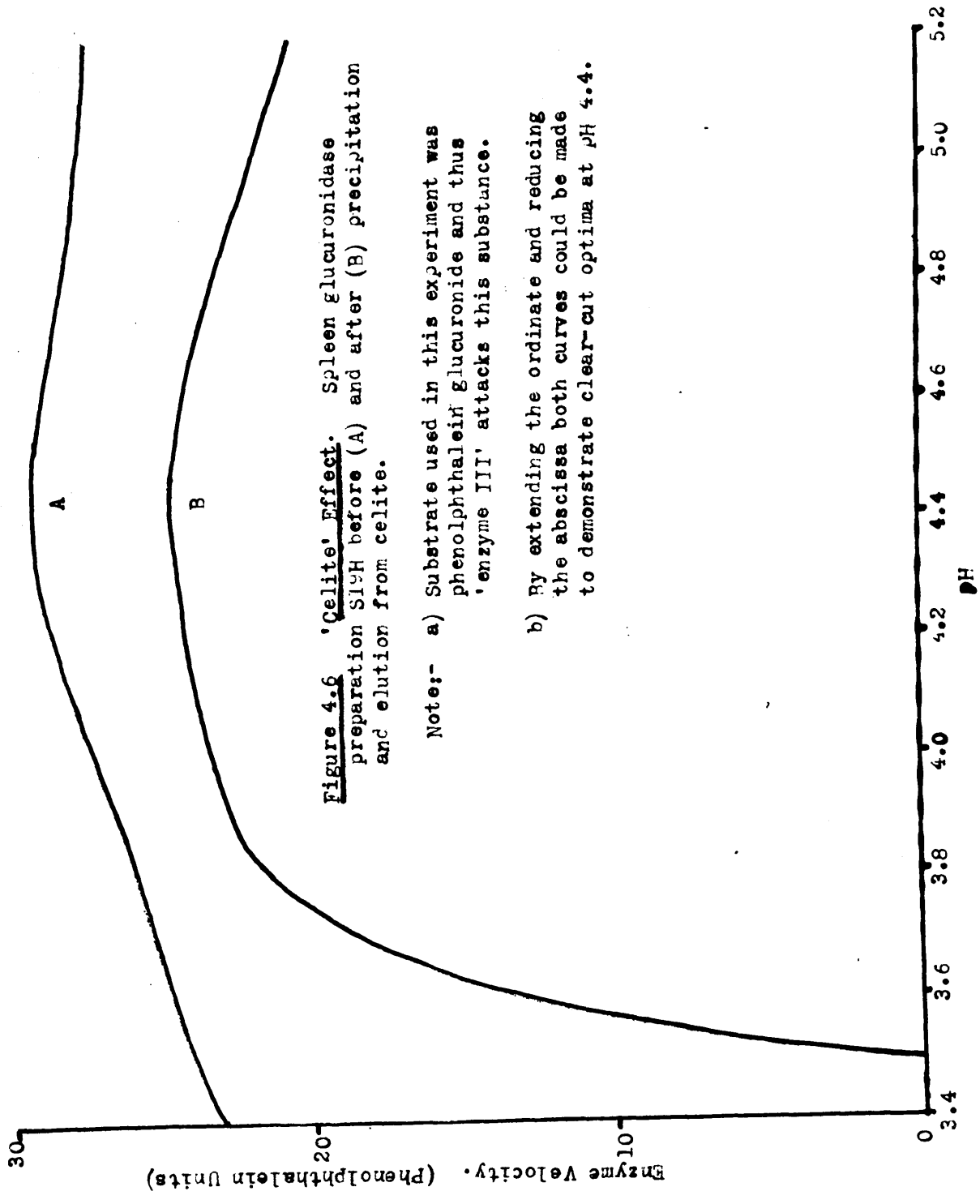
Figure 4.5. Composite Graph of a Fractional Elution Analysis.

- Note:-
- Phase changes as in the variable solvent solubility test.
  - No significant quantitative variations between phases at different pH's of assay.
  - Almost complete disappearance of activity at pH 3.4.

picture similar to a variable solvent solubility test curve. However, the relative amounts of enzyme at the three different pHs of assay remained relatively constant throughout this very exacting fractionation.

Superficially it appeared that these results were also disappointing but an interesting phenomenon soon became obvious. As shown in fig 4.1 high glucuronidase activity had been found at low pH values but after fractional elution it was found that this activity had disappeared and that activity fell to zero at pH 3.4. This dramatic effect is illustrated in fig. 4.6. These preparations had been assayed without dialysis due to a shortage of dialysis tubing at the time. Later it was observed (by Miss E.E.B. Smith) that dialysis reversed this inhibitory effect. Thus, although the method was only very partially, successful at the best of times in demonstrating separation of three enzymes it did provide good evidence for the independence of enzymatic activity at low pHs from that at higher pHs and also provided some possible indication of the reasons for other workers inability to demonstrate the existence of glucuronidase activity at lower pHs.

In further attempts to separate the three enzymes the method of alcohol-low temperature fractionation as employed by Cohn/



**Figure 4.6** 'Celite' Effect. Spleen glucuronidase preparation SIPH before (A) and after (B) precipitation and elution from celite.

Note:- a) Substrate used in this experiment was phenolphthalein glucuronide and thus 'enzyme III' attacks this substance.  
 b) By extending the ordinate and reducing the abscissa both curves could be made to demonstrate clear-cut optima at pH 4.4.



Cohn and his co-workers (Cohn et al, 1946) was tried. In conjunction with Dr Mills two experiments of this nature were performed and again no satisfactory separation was obtained. However, subsequently Dr Mills and Miss Smith have investigated this method at greater length and have achieved some separation, although unfortunately it is not yet clear to what extent this has been due to selective denaturation. These workers have also investigated the possibilities of electrophoretic separation of the enzymes, both by the orthodox method and by the method of filter-paper electrophoresis (Mills & Smith, 1951), and their results, though suggestive cannot yet be considered conclusive. An electrophoretic picture of a glucuronidase preparation is presented in fig 4.7 by courtesy of Dr Mills and Miss Smith. Enzymatic assay indicated that glucuronidase activity was only associated with the broad peak marked and the other peaks probably represent other proteins. Although different fractions of this peak did not show very different pH activity curves the breadth of the peak suggests that it does not represent a homogeneous protein.

#### Kinetic studies on glucuronidase.

Although it was not possible to obtain three distinctly separate proteins with distinctly different pH optima by design in/

Ox Spleen  $\beta$  Glucuronidase

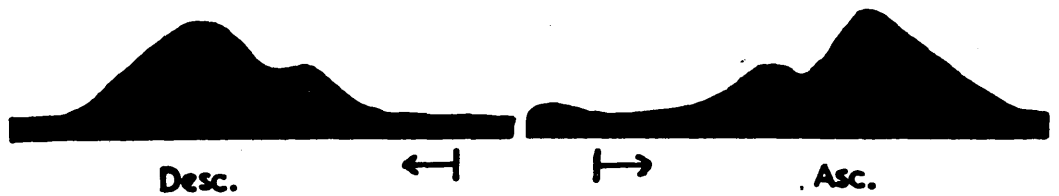


Figure 4.7

Anodic migration at pH 8.0 in phosphate buffer,  
ionic strength 0.20.

Main peak (88%) = Glucuronidase

Subsidiary peak = No activity

in the above experiments it was possible in the course of the study to obtain odd preparations in which one enzyme predominated to an overwhelming degree. Employing such preparations, which had been freed almost completely of extraneous protein, kinetic studies were made. Previously Mills (1948) is the only investigator who has attempted to distinguish between these different enzymes in kinetic studies. The original studies of Masamune and Oshima and those of Fishman (1940) and Kerr et al (1948) were made on preparations which probably contained mixtures of enzymes in unknown proportions. Moreover, those of Kerr et al and Karunairatnam and Levvy (1949) were conducted in citrate buffers and this, as will be shown, is an inhibitor of glucuronidase and indeed was described as such by Oshima (1936). Most of the findings now to be described have been published (Mills and Paul, 1949; Mills, Paul and Smith, 1949).

#### Effect of pH

On the purest preparations available the following optima for the enzymes 'I' and 'II' as originally described by Mills and enzyme 'III' (Mills and Paul) were obtained. (Table 4.1)

In addition, evidence has been found for the presence of a fourth glucuronidase with an optimum pH of about 6.5. This is/

Table 4.1

pH optima of  $\beta$ -glucuronidases

Substrate	I	II	III	IV
1-menthylglucuronide	4.5	5.0-5.1	-	-
phenylglucuronide	4.5	5.2-5.3	3.4	-
phenolphthalein glucuronide	4.5	5.2-5.3	3.4	ca. 6.5

is probably the enzyme which accounts for the glucuronidase activity of liver-slices in Krebs-Ringer buffers at pH 7.4 (see later) and may represent the actual enzyme responsible for glucuronidase activity in vivo.

#### Enzyme concentrations

The rate of hydrolysis of substrate by glucuronidase has been found to be directly proportional to the concentration of the enzyme (Florkin et al, 1942; Talalay et al, 1946) and we have made similar observations except that in the case of very strong enzyme preparations or impure preparations a deviation from linearity has been observed.

#### Substrate dissociation constants.

Relation of initial velocity of hydrolysis to substrate concentration has been studied by a number of workers (Fishman 1939; Kerr et al 1948; Talalay et al, 1946). It is dubious whether their figures are of any significance since the assays were carried out on different organs in a number of different species and even at different pHs. The substrate dissociation constants reported below were calculated from data obtained from preparations of ox-spleen glucuronidase of the type referred to earlier. In most cases the results are means of assays carried out on different preparations at different times and/

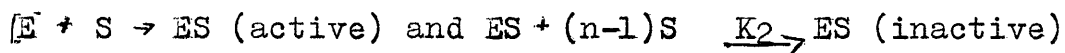
Table 4.2  
Enzyme-substrate dissociation constants (38°C)

Substrate	I	II	III	IV
l-menthylglucuronide	0.0047	0.019	-	-
phenylglucuronide	0.0020	0.0051	0.0012	-
phenolphthalein glucuronide	0.0008	0.0023	0.0010	0.0015 (⊖)

(⊖) - pH 7.4

and by different workers (Dr Mills and myself). There were no great variations between individual figures. (Table 4.2)

The object of the study was to demonstrate the different behaviour of the different enzymes. In these preparations there was little, if any, evidence of substrate inhibition using phenyl glucuronide as substrate but with phenolphthalein glucuronide, although there was little or no substrate inhibition of the enzyme 'IV', there was considerable evidence of inhibition in the case of the other three, in the order III > I > II.  $K_S$  values were determined according to the methods proposed by Lineweaver and Burke (1934) (see appendix). These authors also developed an equation to express substrate inhibition, assuming the equilibria



Analysis of the data by this means gave the following values.

Table 4.3

Enzyme	n	K2
III	3	0.0006
I	2	0.00055

At this point it may be mentioned that, due to the different values of these constants, it is possible in an unfractionated/

unfractionated glucuronidase preparation to obtain entirely different pH activity curves simply by varying the substrate concentration. Thus with low substrate concentrations enzyme III is particularly prominent whereas at high substrate concentrations the optimum pH is nearer pH 6. Values between these two may be obtained simply by selecting an appropriate substrate concentration. This is particularly true in the case of phenolphthalein glucuronide where the effect of substrate inhibition aggravates the changes. This fact probably explains a number of discrepancies which have occurred in such studies previously.

#### Activation energies.

Values for these are shown in table 4.4. It may be noted that there is a close agreement between the figures obtained with different substrates and that there are fairly marked differences for the three enzymes.

#### Inhibition Studies.

As early as 1936 Oshima demonstrated that malic, tartaric and citric acids inhibited glucuronidase activity. Karunairatnam and Levvy (1949) later described saccharic acid as an inhibitor and it is the most powerful specific inhibitor for glucuronidase yet found. Becker and Friedenwald (1949) described inhibition of glucuronidase/



Table 4.4

Activation energies for -glucuronidase

Substrate	I	II	III
Phenylglucuronide	14,700	19,300	16,900
Phenolphthalein glucuronide	14,400	18,900	16,200

glucuronidase by heparin and ascorbic acid and Fishman, Altman and Springer (1948) described an inhibitor in serum. In order to attempt a correlation of these disconnected observations the following study was undertaken.

Carboxylic acids.

Reference has already been made to inhibition by citrate. This particular form of inhibition interested us originally because it inhibited enzymes I and III but not enzyme II. In view of this observation and those made by others it was considered advisable to investigate a large series of carboxylic acids and their effects on glucuronidase and to investigate more fully the kinetics of action of some of these. In table 4.5 are shown the effect of a series on enzyme III. It is interesting to note that gluconate and galacturonate are not such powerful inhibitors as mucate and consequently it would seem that although similarity of the molecule is of some importance, much stronger inhibition is obtained in the presence of two carboxyl groups. Since all carboxylic acids tried have an inhibitor effect it would suggest that possibly the carboxyl group of glucuronic acid might represent its point of attachment to the active grouping of the enzyme.

That the three enzymes vary considerably in their sensitivity/



Table 4.6

% inhibition by saccharate  
(Phenylglucuronide 0.005 M)

Conc. M	Glucuronidase		
	I	II	III
$10^{-3}$	100	100	100
$2 \times 10^{-4}$	100	70	100
$10^{-4}$	60	20	100
$5 \times 10^{-5}$	20	0	90
$10^{-5}$	0	0	25

sensitivity to saccharate is demonstrated in table 4.6.

More intimate studies concerning the nature of the inhibition were carried out using mucate, citrate, oxalate and saccharate, the method of Lineweaver and Burke (1934) (appendix) being used to analyse the results. The findings are shown in table 4.7 and some of the data from which the conclusions were reached are shown in figs 4.8 and 4.9.

From these figures it was also possible to calculate the enzyme-inhibitor dissociation constants for those inhibitors which behaved competitively (Table 4.8).

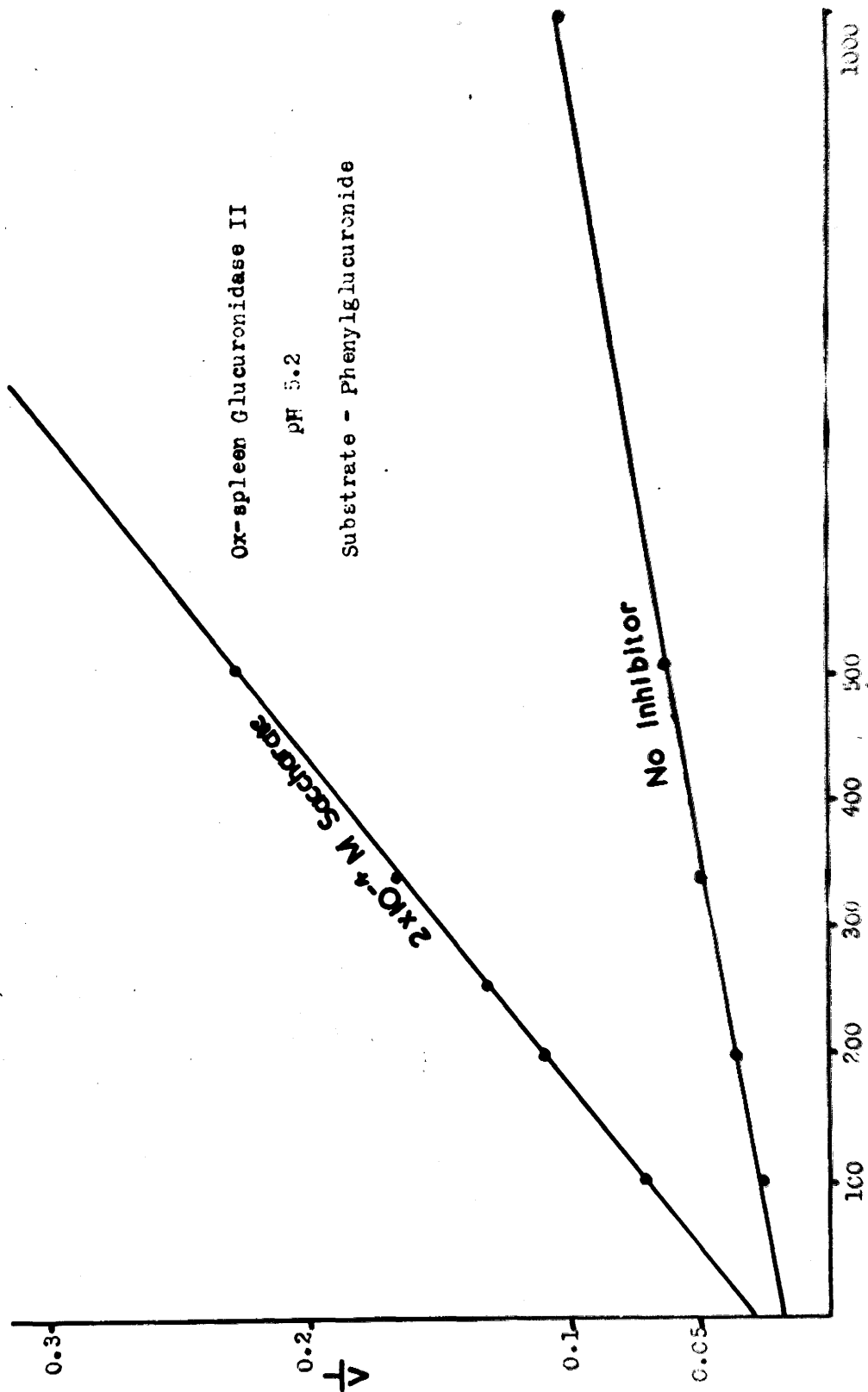
#### Sulphonic Acid Inhibitors.

Wills and Wormall (1949) showed that suramin would inhibit certain enzymes on the acid side of their isoelectric points but not on the alkaline side except in a few cases where inhibition occurred at neutral pHs. As demonstrated in fig. 4.10 this also applies to glucuronidase. In the particular experiment illustrated suramin was added to tubes containing solutions of glucuronidase and buffer at the different pHs indicated. In one set of tubes the pH was then raised to pH5.2 after five minutes, substrate was added and the glucuronidase activity was assayed at this pH. The second set of tubes was treated/

Table 4.7

Type of inhibition exercised by various inhibitors.

Inhibitor	Glucuronidase		
	I	II	III
Citrate	Competitive	No inhibition	Competitive
Oxalate	Very slight	No inhibition	Competitive
Mucate	Competitive	Non-competitive	Competitive
Saccharate	Competitive	Non-competitive	Competitive



[5]  
Figure 4.8

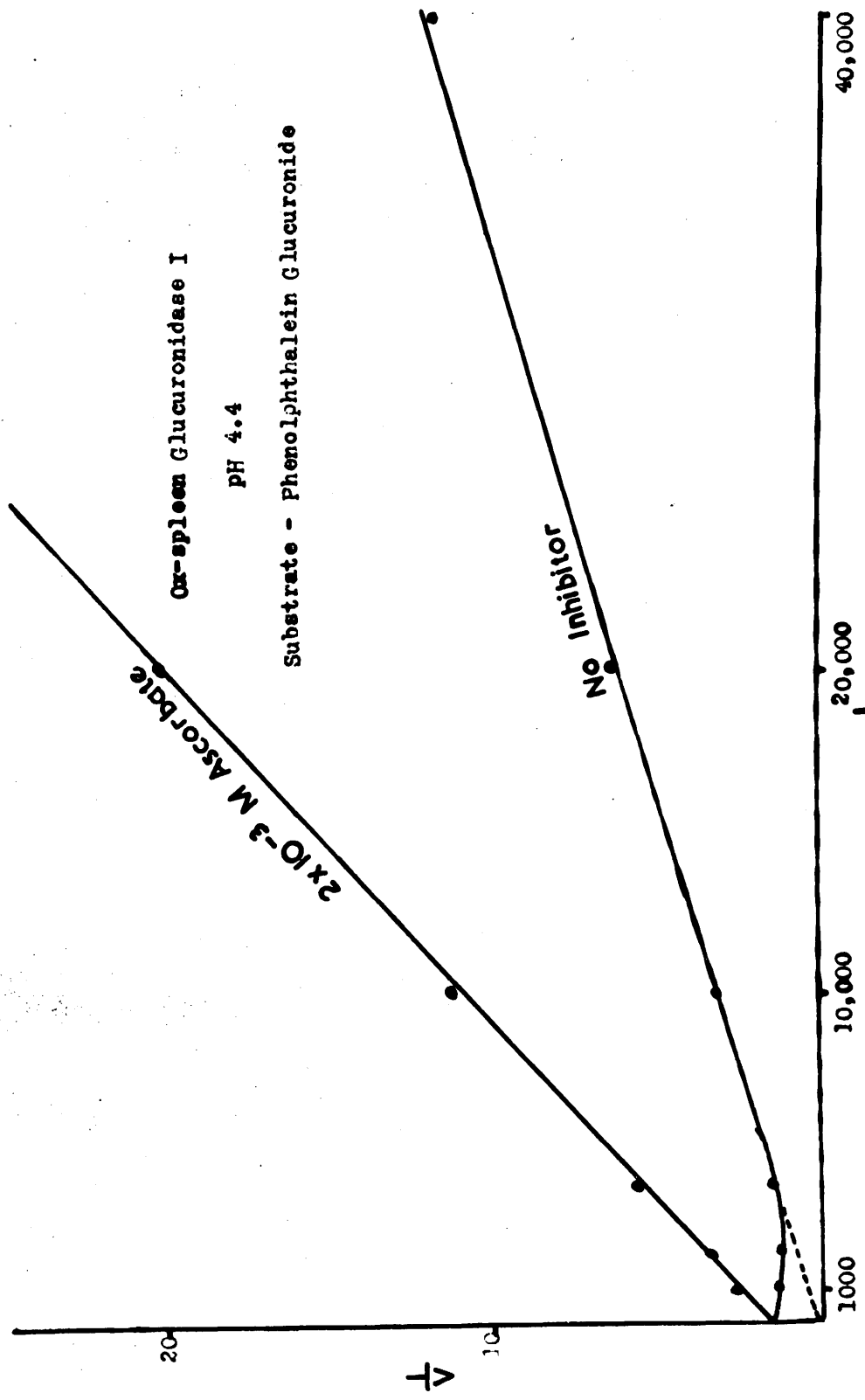


Figure 4.9



Table 4.8

Enzyme-inhibitor dissociation constants (38°C) for competitive inhibitors.

Inhibitor	Glucuronidase	
	I	III
Citrate	0.123	0.072
Oxalate		0.074
Mucate	0.048	0.00041
Saccharate	0.020	0.000057

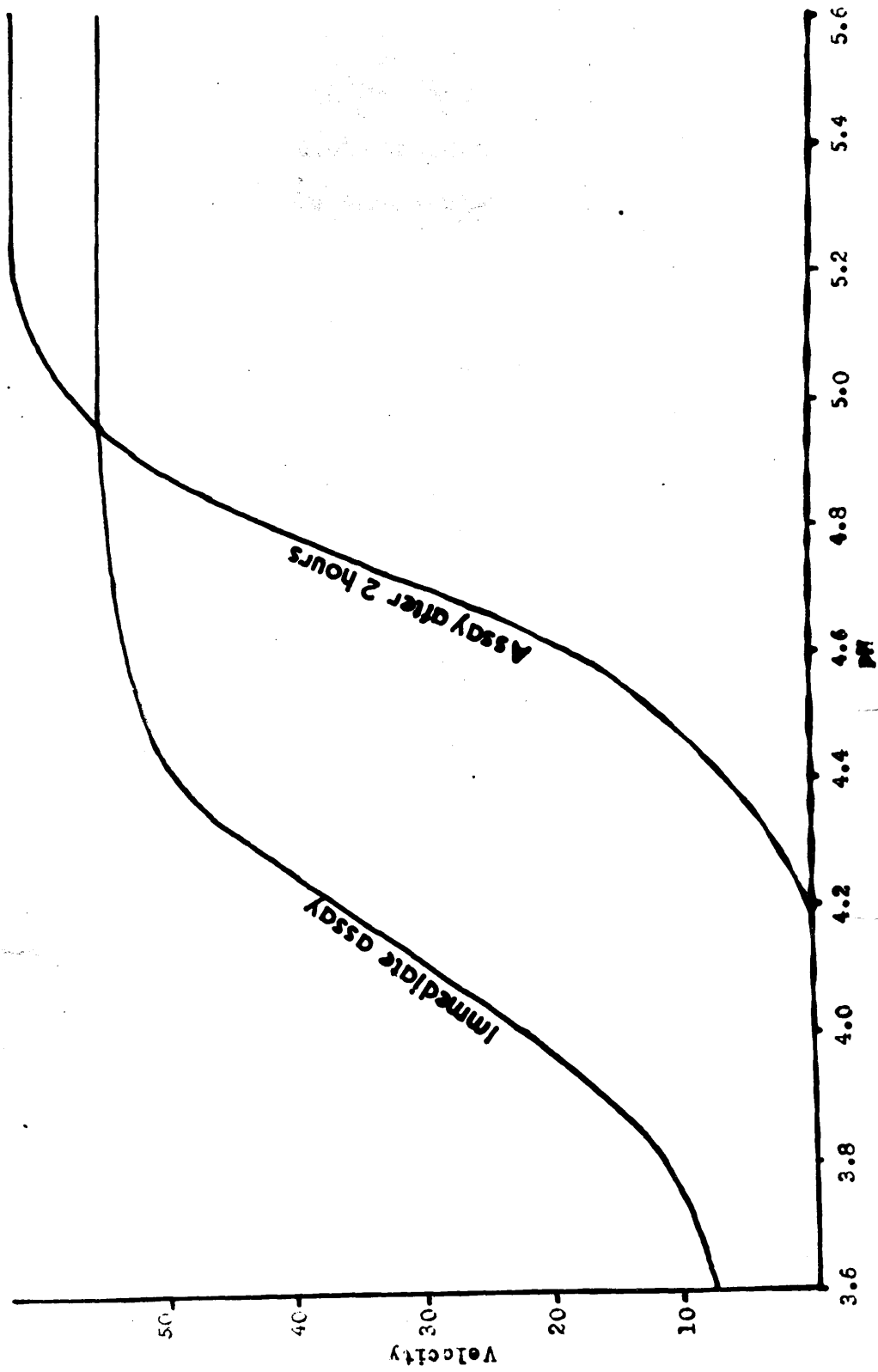


Figure 4.10 Inhibition by Suredin.

treated similarly but the pH was not raised until some hours had elapsed. The experiment demonstrated that a) the combination between suramin and protein was irreversible on raising the pH and 2) it required an appreciable time for suramin and enzyme to react and thus unreliable results would be obtained unless time was allowed for the reaction to proceed to completion. On the hypothesis of Wills et al glucuronidase would have an isoelectric point of about pH 4.7 (50% inhibition). Becker and Friedenwald (1949) had found that heparin inhibited glucuronidase to about 50% at pH 4.5 and that an increasing amount of heparin did not increase the degree of inhibition. Similarities of structure between suramin and heparin suggested that heparin might behave in the same way as suramin in virtue of its sulphonic acid groupings. As demonstrated in fig. 4.11, this proved indeed to be the case. There was one difference inasmuch as heparin still exerted a considerable inhibitory effect at pH 5.3 and this is possibly due to the additional presence in the molecule of carboxyl groups of glucuronic acid.

#### Ascorbic acid

Becker and Friedenwald (1949) also described this substance as an inhibitor of glucuronic acid and it is described separately/

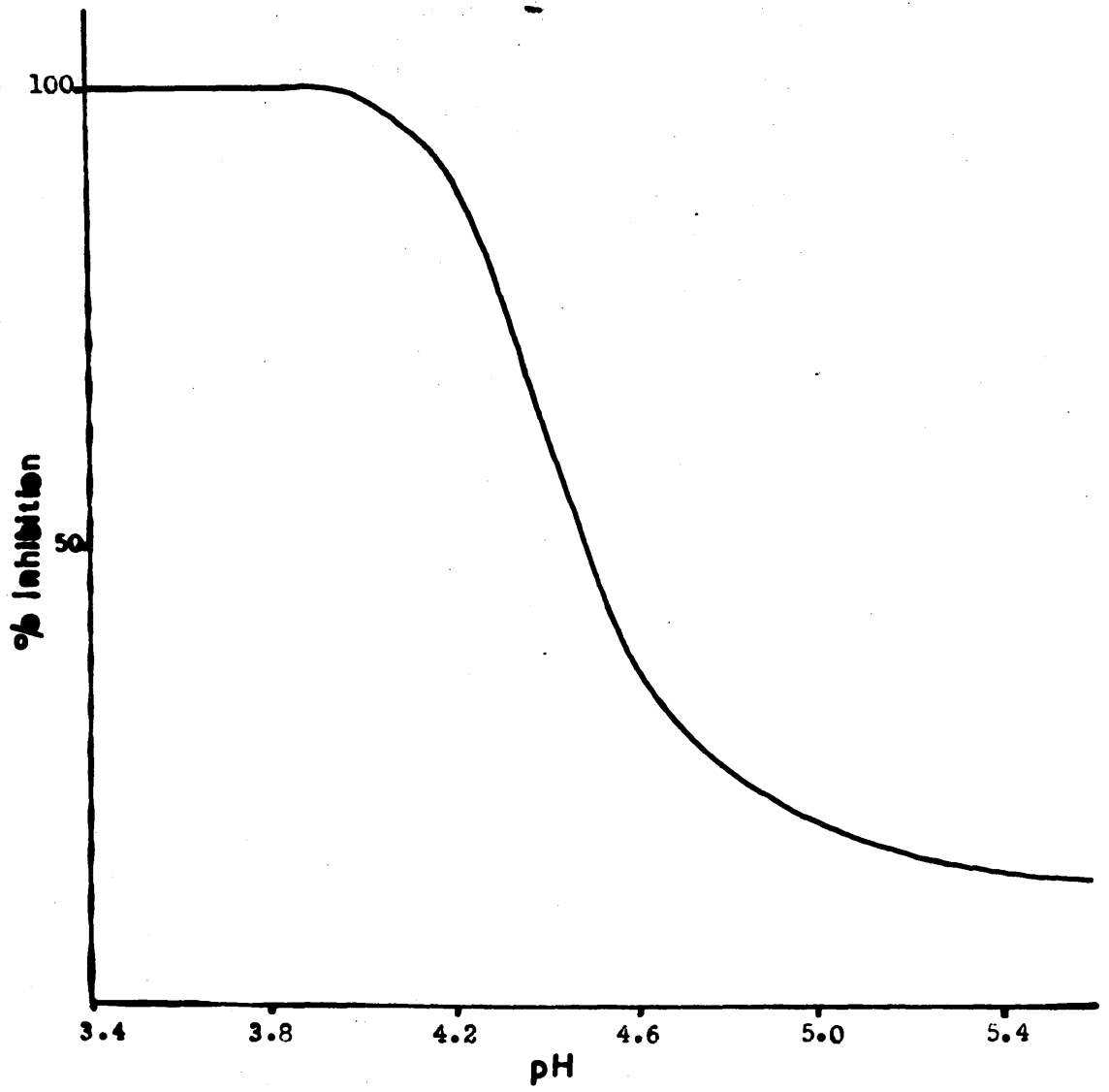


Figure 4.11      Inhibition by Heparin.

separately since it does not fit into either of the above groups. Ascorbic acid proved to be a non-competitive inhibitor for enzymes I and III and hence differed from the carboxylic acids which were competitive inhibitors for these two enzymes.

Oxalic acid.

This substance is also described separately for, although not a powerful inhibitor, it displays a peculiar phenomenon in the presence of substrate inhibition of enzyme I due to phenolphthalein glucuronide. As is shown in the velocity curve in fig 4.12 while the enzyme is inhibited by oxalic acid to a slight degree in the ascending part of the curve, the oxalic acid curve crosses over the normal curve when substrate inhibition commences and in the descending part oxalic acid is actually working as an activator.

The serum inhibitor.

Friedenwald and Becker had suggested that the serum inhibitor of Fishman et al, and heparin might be identical, and obviously many of the above substances might also be related to it. However, pH activity curves in the presence and absence of serum indicate that it is quite distinct from any of those described since inhibition by serum is almost uniform throughout the entire pH range (fig. 4.13). Another interesting and characteristic/

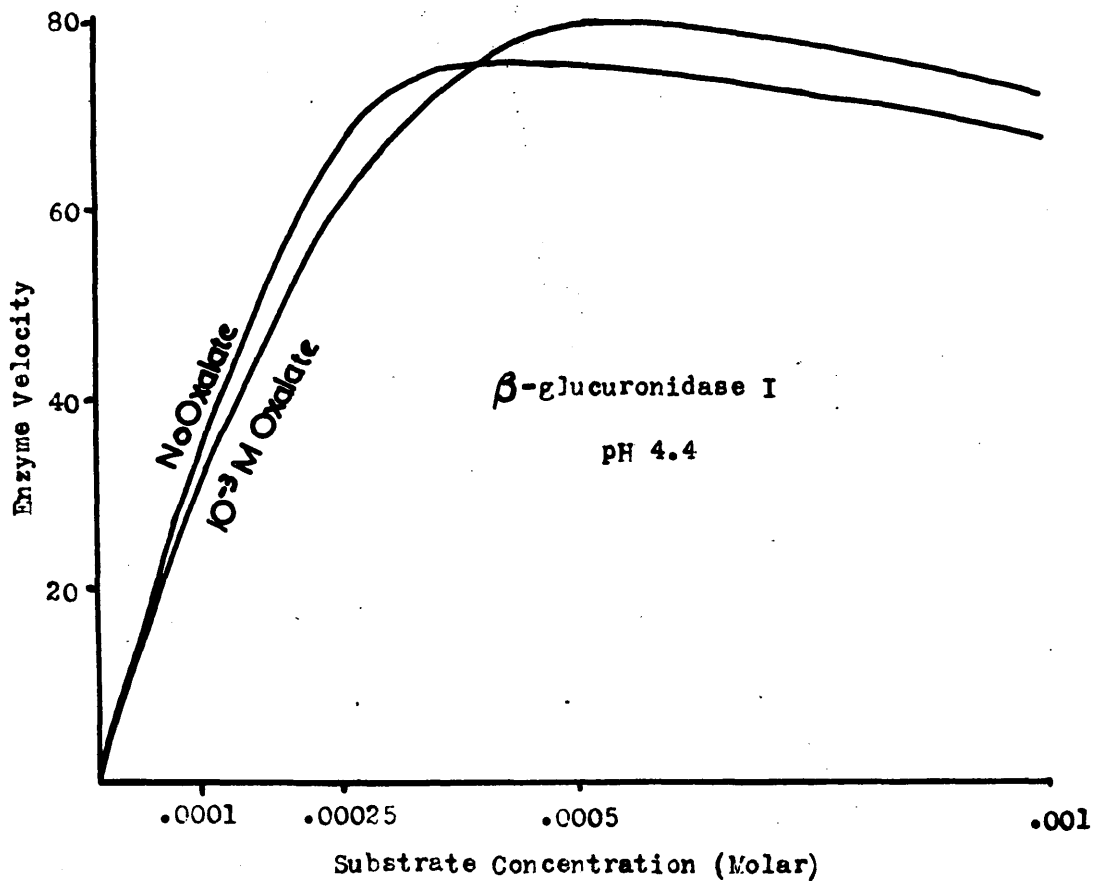


Figure 4.12 Effect of Oxalate in the Presence of Substrate Inhibition.  
(Substrate - Phenolphthalein glucuronide)

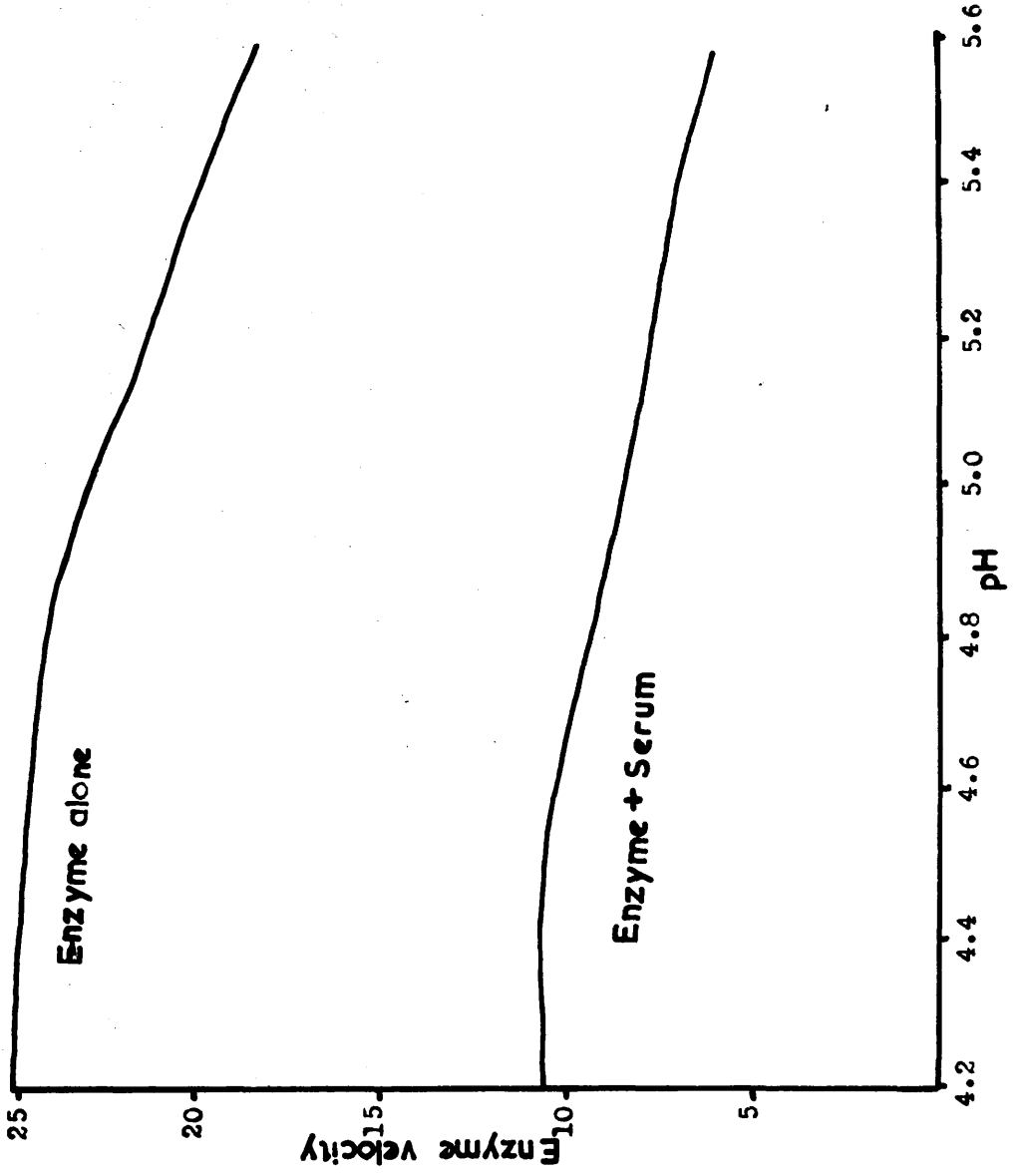


Figure 4.13 Inhibition by Serum.

characteristic feature of this inhibitor revealed by some later studies was that whereas inhibition of a given preparation of glucuronidase was remarkably constant for serum from widely different sources yet there was an enormous variation in the degree to which serum would inhibit different glucuronidase preparations. Thus it would seem that inhibition of glucuronidase by serum depends to some extent on the physical state of the enzyme itself.

Glucuronidase activity of liver slices.

Although many theories had been advanced regarding the possible function of glucuronidase in vivo it had never been satisfactorily demonstrated to have any function at what are generally considered to be physiological pH levels and hence the following experiments were performed.

Phenolphthalein glucuronide was used as substrate, the method of Talalay et al (1946) being used with some slight modifications. Tissue slices were cut, weighed and put into flasks containing Krebs-Ringer-Phosphate saline at pH 7.4 to which had been added phenolphthalein glucuronide in the appropriate concentration. At the end of the experiment the slices were removed and phenolphthalein estimated in the same fashion as before.

Table/



Table 4.9 shows that quite considerably hydrolysis did occur in these circumstances and that hydrolysis was inhibited by saccharate but not by heparin or suramin. It was not significantly increased by the addition of fluoride to inhibit synthesis and was not significantly decreased by citrate. The velocity curve showed no substrate inhibition (fig 4.14).

A homogenate of the same rat's liver showed a similar degree of activity at this pH, the enzyme exhibiting similar properties. Thus the intact cell was not necessary for glucuronidase activity at this pH and the most likely explanation of the activity at this level is the presence of an enzyme with a higher optimum pH than those previously described by Mills (1948) and Mills & Paul (1949).

Table 4.9

Hydrolysis of phenolphthalein glucuronide (0.002 M)  
by surviving rat-liver slices at pH 7.4

Effect of glucuronidase inhibitors etc.

Expt.	Ringer-phosphate saline containing phenolphthalein glucuronide and	$\mu\text{g}$ phenolphthalein liberated/g. tissue/hr.	Effect on hydrolysis
1.	No addition Saccharate 0.01 M Heparin (5 $\mu\text{g}/\text{ml}$ )	468 0 590	% -100 +26
2.	No addition Fluoride 0.01 M Saccharate 0.005 M	474 512 137	+8 -71
3.	No addition Suramin 0.0025 M	539 591	+10

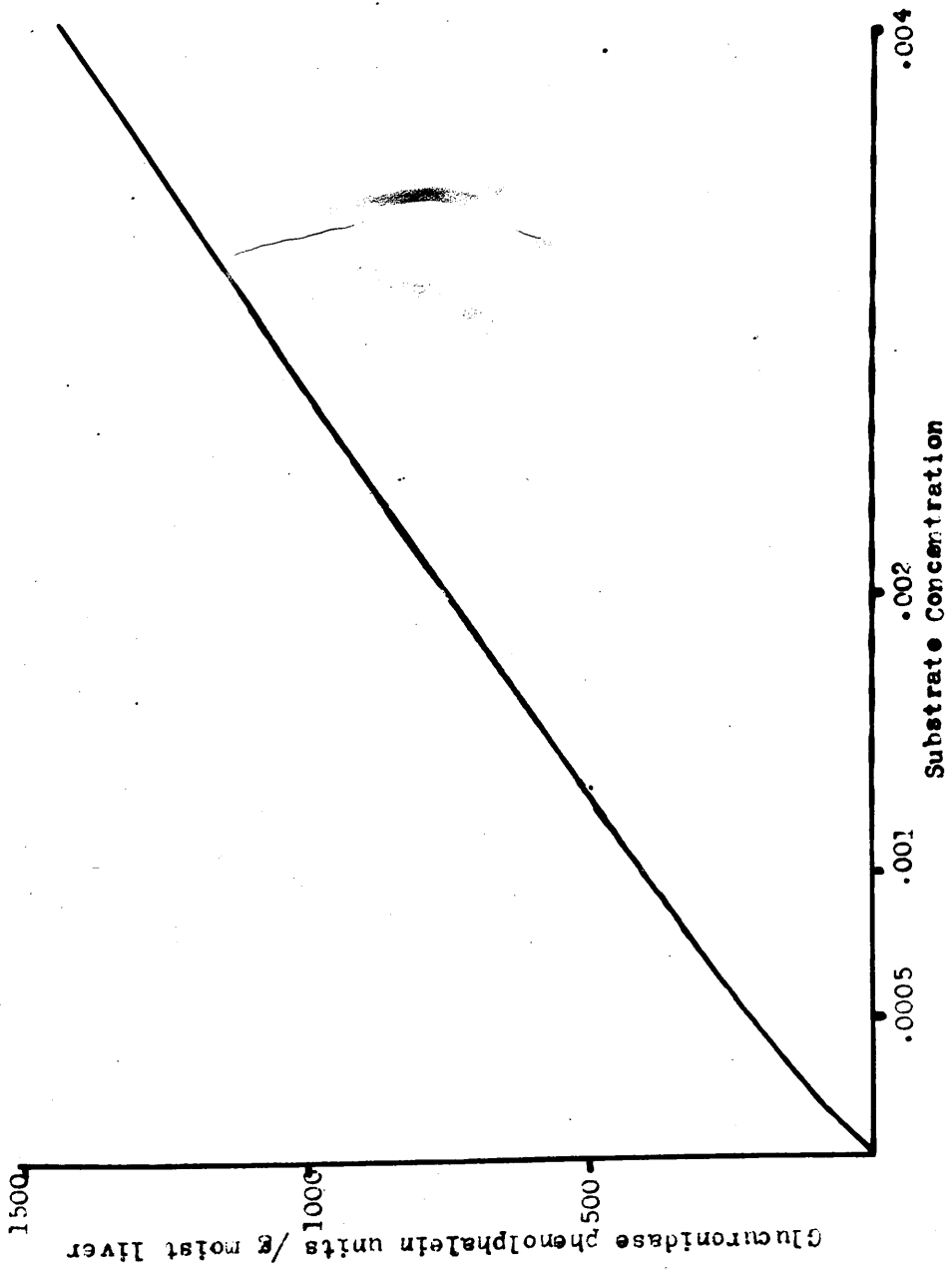


Figure 4.14 Hydrolysis of Phenolphthalein Glucuronide by Surviving Liver Slices.

The Function of  $\beta$ -glucuronidase.

In this field at the moment there is a considerable measure of disagreement, the reasons for which it is proposed to discuss later.

Whether glucuronidase is concerned with glucuronide conjugation is, of course, a matter of fundamental importance and most of the work in connection with this aspect of the subject has already been reviewed. Most recent work has been concerned with other possible relationships and there are two main schools of thought. Fishman (1940) originally claimed to have demonstrated increases in glucuronidase activity in a number of organs after feeding menthol and later Fishman and Fishman (1944) reported similar increases in response to oestrogens. A number of Fishman's colleagues, notably Odell et al (McDonald and Odell, 1947; Odell and McDonald, 1948; Odell and Fishman, 1950) pursued this aspect and claimed to have established a close relationship between glucuronidase activity and oestrogen action. Kerr and Levvy (1948) verified some of Fishman's findings while Levvy, Kerr and Campbell (1948) reported increased glucuronidase activity in the livers of mice regenerating after hepatectomy and also reported that the glucuronidase activity of the livers of young mice was higher than that of adults. They interpreted their results in quite a different/

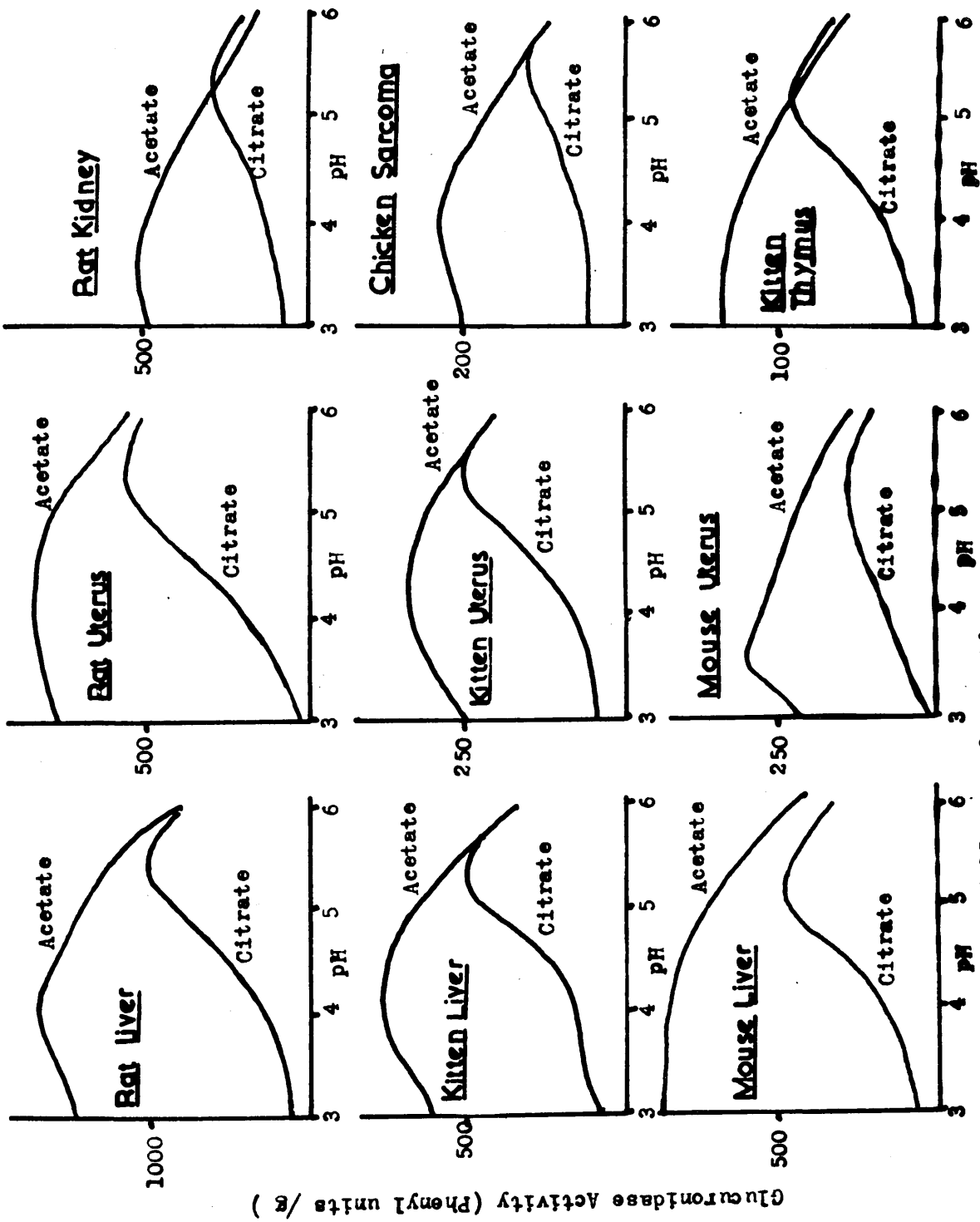
different way from Fishman, considering that their experiments indicated a close relationship between glucuronidase activity and cell proliferation. Recently Fishman and Anlyan (1947, 1947a, 1947b, 1947c ) have reported increased glucuronidase activity in cancer tissue, a finding which they interpret according to their own theories while Levvy et al consider it to support their theory. It will be necessary to comment on these findings later. Our own studies have not permitted us to agree with the theory postulated by Levvy et al although some of their findings have been confirmed.

Distribution of glucuronidase.

Practically every possible animal tissue has been examined for glucuronidase activity and to date a tissue has not been found which is totally devoid of it (Oshima, 1934; Fishman, 1940; Talalay, Fishman and Huggins, 1946; Mills, 1946; Rossiter and Wong, 1950). In view of our demonstration of the existence of at least three active enzyme groupings it was considered advisable to investigate a number of tissues for these, particularly in view of the claim of Levvy and Kerr (1948) that there was only one enzyme in mouse uterus. It has been observed that, after treating enzyme preparations with Celite in the presence of high salt/

salt concentrations, there remained no activity below pH 3.4 and hence activity below this level was taken to indicate the presence of glucuronidase 'III'. Secondly, at the substrate concentrations used by us, both enzymes 'I' and 'III' were inhibited by citrate buffer and consequently a peak of activity at pH 5.2-5.3 in this buffer was held to indicate the presence of enzyme 'II', while a normal peak of activity in the region pH 4.2-4.5 was considered to indicate the presence of enzyme 'II' as it was not possible to conceive of a summation effect of enzymes 'I' and 'III' which would account for such a peak (the shape of their pH activity curves being known from previous studies). By these criteria in every tissue examined there was evidence for the existence of all three enzymes. Some of the pH activity curves obtained are illustrated in fig 4.15. The following organs have been examined:- rat liver, rat kidney, rat spleen, rat uterus, cat liver, cat uterus, mouse liver, mouse uterus, rabbit liver, human serum, kitten thymus, ox spleen, ox liver, and a chemically induced chicken sarcoma.

It may be observed in passing that a clean pH activity curve with an optimum at pH 5.2-5.3 is obtained in citrate buffer whereas in most cases with phenylglucuronide as substrate a broad pH optimum between pH 4.0 and 4.5 in acetate buffer is found/



**Figure 4.18** Glucuronidase Activity in Various Organs.

found. Some authors have been guilty of distorting the pH activity curves by extending the ordinate and shortening the abscissa and it is therefore easy to understand how, in these cases, there appears to be a single enzyme present.

Species differences.

By reference to fig 4.15 it may also be observed that, while there is little qualitative difference in the glucuronidase picture from different species there are fairly marked quantitative differences. These quantitative differences are not confined to different species because we find equally marked differences between different varieties of rats. In the following experiments two varieties were used, hooded rats and albino rats. For hooded rats the average liver glucuronidase activity expressed in phenyl units was found to be 1913 ( $\pm$  574) phenyl units /g. whereas in albino rats the figures were 3110 ( $\pm$  665) phenyl units /g. A more fundamental difference than this, however, is that which apparently exists in the response of the organism to various forms of interference and, in particular, there exists the marked difference between the results obtained by Levvy et al (1948) on growing mice, verified by us, and the results which we obtained on growing rats. These will/



will now be discussed.

Variations in glucuronidase activity during growth.

In order to try to verify the results of Levvy et al (1948) the liver glucuronidase activity of a large series of rats at all ages was investigated. The rats used in this experiment were hooded rats of the same strain. The enzyme was prepared by method 2 and assays were performed with phenylglucuronide according to the method already described. The experimental results are displayed in table 4.10.

It may again be observed that there was no significant alteration in the shape of the pH activity curves in the course of the experiments. Parallel with these assays Miss Smith performed assays using phenolphthalein glucuronide as substrate and obtained essentially identical results. Subsequently, another investigation was undertaken (Mills, Smith, Stary and Leslie, 1950) in which the same findings were obtained. There is obviously no measure of agreement between these findings and the results reported by Levvy et al (1948) in which the glucuronidase content of the liver was found to be highest at birth. In order to exclude differences due to different methods some infant mice and adult mice were killed and their livers assayed similarly. Table 4.11. shows that the results were, on this/

Table 4.10  
Liver glucuronidase activity during growth.  
(Phenyl units/g. of moist tissue)

Age days	Liver wt. (g)	pH Acetate buffers											Citrate pH 5.3
		3.0	3.6	3.8	4.0	4.2	4.4	4.6	4.8	5.2	5.4		
1	0.25	1000	975	986	1114	1152	1225	1175	1125	938	848	788	
2	0.28	882	740	736	744	744	759	740	702	686	605	510	
3	0.252	-	1343	-	1920	-	2277	-	-	1520	-	1085	
5	0.290	722	675	750	827	885	978	1048	1011	955	855	862	
10	0.430	1125	1236	1216	1213	1254	1298	1254	1216	1004	954	874	
15	0.690	-	356	345	356	348	345	345	329	208	281	250	
16	0.960	1851	1916	1981	1997	2030	2045	1994	1933	1580	1412	1300	
17	0.900	2035	2067	2170	-	2235	-	2170	2100	1702	1548	1514	
20	1.240	2017	1960	2160	2390	2475	2635	2617	2645	2246	2103	1832	
25	2.000	1417	1506	1527	1548	1540	-	1444	1382	1131	1026	994	
31	3.300	-	1761	1845	1948	2015	1998	1982	1874	1597	1439	1128	
35	3.690	839	-	812	-	-	1046	-	-	1004	-	-	
41	3.590	2751	3037	3150	3185	3333	3295	3185	3037	2595	2410	2020	
52	6.610	1243	1281	1394	1351	1507	1500	1432	1356	-	1017	923	
75	8.200	2016	2226	2440	2382	2420	2363	2305	2212	1886	1735	1392	
125	10.50	1586	1564	1640	1736	1754	1677	1620	1582	1392	1240	1052	
150	13.55	1461	1480	-	1642	-	-	1642	-	1350	-	1035	

In most cases each set of figures represents the mean obtained from a batch of four or five animals.

Table 4.11

Mouse-liver glucuronidase activity in young and adult mice.

Preparation	Phenyl units/g. moist tissue	
	Acetate buffer pH 4.4	Citrate buffer pH 5.3
5-day A (5 mice)	1235	749
5-day B (5 mice)	1136	713
Adult 1 (male)	544	312
" 2 ( " )	488	259
" 3 ( " )	585	338
" 4 (female)	730	365
" 5 ( " )	860	504
" 6 ( " )	680	382

this occasion, in agreement with those of Levvy et al (1948).

In considering relationships between cell proliferation and other factors it is necessary to plot the experimental figures in relation to the specific growth rate of the tissues (appendix) It may be seen from fig 4.16 that there is no relationship between this curve and the curve of glucuronidase activity of the liver. The relationship between glucuronidase activity and body weight was then examined by the allometric method of Huxley (1924) (see appendix), which excludes the necessity for considering the time factor. As seen in fig 4.17 when the logarithms of both variables were plotted against each other the result was a straight line whose slope was significantly greater than 1. The latter finding would indicate that, in the case of the hooded rat, the results are to be explained on an increasingly important role for glucuronidase as the liver approaches adult size, a finding not in any way in accord with the theory of Levvy et al.

#### Partial Hepatectomy experiments

Two hepatectomy experiments were performed.

In the first experiment male hooded rats of about 250 g. were employed. About two-thirds of the liver was removed at hepatectomy/

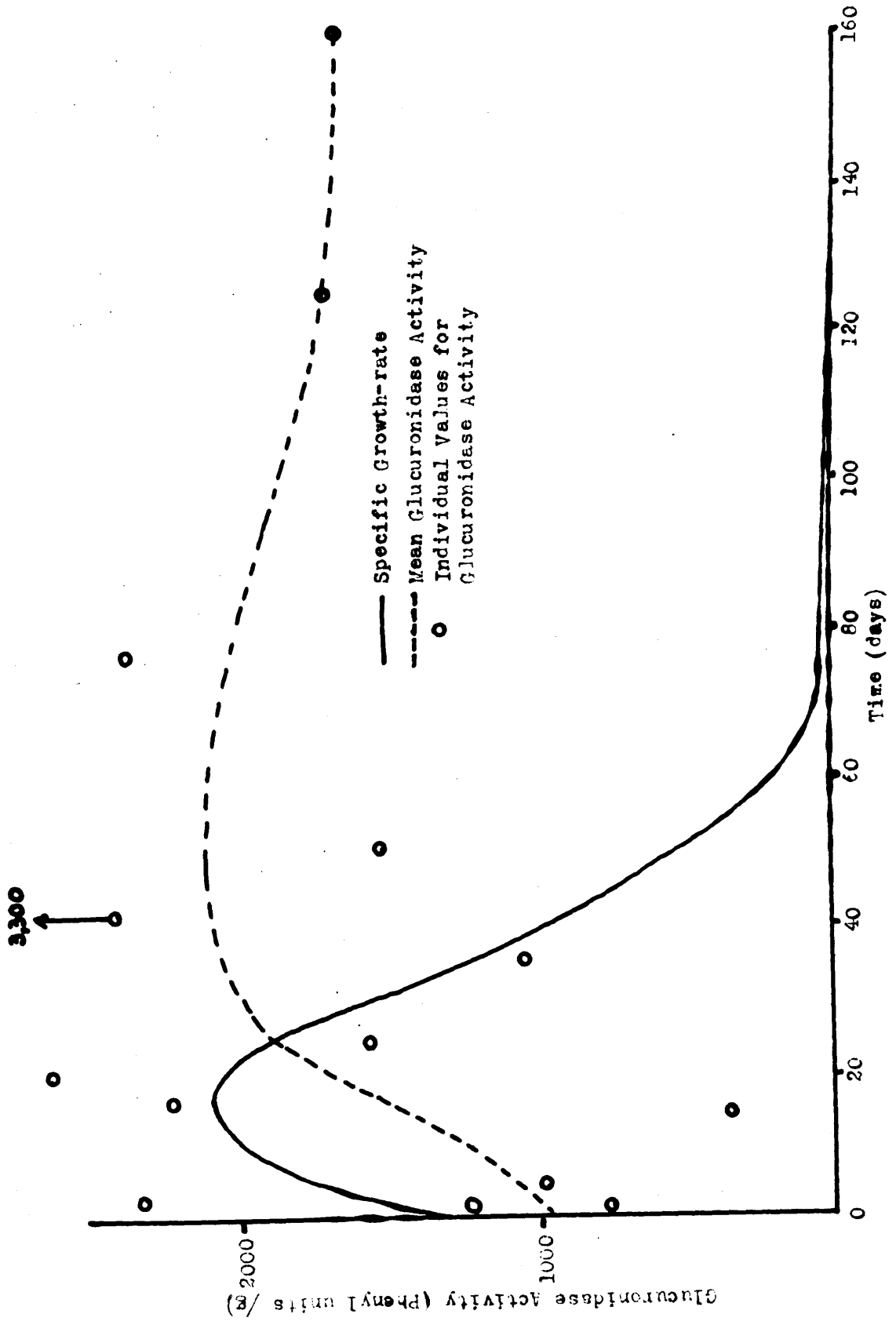


Figure 4.16 Glucuronidase Activity in Relation to the Rate of Cell Proliferation in Growing Rat Liver.

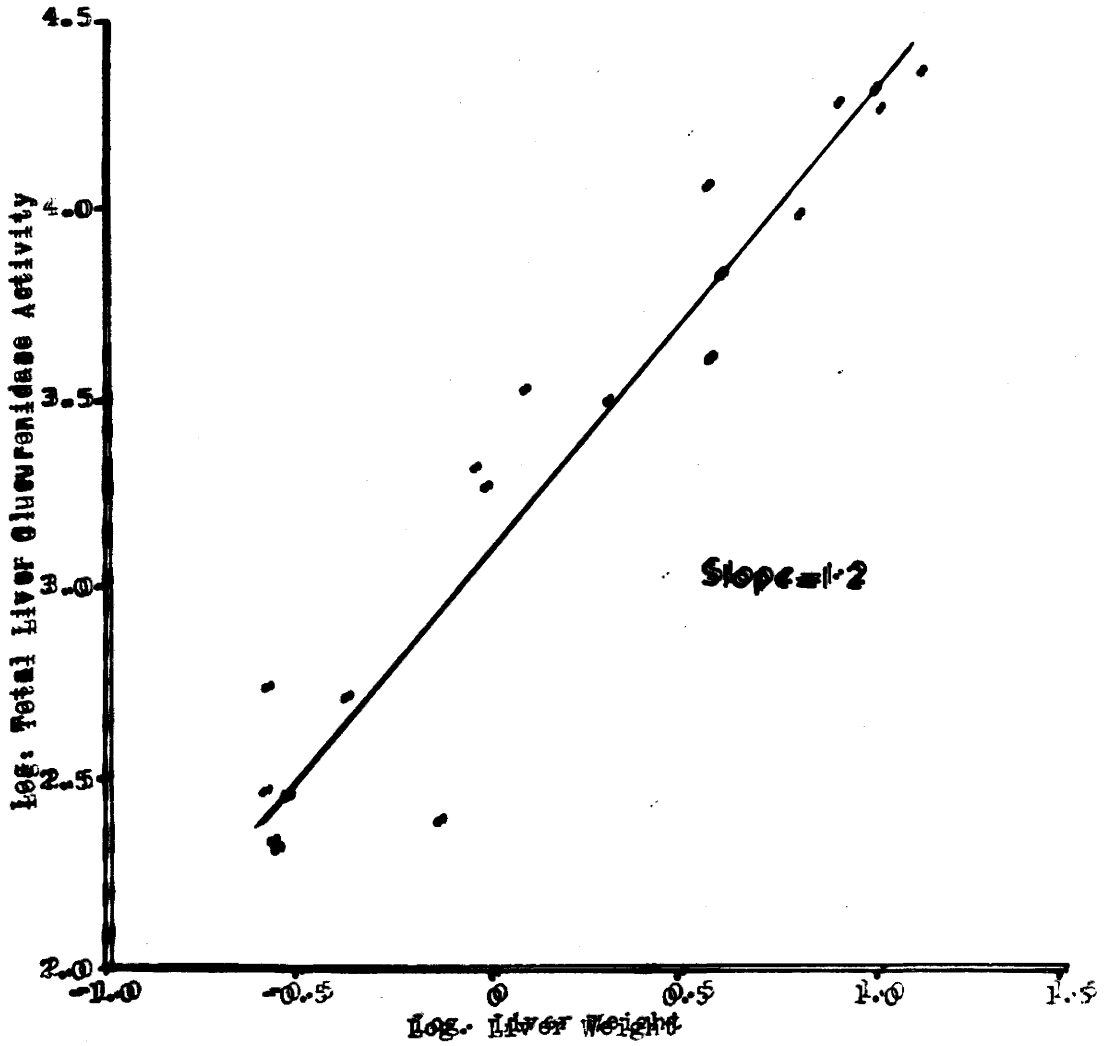


Figure 4.17

Allometric Analysis of the Data in Table 4.10.

hepatectomy by the method of Higgins and Anderson (1931) and these specimens were assayed to give control readings. At intervals up to six days the surviving rats were killed and their livers were assayed for glucuronidase activity. The results are expressed graphically in fig 4.18. The value obtained for P (0.07) is such that the deviations from control values cannot be regarded as significant (appendix). At the same time such deviations as do occur tend towards a decrease and if we assume the validity of Levvy's hypothesis and analyse the results according to their agreement with an allover increase of 10% we obtain a value of P that indicates a very significant deviation from this possibility. In fact, if the one case in which the liver glucuronidase activity was increased is excluded the results demonstrate a significant decrease. From this experiment we can conclude that in hooded rats there was no tendency for the glucuronidase content per gram of liver tissue to increase within six days of hepatectomy.

The second experiment was performed on albino rats. A batch of animals which had received no treatment such as might affect glucuronidase activity was used as controls. The test animals were killed and their livers assayed for glucuronidase activity at two, four and six days. The results are expressed in/

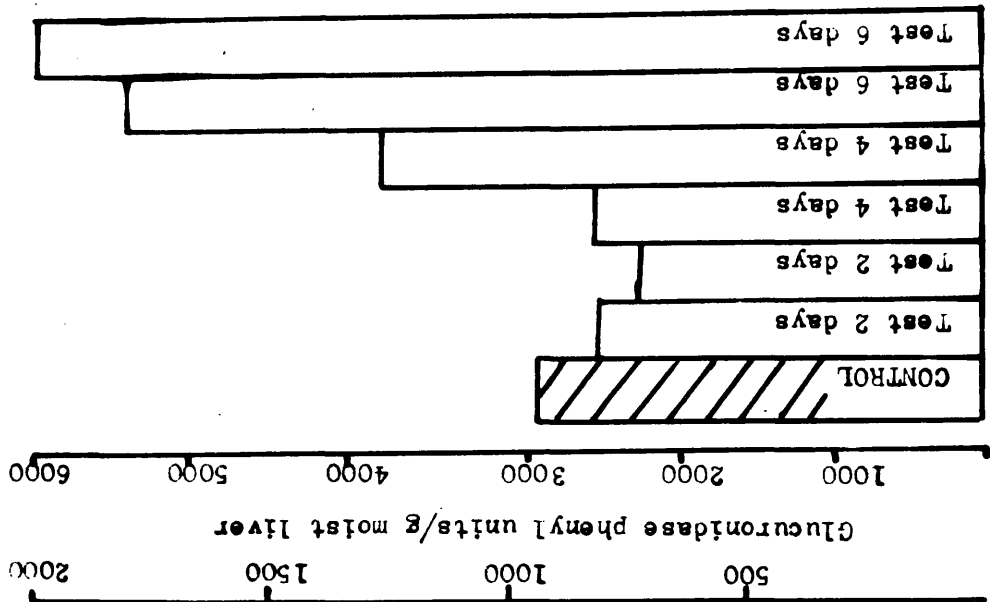


Figure 4.19 Hepatectomy experiment 2.  
(Albino rats)

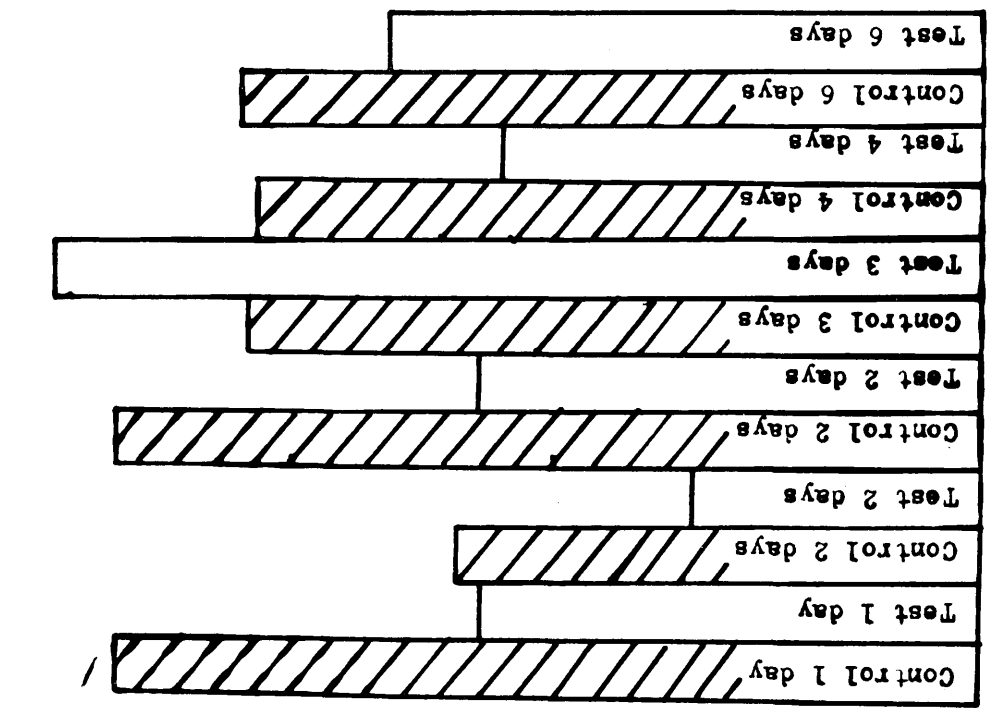


Figure 4.18 Hepatectomy experiment 1.  
(Hooded rats)



in fig 4.19. The statistical analysis of these results was as follows.

(Coding - figures signify 100s of phenyl units)

	Controls	2 day	4 day	6 day
No of rats	21	2	2	2
d.f.	20	1	1	1
Total d.f.		21	21	21
Mean	31.1	33.6	29.7	54
Sum of squares	883.5	9.7	12.5	15.7
$\sum x^2$		7.5	1.4	22.9
$\sum Sx^2$		891	884.9	906.4
Pooled variance ( $S^2$ )		42.4	42.1	43.2
$S \bar{x} = S^2(n_1 - n_2) / n_1 n_2$		4.9	4.85	4.92
t		1.53	0.288	4.66
p		0.15	0.5	0.01

(d.f. = degrees of freedom)

The values for P indicate that while the apparent fall in glucuronidase activity at 2 days is not significant the rise at 6 days is definitely significant.

The apparent discrepancy between the two sets of results may be explained in a number of different ways. In the first place it may be that a rise in glucuronidase activity after hepatectomy is not a constant occurrence and may depend on such factors as species or even extrinsic factors such as diet. On the other hand it may be that the rise in glucuronidase activity does not take place until six days or more after hepatectomy in rats and that in the first series of experiments on hooded rats this rise had/

had not yet occurred by the end of the experiment. That this is indeed the case has been suggested by recent results obtained by Mr Goodlad working with Dr Mills. In these experiments rat livers were assayed up to twenty days after hepatectomy and the peak of activity was found at ten days. Thus there would seem to be confirmation of the observation of Levvy et al (1948) that a rise in glucuronidase activity occurs in the liver after hepatectomy but the findings conflict with the explanation suggested by these authors since the peak of growth in these livers occurs at about the third day

Injection of various substances.

In a further attempt to verify some of the observations of Levvy, Kerr and Campbell (1948), in which increased liver glucuronidase activity was found after the administration of a variety of toxic substances to mice, the following experiments were performed. In these experiments also the animals were killed off during the period of rapid regeneration of the liver and it is possible that a rise in activity may also have occurred later in some cases.

Injections of carbon tetrachloride.

18 adult male albino rats were each injected with 0.5 g. of carbon tetrachloride/100 g. body weight, olive oil being used/

used as a vehicle for the injections. 6 rats simultaneously received 1 ml of olive oil/100 g. body weight and these served as controls. Of the animals which had received carbon tetrachloride five died. The remaining fifteen rats were divided into three groups and one group was killed 2 days after injection, another at 4 days and the third at 6 days, two control rats being sacrificed with each group. The livers were pooled in groups as indicated and assayed over a range of pH levels. The results are collected in table 4.12.

It can be seen that there was no significant alteration in the glucuronidase content of the livers despite the very great structural changes to be seen in the microphotographs from typical members of this series (fig.420)

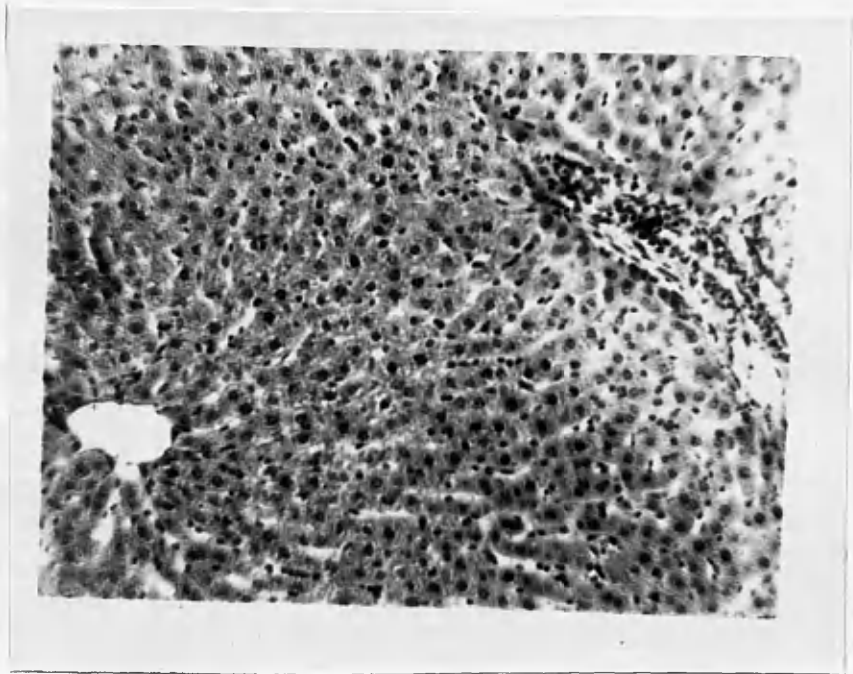
#### Injection of Thiourea.

Most of the substances injected by Levvy and his co-workers were such as would lead to fatty degeneration of the liver. In an attempt to produce liver damage without fatty degeneration thiourea was injected into 12 albino rats. The eleven survivors were killed in batches at one, two, three and four days after injection and the livers assayed for glucuronidase activity. Three untreated animals served as controls. The livers were examined histologically and there occurred/

Table 4.12

Effect of carbon tetrachloride injection on liver glucuronidase activity. (In each case pH activity curves were determined in acetate buffer and an estimation was performed in citrate buffer at pH 5.3. Since no significant changes in the shapes of the curves occurred, only the value at pH 4.4 in acetate buffer is given.)

Days after injection	Test group A CCl <sub>4</sub>	Test group B CCl <sub>4</sub>	Controls Olive Oil
2	3140	2905	2301
4	2220	2627	3380
6	2150	2102	1913



Normal Rat Liver.



4 days after Carbon Tetrachloride Injection.

Figure 4.20 Effect of Carbon Tetrachloride Injection  
on Rat Liver.

occurred a marked necrosis with little evidence of fatty change and little evidence of regeneration in the course of the experiment. In this case (table 4-13) figures on the first day appeared surprisingly high, particularly in view of the uniformly low figures for the next three days. However, there was in fact no really significant alteration of the glucuronidase activity of the livers of these animals in the course of the experiment, this isolated observation being hardly valid by itself.

#### Injection of olive oil.

At this time it had been observed that occasionally rats killed shortly after the injection of olive oil yielded rather high values for glucuronidase activity. A series of animals was therefore investigated, some completely untreated, others two, four and six days after olive oil injection (1.5 ml) and others, as control animals, two and four days after injection of liquid paraffin (1 ml). The results are shown in table 4.14.

There is obviously no significant deviation from the normal but again one is struck by the tendency for the higher figures to appear within two days of injection.

In the above experiment albino rats were used and a similar/

Table 4.13

Effect of liver necrosis caused by thiourea on liver glucuronidase activity. (Albino rats)

(Each reading represents a pH activity curve, no significant alteration in the shape of these having occurred).

Days after injection	phenyl units/g. moist tissue			
	Test 1	Test 2	Test 3	Controls (untreated)
1	5270	4690	-	3790
2	2200	2377	2512	4260
3	3258	2437	3426	4370
4	3025	3936	3192	2827

Table 4.14

Effect of injecting liquid paraffin and olive oil on the glucuronidase activity of rat liver. (Albino rats).  
(Only assays in acetate buffer at pH 4.4 reported).

Days after injection	phenyl units/g. moist tissue		
	Inj. liqu. par.	Inj. olive oil	Uninjected
2	5310	5610	3565
	2670	4720	4370
4	3680	2560	4260
	2827	2877	
6		3490	
		2952	



Table 4.15

Effect of injecting olive oil on the glucuronidase activity of rat liver. (Hooded rats).

Days after injection	p-phenyl units/g.		P (controls pooled)
	Test animals	Uninjected	
2	2075	1882	28%
	1714		
3	1802	1936	50%+
	3007		
4	1336	2240	38%
	2097		
	3080	2950	
	2790		

similar experiment was performed on hooded rats. After injection of 1.5 ml of olive oil subcutaneously to nine rats three each were killed at two, three and four days, two untreated rats also being killed on each occasion. The results of this experiment are shown in table 4.15 and there is obviously no significant difference between controls and injected animals.

Variations in the serum inhibitor.

If, as Fishman et al. (1948) suggested, the serum inhibitor was involved in the regulation of glucuronidase activity in the tissues it seemed possible that simultaneous assays of the serum inhibitor after treatment of the types mentioned above might provide some information on the point. It has already been mentioned that the inhibiting effect of serum was found to vary with the glucuronidase preparation used for its assay and the extent of the variation may be observed in these studies. In each experiment a single glucuronidase preparation was used and the assays were performed simultaneous

The assays were performed as follows. To a test-tube were added 0.5 ml acetate buffer at pH 4.4, 0.2 ml of an enzyme preparation and 0.1 ml of the serum to be tested. The assay was commenced by adding 0.2 ml of 0.005 M phenolphalein glucuronide/

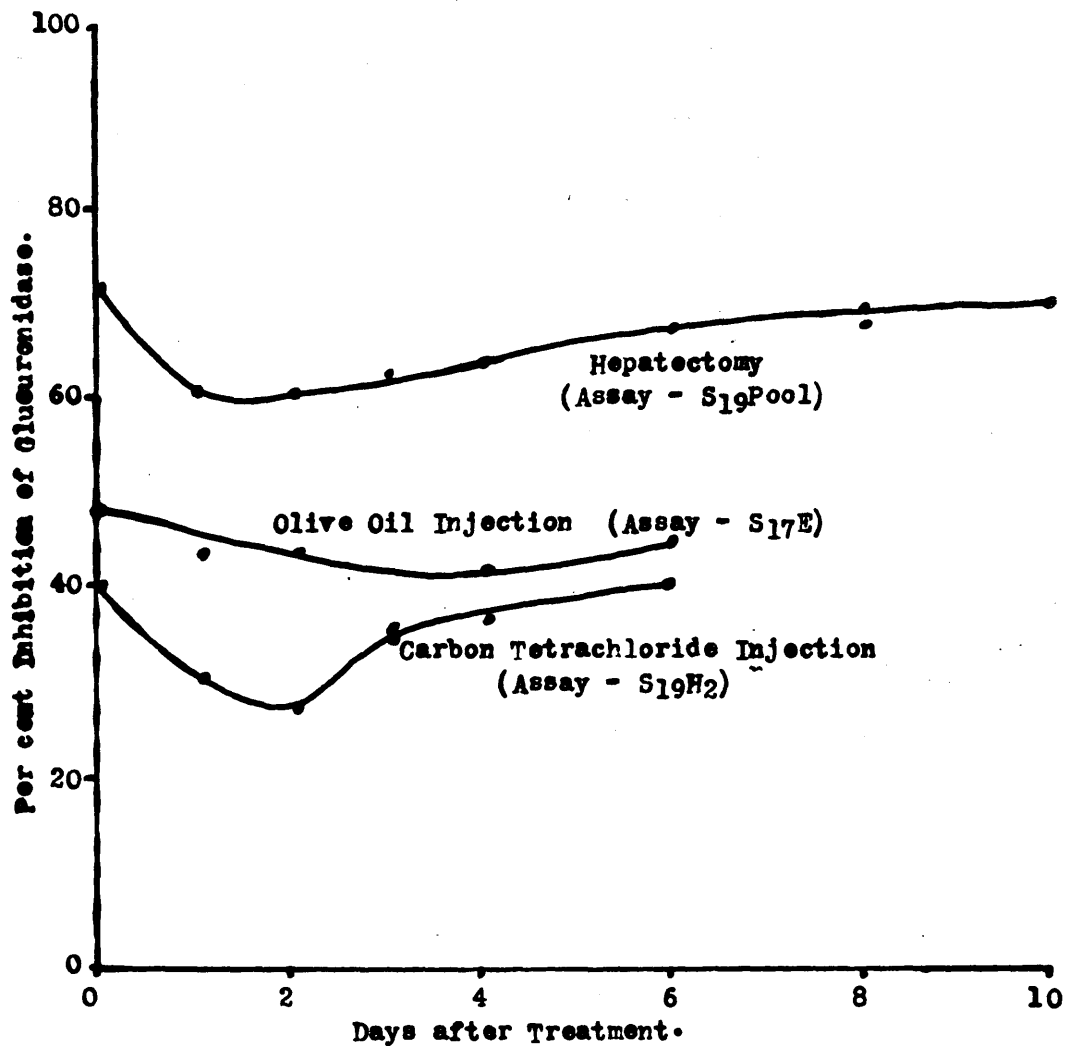
glucuronide (final substrate concentration - 0.001 M). At the end of one hour's incubation at 38°C the reaction was stopped and phenolphthalein estimated by the method of Talalay et al. (1946) Control tubes containing no serum were run simultaneously.

The results are represented diagrammatically in fig 4.21 for alterations in serum inhibitor activity following the injection of carbon tetrachloride (I), olive oil (II) and following hepatectomy (III)

If the variations observed are compared with the results of the assays for glucuronidase activity it will be seen that there is no evidence for a reciprocal relationship between them. On the other hand, after hepatectomy the serum inhibitor level immediately falls and does not return to normal until about ten days, when the liver is almost completely regenerated. Also, after carbon tetrachloride injections the serum inhibitor levels fall and then gradually return to normal between six and eight days. These results would suggest that the liver is the source of the serum inhibitor.

The results obtained after olive oil injection are difficult to understand but there can be no doubt about their significance.

The/



**Figure 4.21** Effect on the Serum Inhibitor of Various Forms of Treatment.

The code number of the glucuronidase preparation used in each set of assays is indicated in parentheses.

The value obtained for  $t$  was 4.7, considerably greater than the tabular  $t$  of 3.012 (d.f. 13) and thus the possibility of the differences observed between controls and treated animals being due to chance is estimated at considerably less than 1%.

## S E C T I O N 5.

### Discussion.

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CHEMISTRY OF GLUCURONIC ACID.

The Behaviour of Glucurone in Alkali.

The production of yellow colours on dissolving carbohydrates in strong alkalies is not uncommon, though heat, or some time for reactions to occur, are usually necessary, and it may therefore be doubted whether the observation that glucurone forms a yellow colour immediately in strong alkali has any particular significance. However, Owen, Peat and Jones (1941) prepared both methylglucopyruronolactone and methylglucofuronolactone by the Fischer synthesis and found that, whereas the pyranoside behaved as other ether glucuronides in alkaline solution, the molecule with the furan ring behaved anomalously. It immediately formed a yellow colour, and, despite the fact that the hemiacetal group was blocked, the solution displayed powerful reducing properties. The yellow colour was associated with a band in the absorption spectrum at 416 m $\mu$ , which faded fairly rapidly, while there was another strong absorption band at 290 m $\mu$ . which remained unaltered. In section 2 it was noted that glucurone behaved similarly, the absorption bands being at 420 and 270 m $\mu$ . The similarity in behaviour of the two compounds suggests an analogy between

the reactions occurring.

An explanation of the behaviour of methylgluco-  
furanolactone was offered by Pigman and Goepp (1948), who  
suggested that it might indicate behaviour similar to that of  
the dilactones of the saccharic acids. These behave in a  
similar manner in the presence of alkali, and the explanation  
offered is that, in strong alkali, one of the two five-  
membered rings is split and the necessary hydrogen ion is  
taken from the neighbouring carbon atom rather than from the  
solution. This would result in the formation of an enolic  
structure, which might be expected to display the abnormal  
properties observed. It might be expected that glucofuranono-  
lactone would behave in a manner similar to methylglucofuranono-  
lactone, whereas glucopyranonolactone would behave in a manner  
analogous to methylglucopyranonolactone. Thus, if the hypo-  
thesis is valid, such behaviour would be more in accord with  
a furan structure for glucurone than with a pyran structure.  
The possible nature of the reactions involved is illustrated  
in figure 5.1.

Now, if we observe the behaviour of glucurone in  
paper chromatograms employing an alkaline solvent, a further  
point arises. Two spots appeared in such chromatograms, and  
Partridge (1948) suggested that these represented glucurone



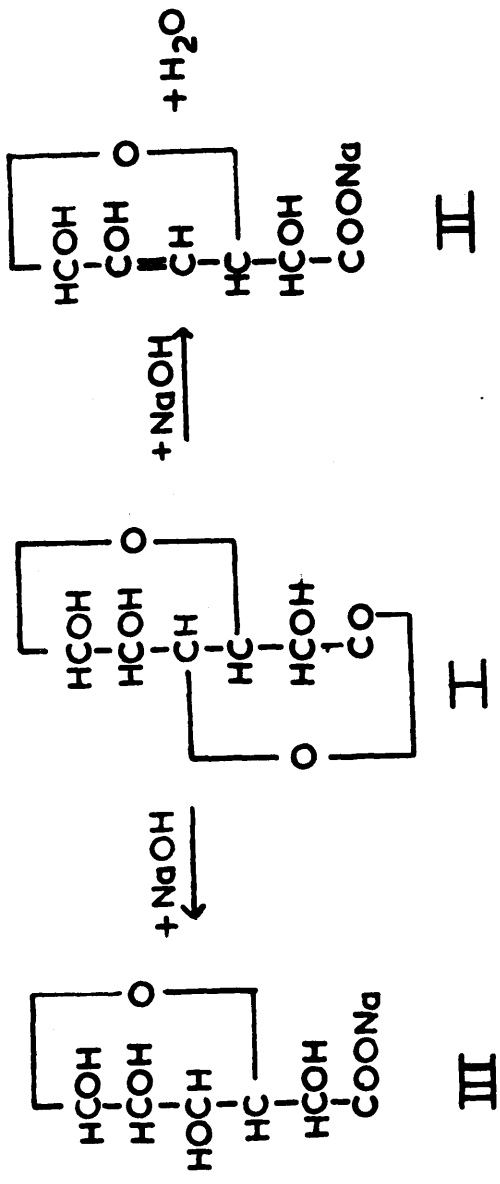


Figure 5.1 Postulated action of alkali on glucurone.

and glucuronic acid. However, a little reflection makes it obvious that this theory, though plausible, is unlikely to be true, since one would expect that hydrolysis of the lactone ring of glucurone would proceed in alkaline solution until eventually complete, and, in such a case, one would expect, not two discrete spots, but a streak due to glucuronic acid. On the other hand, if glucofuranolactone behaves in the manner proposed above, the two spots would be more likely to represent the structures shown in II and III in fig.5.1.

It should be added that, in chromatograms in acid solvents, two spots are often obtained, but in this case it would be valid to conclude that they probably represent glucuronic acid and glucurone, since both are relatively stable in weak acid.

#### The Chemical Synthesis of Glucuronic Acid.

A convenient chemical synthesis for glucuronic acid would be of great value to the biochemist in this field as well as to the carbohydrate chemist. The disadvantage of methods employing the reduction of glucosaccharic acid is that they require a preliminary synthesis of this material. Methods based on oxidation of the primary alcohol group of glucose are more likely to be useful, provided the synthesis

of intermediates does not involve too much difficulty, and the final product does not contain other substances to interfere with crystallisation. Owing to the instability of glucuronic acid, it is also essential that the intermediate products should be readily degraded.

Sell and Link (1938) developed a very successful synthesis of galacturonic acid by permanganate oxidation of the diacetone compound of galactose and a similar synthesis for glucuronic acid immediately suggests itself. Florkin (1947) actually made the statement that glucuronic acid could be synthesised this way, but he gave neither references nor results, and also showed an erroneous structure for diisopropylidene-glucose (diacetone compound of glucose). Since acetone condenses with only two hydroxyl groups in a cis relationship to each other, it is possible to form 1,2-3,4-diisopropylidene-galactose in which the primary alcohol group is free for oxidation. However, it is not possible to form a strictly analogous compound with glucopyranose and in this case glucofuranose reacts to form 1,2,5,6-diisopropylidene-glucofuranose in which the primary alcohol group is not free for oxidation to a carboxyl group. Thus this synthesis is not possible in the case of glucuronic acid. Coles, Goodhue and Hixon (1929) described a very simple way of preparing the pure monoacetone derivative of glucose

(1,2-isopropylidene-glucose) and this might seem a more suitable starting point for the oxidation. However, oxidation of this material has been tried by Ohle, Couticos, and Gonzalez, (1931) and the product was not glucuronic acid but xyluronic acid, as might have been expected due to the ease with which alkaline permanganate is known to rupture carbon-carbon bonds. In view of the claim of Jolles (1911) to have synthesised glucuronic acid from glucose by the direct action of hydrogen peroxide without catalysis, this reagent might be effective for oxidising monoacetone-glucose. Acid oxidising agents are, of course, out of the question in considering a synthesis of this type since the acetone sugars are readily hydrolysed by acid.

In view of the claims made for the high degree of specificity of nitrogen dioxide for the oxidation of primary alcohol groups a synthesis based on this reaction appears at the moment to be the most promising. The sole requirement is to find a suitable compound, easily synthesised or easily obtained in which the hemiacetal group of glucose is blocked, which is resistant to the highly acid conditions of the reaction, but which is readily degraded without too much destruction of glucuronic acid. The attempts of the Author suggest that starch is not too promising as a starting material. In view of the high yields of glucuronic acid claimed by Kiliani

(1921), who oxidised glucose direct with nitric acid rich in nitrous acid, protection of the hemiacetal group may not be necessary, and if this is so, glucose being readily obtainable, the most practical synthesis might simply consist of its direct oxidation by nitrogen dioxide under optimum conditions determined by methods similar to those described by McGee, Fowler, Taylor, Unruh and Kenyon, (1947).

#### Microanalytical Methods for Glucuronic Acid.

It must be re-emphasised that development of micro-analytical methods is all-important before useful research can be done on this subject, and that a great deal of experimental work done in the past is invalid because of unsatisfactory techniques. Most of the relatively specific reactions employed in the past were applied arbitrarily and empirically. In this study, an attempt has been made, with a fair measure of success, to apply some rational principles to the naphthoresorcinol reaction.

The method evolved has an almost complete degree of specificity in relation to similar materials which are likely to cause interference. Only Pyruvic acid and ascorbic acid, of the substances investigated, are likely to constitute sources of error. Only very rarely indeed would concentrations

of pyruvic acid be expected such as might interfere with the reaction and although the same applies to ascorbic acid, the possibility of interference by abnormally high concentrations of this material must always be kept in mind. The reaction described by Dische (1947) was claimed at the time to be more specific than the naphthoresorcinol reaction, and this was probably true in relation to naphthoresorcinol reactions in use up to that time. However, a comparison with some of the results obtained by the naphthoresorcinol reaction using the most specific modification described in section 2 shows the naphthoresorcinol reaction in a more favourable light.

Comparison of Dische and Naphthoresorcinol reactions.

$I_R$  - ratio of the reaction intensity of a compound to that of an equivalent amount of glucuronic acid:

Substance in Solution.	$I_R$ Dische	$I_R$ Naphthoresorcinol
Glucose	av. 0.044	0.0085
Glycogen	0.044	0.0085
Arabinose	0.012	0.000
Xylose	0.013	0.000
Pyruvic acid	0.000	0.021
Ascorbic acid	0.000	0.15

In the presence of ascorbic acid, the carbazole reaction is obviously superior, but this is the only important case in which the naphthoresorcinol test proves inferior.

The naphthoresorcinol reaction is superior in one other important respect. Whereas in the carbazole reaction the colour intensity is not always proportional to the amount of glucuronic acid in compounds being analysed, which necessitates the introduction of correction factors, the naphthoresorcinol reaction (Hanson, Mills and Williams, 1944), produces colours with an intensity proportional to the amount of glucuronic acid present in the solution, provided the heating time is adequate for complete hydrolysis of this to take place. Thus for glucuronides it is the ideal method. However, in some cases, resistance to hydrolysis is too great, and here the carbazole reaction is probably superior. The cases in question are those involving the determination of uronic acids in aldo-biuronides. Howell (1928) originally reported that heparin gave a naphthoresorcinol reaction, but Jorpes and Bergstrom (1937) could not agree. In actual fact the Author has found heparin to give a naphthoresorcinol reaction but, even with several hours heating, the colour intensity was not proportional to the glucuronic acid content. The Dische reaction, on the

other hand, overestimates the amount of glucuronic acid by 60-70% (Dische, 1947). The author has made similar observations with gum acacia and oxidised cellulose. In order to overcome such difficulties Kapp (1940) suggested a preliminary hydrolysis but this is not feasible since much of the glucuronic acid is destroyed by decarboxylation in such a procedure. Meyer, Block and Chaffee (1942) proposed a preliminary methanolysis, and this is probably the most practical suggestion, but, of course, all methanol would have to be removed before conducting a naphthoresorcinol reaction.

As regards the naphthoresorcinol reaction itself, the limitations of most previous modifications have been implied in section 2, while others will be discussed later in this section. Reference may be made to the method of Bisset, Brooksbank and Haslewood (1948), which, according to Jarrige, (1950), is the best to date. These workers added potassium ferricyanide to their naphthoresorcinol reagent in order to "age" it. On inspection, their method shows an increased tolerance over most previous methods, and this is undoubtedly due to the fact that potassium ferricyanide is an oxidising agent, and behaves in the same way as chloramine-T. The reaction is rather less accurate than one might expect,



and this is almost certainly due to the formation of suspensions in the extracting solvent, as has already been commented on.

It should also be noted that mucic acid and gluconic acid are not particularly important sources of interference in this reaction. Statements to the contrary have been perpetuated from the earlier literature where only qualitative experiments were performed.

Some remarks may be appended on the nature of the reaction. The fact that saccharic acid does not react with naphthoresorcinol to a significant degree and that naphthoresorcinol seems to have a considerable affinity for aldehyde groups suggests that the first reaction to take place in the reaction mixture is a condensation between naphthoresorcinol and aldehyde groups. It can be demonstrated readily, in support of this suggestion, that glucose boiled with an acid solution of naphthoresorcinol rapidly loses its reducing properties. Thereafter, since full colour development is not achieved for some hours (Kapp) a reorganisation of the molecule or a further condensation or a splitting of the molecule to form the pigment must occur.

#### Ion-exchange and partition chromatography Techniques:

It is too early to judge the possible value of these

techniques in investigations on glucuronic acid metabolism. It is hoped that the ion-exchange method may permit the isolation in large amounts of the glucuronides normally present in urine. As has been pointed out earlier, however, it is not yet applicable to this problem.

Partition chromatographic methods should prove useful not only for investigating the normal glucuronides in urine but for detecting glucuronides in the urine of animals to which have been administered various materials. They should therefore prove particularly valuable in detoxication studies when the amounts of glucuronides present are too small to be isolated in the pure state. In in vitro experiments, also, the method has already proved useful for proving the conjugation of menthol with glucuronic acid in tissue slices. Better solvents for this purpose may yet be evolved, and in particular, the use of buffered stationary phases and of reversed phases may facilitate the separation of some glucuronides which are not very well separated by the methods developed so far.

#### Natural occurrence of Glucuronic Acid.

It has already been mentioned that little attention has been paid to the possibility of glucuronides being excreted by channels other than the urine. Yet such studies as

have been performed, and such evidence as may be related to the question, tend to suggest the likelihood of glucuronides appearing in the bile, while the preliminary studies reported here indicate such a possibility. This is an important point to establish, because many figures relating to the relative degrees of conjugation of different aglycones with glucuronic acid may be found to have little meaning, if it be the case. As outstanding examples, we may consider the cases of borneol and phenolphthalein. Borneol administered to man appears in the urine as bornyl glucuronide to 80-90% of the theoretical amount. (Pryde and Williams, 1936; Wagreich, Bernstein, Pader and Harrow, 1941; Rosenmund and Esselier, 1947), and rabbits are capable of excreting similarly large amounts as a glucuronide, (Hämäläinen, 1909; Schüller, 1911; Hämäläinen and Sjöström, 1910). This means not only that borneol is conjugated by these organisms to a very high degree, but also that almost all the excretion of bornylglucuronide is by the kidneys, so that excretion by alternative routes must occur to a very small extent, if at all. On the other hand, Di Somma (1940) found that phenolphthalein glucuronide was excreted in the urine of rabbits to a very much smaller degree relative to the initial dose, and moreover, only 45% of the phenolphthalein administered could be recovered from the urine at all. One might interpret

these findings as meaning that phenolphthalein is conjugated to a very much smaller degree than borneol as a glucuronide (which is almost certainly true) and also that some of the phenolphthalein is destroyed in the animal body. However, more of the phenolphthalein may be accounted for by the observation of Abel and Rowntree (1909) that some was also excreted in the bile in the form of a conjugate which could be extracted with ethyl acetate. Ethyl acetate extraction is actually employed in the preparation of phenolphthalein glucuronide, and it is possible that the conjugated phenolphthalein which was observed in the bile was in this form. If this be the case, then it is obvious that the degree of conjugation of phenolphthalein with glucuronic acid has been underestimated and we do not know to what extent this factor of differential excretion in the bile may affect other glucuronides. It is obvious that this point must be established with certainty since, until it is, the value of much of the information gained in the past from quantitative studies of the excretion of glucuronides in urine must remain in doubt

As a corollary, it may be added that if the excretion of glucuronic acid in the bile is established, it renders even more dubious attempts to estimate liver function by determinations of glucuronic acid in the urine.

Apart from this possibility, it must be maintained that a very great deal of the published work on glucuronide excretion in the urine is valueless, due to the use of unreliable methods for the estimation of the glucuronic acid in the urine. If we exclude investigations in which the glucuronide has actually been isolated in the pure state from the urine of animals to which large doses of an aglycone have been given, we find that we must reject almost every other paper published on the subject, on these grounds.

The early 'specific' methods for glucuronides (estimation of laevorotation and the reducing power after Hydrolysis) would satisfy very few investigators to-day, yet it is doubtful whether the methods based on furfural distillation or the naphthoresorcinol reaction used by many workers in the past were any better when applied to urine. The study of factors interfering with the naphthoresorcinol reaction indicates that in all previously used modifications, the results were almost certainly subject to gross errors. Some of these may be listed.

- 1) Samples containing glucose or too much glucuronic acid would be expected to give very low readings instead of very high readings. (fig 2.5.)

- 2) Using non-critical solvents, such as ether,

pigments, due to substances other than glucuronic acid, were almost certainly extracted from the reaction mixture.

3) The administration of many substances to animals gives rise to gross interference in the urine. In this respect, the most notable substance is probably ascorbic acid, but many drugs, (e.g. hexamine, which gives rise to formaldehyde in the urine,) would also invalidate the reaction completely.

4) In addition, there may be naturally occurring substances in the urine of many species, (e.g. the rabbit,) which interfere with the reaction.

Entirely apart from these sources of error, it was pointed out by Senior, (1948) that attempts to determine glucuronic acid in urine which had been kept for some hours without a preservative were futile. In view of the observations of Buehler, Katzman and Doisy, (1949) that coliform bacilli can hydrolyse glucuronides, and of Kay (1926) that members of the coli-typhoid group can ferment glucuronic acid, in association with the fact that unpreserved urine is teeming with coliform organisms in a few hours, there can be little doubt that his remarks are perfectly justified. He recommended mercuric chloride as a preservative, but in view of the effect of metallic ions on the naphthoresorcinol reaction, some other

preservative, such as toluene, might prove more satisfactory. In the present work phenol has been used in some experiments.

In the light of these facts, it can be maintained with justification that the vast majority of investigations based on the determination of glucuronic acid in the urine by the naphthoresorcinol reaction are completely unreliable, and, in fact, they may all be considered as suspect.

Precisely the same criticisms may be applied where the naphthoresorcinol reaction has been used with blood. With the sole exception of the methods of Florin et al., (1940), it is quite obvious that any results obtained were invalidated by the presence of glucose, which would be expected to inhibit pigment formation if the concentrations were high, and to produce an ether-soluble pigment if the concentrations were relatively low.

Whether the modifications proposed in sections 2 and 3 will prove any more reliable than earlier methods when applied to biological materials, only the experience of different investigators will tell. However, the elucidation of the reasons for the behaviour of the pigment in the presence of interfering substances, and the investigation of circumstances which increase specificity should assist in placing the reaction on a sounder and more rational basis than it has enjoyed in the past.

The Significance of Increases of 'Basal' Glucuronic Acid.Excretion.

In the results of glucuronic acid determinations on the urine of a number of chinchilla rabbits, it may be observed that the figures for pregnant rabbits are significantly higher than those for non-pregnant females and males. This tendency was even more marked in a larger series of results which were rejected as probably unreliable. (The results shown are only advanced tentatively as regards reliability). In many similar experiments on the normal glucuronide-synthesising capacity of animals in varying environmental conditions, investigators have concluded that such increases betokened an increase in the ability of the organism to synthesise glucuronic acid. Such a conclusion is completely fallacious. It has been shown time and again that the animal organism normally has a very great reserve capacity for conjugating glucuronides in response to the administration of suitable aglycones. Normal excretion never approaches this reserve capacity and therefore any increase in the glucuronic acid excretion in the urine can only imply an increase in the supply of suitable aglycones for conjugation. In the case of the pregnant animal we know that there does occur an increase in the production of pregnanediol and other steroids which form conjugated glucuronides.



Moreover, p-cresol is quantitatively the most important phenol in urine (Siegfried and Zimmerman, 1911), and there is some evidence to suggest that it may, in part, arise from the breakdown of some steroid hormones. (Williams, 1947; Hey, 1944). p-Cresol is excreted in combination with glucuronic acid as well as with sulphuric acid (Neuberg and Kretschmer, 1911) and consequently, if this reasoning is correct, we should expect an increase of these glucuronides in pregnant urine also. Whether the explanation be correct or not, the point that requires emphasis is the fact that increases in the basal glucuronic acid excretion of normal animals are usually to be explained on an increased production of an aglycone, rather than on an increased capacity for glucuronic acid synthesis and conjugation.

Origin of 'Normal' Urinary Glucuronides. At this point it may be interesting to comment on the possible origins of the glucuronides which appear in normal urine. It is generally accepted that most of the material with which glucuronic acid is conjugated, mainly phenols, arise from bacterial action on amino-acids in the gut, and it seems highly likely that this accounts for a fairly large part of such material. However, the discovery of pregnanediol glucuronide by Venning and Browne (1936), of oestriol glucuronide by Cohen and Marrian (1936) and

recently of adrenaline glucuronide by Dodgson and Williams (1949) indicates that at least some arise from endogenous material.

Apart from these observations, there has been little reliable work to indicate whether the majority of normally produced glucuronides are of endogenous or exogenous origin. Clogne and Fiessinger (1916) found that glucuronic acid increased in the urine after food, and then decreased till the next meal. Such a finding would obviously suggest an exogenous origin for practically all the normal 'aglutones'. On the other hand, Ogata and Takeo (1939) reported that the excretion of glucuronides was increased after exercise, a finding which would tend to the alternative conclusion. The methods employed by each of these groups were subject to most of the criticisms already mentioned, and it is not possible to accept their conclusions without reserve.

#### Synthesis and Conjugation of Glucuronic Acid.

The in vivo experiments of Quick, (1926a; 1926b), Schmid (1936) and Dzewiatkowski and Lewis (1944) provided suggestive evidence for glycogen as the ultimate precursor of glucuronic acid. Most other experiments designed to solve this problem in the intact animal must be interpreted with

extreme caution, since the majority of them employed methods subject to the criticisms stated above, and in addition, many investigators used camphor as a glucuronogenic material. Since camphor must itself undergo oxidation before it can be conjugated with glucuronic acid it cannot be regarded as a satisfactory material for the purpose of investigating glucuronide formation.

The perfusion experiments of Hemingway, Pryde and Williams (1934) provided the first reliable information on some of the factors involved in glucuronic acid synthesis, and, in particular, their demonstration that the process was inhibited by cyanide indicated that intact electron carrier systems were essential for this purpose.

T The studies of Lipschitz and Baeding (1939) provided a great deal more information on the subject and their conclusions have influenced thought on the subject for more than a decade. However, the time has come to revise the significance of their findings, since other workers have failed to verify their most important conclusions. Let us consider first those observations which have been verified.

In the first place, Lipschitz and Bueding verified the previous observations of Hemingway et al, (1934), by showing that the in vitro conjugation of borneol by guinea-pig liver slices was inhibited by anaerobiosis and cyanide. These

results were subsequently confirmed in the work of Storey, (1950) and the present results are in agreement. Secondly, they demonstrated that certain inhibitors of the glycolysis cycle, namely iodoacetate and fluoride were capable of inhibiting the reaction. De Meio and Arnolt (1944) verified that phenol conjugation was inhibited by iodoacetate, while the present results are also in accord with those of Lipschitz and Bueding. These latter workers also found that liver, and to a slight degree, kidney, alone of all the organs tested, were capable of forming glucuronides in the guinea-pig and rat. Storey made similar observations in the mouse. Crépy, (1947) on the other hand, demonstrated conjugation only in the liver of the guinea-pig, with possibly a trace of conjugation in the intestine. The present results are in general agreement, conjugation having been found to take place in liver slices, to a small but definite extent in kidney slices, and possibly a trace in the intestinal mucosa.

Lipschitz and Bueding (1939) claimed to have demonstrated the conjugation of borneol in the rat, but Crépy could not demonstrate glucuronic acid conjugation with menthol or (?)borneol in this animal, and the present author was unable to demonstrate the formation of menthylglucuronide in the rat. Such a discrepancy is readily explained. De Meio (1945)

found marked differences in the ability of different strains of rats to conjugate phenol, while Mosbach, Jackel and King, (1950) have shown that different strains of albino rats (Wistar and Sherman) show marked differences in their basal glucuronic acid excretion. Such an observation (see above) would suggest that some strains are more capable of dealing with toxic materials by other channels and hence fewer aglycones are available for conjugation as glucuronides. It is actually established that different species treat menthol differently, (Quick, 1924; Williams, 1938; Williams, 1947), and that some apparently oxidise menthol quite readily, so that very little glucuronide conjugation occurs.

So far, there has been a fair measure of agreement between different observers and the findings discussed above may be accepted as established.

However, the most significant observation of Lipschitz and Bueding was that glucuronide production was only stimulated by certain 3-carbon compounds, although many materials were tried. No subsequent observer has been able to confirm these findings. In an attempt to find an explanation for the discrepancy, one is led first to examine the methods they employed. A possible source of error that suggests itself is the extraction of pyruvic acid into the extracting solvent

(ether) along with the glucuronide. They added sodium bisulphite to the aqueous solution in large amounts to form the bisulphite compound of pyruvic acid, acidified the solution and extracted with ether for 2 hours. Since the bisulphite compounds are unstable in acid, one would expect some pyruvic acid to be extracted by the continuous flow of ether.

In fact, it has been found in the present work that on direct extraction of such a solution with ethyl acetate, this solvent removed small amounts of pyruvic acid. Although these workers used benzene as the extracting solvent for the pigment produced in the naphthoresorcinol reaction, they also used an alcoholic solution of naphthoresorcinol (which has been found to increase the solubility of the pigment in benzene) and consequently pyruvic acid would be expected to give a considerably elevated reading. Despite such reasoning, however, it must be admitted that the controls and recoveries reported by these workers answer it completely. We must look elsewhere, and (apart from species difference) there seems only one other possible explanation for the discrepancy. Lipschitz and Bueding reported that their experiments were carried out in a saline medium, but no details of the constitution of the saline were offered. In the one experiment in

which the present author found somewhat similar results to those of Lipschitz and Bueding, magnesium had been omitted from the medium. However, even in this case, I found stimulation by glucose and glucurone as well as by pyruvate, whereas in the other case, using a complete medium, no activation by any carbohydrate addition was achieved.

Crépy described no new factors likely to be involved in this reaction, but Storey made the interesting observation that glucuronide conjugation was vastly increased by using a bicarbonate Ringer medium rather than a phosphate Ringer medium, an observation which the present author has amply verified. He tentatively suggested the possibility of carbon dioxide fixation being involved in the synthesis of glucuronic acid. However, the experiments of Mosbach and King, (1950) using  $^{14}\text{C}$  suggest very strongly that this is not the case. It is possible to explain the phenomenon in another way. The synthesis of glucuronic acid has been shown to depend on aerobic processes in the cell, and these may, in actual fact, be the limiting factors in synthesis in many experiments. It is known (Barcroft, Haldane, quoted by Samson Wright, 1940) that carbon dioxide tension affects the dissociation of some of the respiratory pigments, and thus the increased synthesis

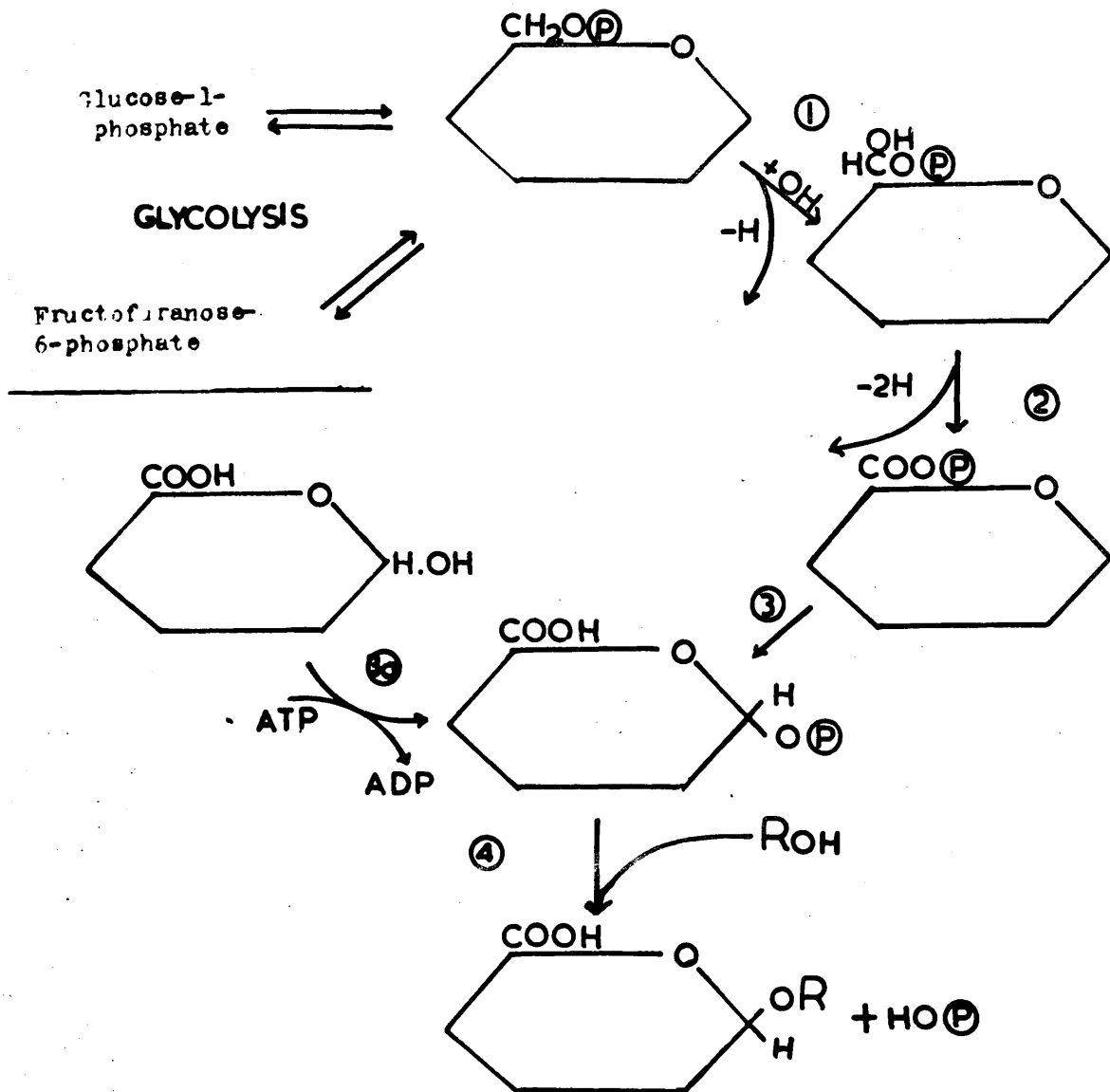
of glucuronic acid in the presence of carbon dioxide may simply be due to non-specific factors of this nature.

The observation of De Meio and Arnolt (1944) on the reversibility of iodoacetate inhibition of phenol conjugation by glucurone has been verified and extended by the observation in the present work that the formation of menthylglucuronide can be inhibited by iodoacetate and that this inhibition can be reversed by glucurone. The valid criticism has been advanced (Fishman, 1950) that no attempt has been made previously to distinguish between synthesis of glucuronic acid and conjugation of glucuronides. This experiment would seem to provide a tool for distinguishing the two. Obviously, unless glucurone has a non-specific action of some sort, the stages between free glucuronic acid and menthol, on the one hand, and menthylglucuronide on the other must be very few, and, in fact, there would seem to remain only one problem to be solved - whether glucuronic acid is conjugated directly with the aglycone, or whether it is first converted to an 'active' intermediate. In analogy with other carbohydrate synthetic processes, it seems not unlikely that an intermediate, possibly glucuronic acid-1-phosphate, is formed, and that this then conjugates with the aglycone, the enzymes involved not being inhibited by iodoacetate. The observation is totally incompatible with



intermediate glucoside and trioside hypotheses.

What, then, is the likely path of formation of glucuronic acid-1-phosphate? Such experiments as provide a shred of reliable evidence suggest that the source of glucuronic acid is also a precursor of glucose. Earlier work suggested that it was probably glycogen, but Mosbach and King (1950) using radioactive tracer techniques, have suggested that the source may actually be glucose itself, and is probably not so distant as glycogen. (Their degradation studies are not very satisfactory. Having found that one-sixth of the radioactivity appeared in the carbon dioxide evolved in the presence of strong mineral acid, they assumed that radioactivity was incorporated evenly in all six carbon atoms of the glucuronic acid formed from administered glucose. Though this may well be true, it is not acceptable evidence). There may be a germ of truth in both schools of thought, for the precursors of glucuronic acid may well be phosphorylated glucose intermediates. (Crépy suggested glucose-1-phosphate). It is not difficult to produce a hypothetical scheme originating from glucose-6-phosphate, as outlined in figure 5.2. Such a scheme has close analogies with reactions already known to occur in the course of glycolysis. It would explain why intact oxidative enzyme systems are essential for the reaction, why



**Figure 5.2** Hypothetical scheme for the synthesis of glucuronides in vivo.

Possible Types of Enzymes Involved in the Reactions

- ① Dehydrogenase.
- ② Triosephosphate dehydrogenase type. ? inhibited by iodoacetate.
- ③ Phosphoglucomutase type.
- ④ Hexokinase type.
- ④ Probably specific "glucuronoconjugase".

iodoacetate inhibition of the reaction can be reversed by glucuronic acid, why the system is inhibited by inhibitors of phosphorylating enzymes and why glucose precursors, though not glucose itself, appear to be immediate precursors of glucuronic acid. It is necessary to postulate a Walden inversion in the act of conjugation since glucuronides belong to the  $\beta$ -series, whereas the glucose intermediates mainly belong to the  $\alpha$ -series. On the other hand, of course,

$\beta$ -glucuronic acid-1-phosphate could be formed at stage 3. The scheme is advanced merely as a suggestion of the types of reactions that may be involved in the synthesis of glucuronides and it must be admitted that the evidence to support such a scheme is rather slim at the moment. Many alternatives to such a scheme could be proposed. For instance, the synthesis of glucuronic acid-1-phosphate might commence with the formation of glucose-1-6-diphosphate, in which case stage 3 could be omitted. Also, if it can be shown that glucuronic acid is directly conjugated with its aglycone without the necessity for a phosphorylated intermediate, the scheme could be simplified still further.

### The Physical Properties of Glucuronidase.

Owing to the differences which have existed in relation to many aspects of the behaviour of glucuronidase, it is necessary first to consider the methods which have been used to prepare the enzyme for assay.

Possibly the most significant observations in relation to this problem are those which have been made recently by Kerr and Levvy, (1950). These workers found that glucuronidase was distributed between the mitochondria and the general cytoplasm of the cells and that, whereas by treatment with citrate buffer at about pH 5 this distribution was not affected, when acetate buffer was used, practically all the glucuronidase activity appeared in solution after 4 hours incubation. This implies that by their method of preparation using citrate buffer only that part of the glucuronidase activity which was not associated with the mitochondria was actually estimated, while with the method of preparation employed in the present work, the total glucuronidase activity was assayed. It remains to be decided whether it is more useful to assay the 'soluble' glucuronidase or the total glucuronidase, and whilst Kerr and Levvy provide some arguments to justify their procedure and while investigation of this moiety might prove interesting as a separate study,

it seems most logical that the total amount of the enzymatic activity should be estimated if one desires to obtain evidence relating to its possible function.

It should also be pointed out that in at least some of their estimations these workers used a blue filter in estimating the optical density of the blue solutions formed with the Folin-Ciocalteu reagent, (Kerr, Graham and Levvy, 1948.) The correct filter for use with this colour is the Ilford red 608 filter. While this undoubtedly reduced the sensitivity and probably the accuracy of their method, it is unlikely to have affected the comparative aspects of their work and explanations of discrepancies must be sought elsewhere.

A recent attempt to prepare glucuronidase by the method employed for crystalline catalase was made by Sarkar and Sumner, (1950).

Their method compared favourably with other methods when applied to liver, as regards purity. It is unfortunate that Sarkar and Sumner, (1950) should have chosen to introduce a rather clumsy unit of glucuronidase activity based on the 100 reading of their own colorimeter when the very convenient phenolphthalein and phenyl units proposed by Fishman et al, (1946), Kerr et al, (1948) and Mills, (1948), are in common use.

These authors and also Talalay et al (1946) were unable to verify the claim of Mills (1948) to have demonstrated the existence of two different enzymes in glucuronidase preparations. It is remarkable that points on the pH activity curves which Sarkar and Sumner (1950) produce to support their statement could be made to fit either flat-topped curves or double-peaked curves rather better than the round-topped curves they illustrate. Moreover the substrate concentration used by these workers favours the 4.4 enzyme (glucuronidase I). The same may be said about the figures quoted by Talalay et al and it may also be mentioned that these workers used a pooled extract from a number of different organs for their kinetic studies.

Kerr, Campbell and Levvy (1949) on the other hand, were able to verify the results reported by Mills, (1948). The present studies, repeated by many different workers in this laboratory, indicate that there almost certainly exist at least three and possibly four separate optimum areas of glucuronidase activity between pH3 and pH8. The evidence proposed in favour of this contention may be summarised as follows:

- 1) pH activity curves with quite different optima for the same substrate in the same concentration may be obtained.

2) Substrate-enzyme and inhibitor-enzyme dissociation constants are quite different at these separate optima.

3) The type of inhibition by various inhibitors is different at these separate optima.

4) Activation energies of the enzyme at the separate optima are different.

The problem that now confronts us is whether these different optima represent separate proteins or whether they represent difference of some kind in the function or relationships of the prosthetic group. Some evidence for the reasons for the behaviour at different pHs. may be deduced from the experimental results in section 4.

Let us consider the following observations:

1) The isoelectric point of the enzyme calculated from the suramin method (Wills and Wormall, 1949), corresponds to the isoelectric point calculated from electrophoresis experiments.

2) All the inhibitors described are acids.

3) The affinity of these and of the substrates for the different enzymes increases as the pH decreases. Moreover, the type of inhibition on the alkaline side of the

isoelectric point differs from that on the acid side in many cases, and, in fact, some inhibitors have no action on the alkaline side but are potent inhibitors on the acid side.

We may also consider some of the phenomena observed in relation to the uptake of glucuronide anions by ion-exchange resins in section 2. These may be summarised as follows:

1) Large anions may be adsorbed on to cation exchange resins by van der Waals forces provided the resin remains unionised, but if the resin becomes ionised, the anions are repelled.

2) A similar phenomenon may be observed with anion-exchange resins, but in this case, the capacity of the resin is much greater, since electrostatic forces also assist in binding anions to the resin.

Let us now consider the possible structure of a typical enzyme. It seems likely that it comprises an active or prosthetic group, which itself consists of affinity groupings and functional groupings, and a carrier molecule or apoenzyme of protein nature, probably necessary for energy exchanges,

Now, in view of the fact that all the specific inhibitors and substrates for glucuronides are acids, it seems



not unlikely that part, at least, of the affinity groups of the enzyme are of basic nature. Moreover, these being bases, one would expect them to combine readily with sulphonic acid inhibitors, such as suramin. If this were the case, we would expect such inhibition to occur on the acid side of the isoelectric point. Now we find that the value obtained for this from suramin inhibition corresponds quite closely with the isoelectric point calculated from electrophoresis experiments and thus it seems not unlikely that the affinity groupings in the case of this enzyme correspond to the free basic groupings of the complete protein, i.e. amino-groups mainly.

Let us now consider the behaviour of weak acids towards the enzyme in the light of such a hypothesis. At high pHs where the acid groupings of the protein would be expected to be highly ionised, such weak acids would tend to be repelled by the similar charges on the protein molecule, and these forces would cancel out any tendency to be attracted by the oppositely charged amino-groups. On the other hand at low pHs where the protein carboxyl groups are unionised, one would expect a greater affinity for ionised carboxyl groups in the surrounding solution due to attraction by the ionised amino-groups. At lower pHs still this effect would

disappear due to the free acids themselves ceasing to be ionised. Moreover, mole for mole, dicarboxylic acids would be expected to be attracted more strongly in fairly acid solution than monocarboxylic acids. Thus we have an explanation for the increasing affinity of the different active groups for organic acids as the pH decreases, and also an explanation of the great affinity of dicarboxylic acids for the enzyme, in association with the inhibiting effect of suramin.

It is now necessary to modify this theory somewhat. In addition to affinity groups of this basic type it seems likely that there must be other affinity groups responsible for the specificity of the enzyme and probably associated with a particular configuration of the active area of the enzyme. Some basic groups could conceivably be responsible for seizing the carboxyl group of the substrate and this would explain why organic acids act as competitive inhibitors on the acid side of the isoelectric point. If we concede the necessity for other specific groups, however, it also explains why citrate and oxalate do not inhibit the enzyme on the alkaline side of the isoelectric point, since they have little configurational resemblance to the substrate, and could not be expected to be attracted strongly to the protein at high pHs owing to the high proportion of ionisable carboxyl

groups in the molecule which would tend to be repelled by similarly ionised groups of the protein. Saccharic and mucic acids on the other hand have a fairly close configurational resemblance to glucuronic acid and hence some sort of specific inhibition on this account might be expected. On these grounds alone, however, it is not easy, if indeed possible, to explain why saccharic and mucic acids should act as non-competitive inhibitors on the alkaline side of the isoionic point.

A further point may be made. While such factors as those described may influence the collision rate between molecules of substrate and molecules of enzyme, the ultimate velocity of enzyme action probably does not depend on them at all but rather on the turnover rate of the functional groupings. Thus the effect is to enable maximum velocity to be reached at a lower substrate concentration than would be expected if the factors did not operate.

From a consideration of these points, it might seem possible to explain the occurrence of three or four peaks of activity simply on the basis of such factors, associated with the complications arising from ionisation of different groups of the enzyme, substrate and inhibitors at different times. Thus the entire pH activity curve might be considered to be

an artefact with little biological meaning, but this cannot be true in face of the following facts:

1) On this explanation, one would expect always to obtain the same pattern of pH activity curve on enzyme preparations from the same source, using the same substrate concentration. However, it is possible to obtain preparations with different pH activity curves under these circumstances.

2) The 'Celite' effect finds no simple explanation on such a hypothesis.

3) Salting out curves and electrophoresis experiments indicate that the enzyme is not a homogeneous protein.

Such evidence seems to suggest that there exist several different proteins with glucuronidase activity. Whether these may be produced in the course of preparation of the enzyme or even by altering the pH, which might conceivably cause the proteins to dissociate, or whether they occur naturally remains to be decided. To date the separation of the different proteins has proved exceedingly difficult, and this would suggest a close similarity between them, a hypothesis which in itself does not appear highly improbable.

Many experimental observations from time to time have suggested the possibility of coenzymes in connection with glucuronidase. For instance, the 'Celite' effect, in which

all enzymatic activity is lost below pH 3.4 on passing a solution of enzyme in a high salt concentration through Celite 519A, suggested the loss of a coenzyme of some sort. The fact that activity was not lost, but was in fact regained after 'celiting', on dialysis suggested that if such a coenzyme existed, it was probably a large molecule. There is, of course, no a priori reason why protein coenzymes should not exist. Bernfeld and Fishman (1950) have also adduced some evidence for the existence of a coenzyme. Their interesting observation was to the effect that the ratio (enzyme activity/protein concentration) did not remain constant when a highly purified enzyme preparation was assayed in increasing dilutions, but decreased quite markedly. They claimed that a boiled extract of the enzyme or deoxyribonucleic acid added to the medium abolished the effect. If it may be deduced from these observations that glucuronidase has a coenzyme, then it may also be deduced that the coenzyme is a large molecule, for their observation implies that the enzyme dissociates spontaneously to a degree which limits its activity in dilute solution, and if such spontaneous dissociation occurs one would expect a loss of activity on dialysis unless the molecule was too large to escape. This explanation is not too satisfactory either for one might expect some separation of

coenzyme and apoenzyme during ammonium sulphate fractionation or during electrophoresis if it were true.

It has, however, been observed that when enzyme preparations are kept for some weeks or when they are subjected to fractionation by alcohol or other organic solvents at low temperatures or when they are purified by electrophoresis, all enzymatic activity disappears except that due to glucuronidase II (i.e. at pH 5.2). A possible explanation of such a phenomenon is the existence of a basic glucuronidase molecule with a pH optimum at about 5.2, the appearance of optimum activities at other pHs being due to the presence of more labile, modifying molecules which are readily dissociated from the parent. The parent molecule itself need not necessarily have any glucuronidase activity, of course, and might correspond to the apoenzyme, the coenzyme for activity at pH 5.2 being firmly bound to it.

#### Functional Studies on Glucuronidase.

There has been more controversy in this particular field of late than in any other part of this subject, and it may be worth while to attempt to elucidate the points at issue.

The first theory to be considered is that advanced

by Levvy et al, relating glucuronidase activity to cell proliferation. The results on which Levvy, Kerr and Campbell (1948) based their conclusions were rises in glucuronidase in the liver following partial hepatectomy and the administration of a number of toxic substances and during growth. Their experiments were conducted on mice, whereas rats were used in the present work. In many cases the present experimental results agree with theirs, with the exception of the trends in activity in the liver of the growing rat, but in no case can their conclusions be accepted. There are certainly gross quantitative differences in the glucuronidase activity of organs of different species (section 4) and even strains (Morrow, Greenspan and Harrow, (1949; section 4), and it also seems likely that the differences in the behaviour of liver glucuronidase during growth in rats and mice is a species difference. However, it must be argued that the process of cell proliferation is common to both animals and therefore a generalisation relating glucuronidase activity to cell proliferation cannot be true. Even more important, the conclusions arrived at by Levvy et al (1948) are not supported by their own experimental findings. It is almost superfluous to point out that after the forms of injury inflicted on their experimental animals,

the alterations in biochemical function in the tissues must be legion, and in order to prove a relationship between any one variable and another, it is necessary to have very good evidence indeed. Intimate study of the paper by Levvy, Kerr and Campbell, (1948) indicates that in only a few cases did the peak of glucuronidase activity coincide with the peak of cell proliferation, and in many cases it was markedly different. For instance, after injecting menthol, glucuronidase activity had greatly increased and almost reached its maximum in 24 hours, and yet cell division was reported as zero then and was not reported as high until the third day. The following table shows some results, adapted from their paper, which illustrate the point:

Substance Administered	Day after administration showing maximum			
	Glucuronidase activity.	Tissue damage	Cell Division	Tissue Repair.
Carbon tetrachloride	1	1	7	7
Chloroform	1	1	1 & 8	8
Yellow phosphorus	5	2	5	10

It was noted that high figures for glucuronidase activity shortly after treatment were reported in many of these investigators' findings and the same tendency was noted in some of the experimental results quoted in section 4. It seemed



possible that interference alone might be responsible for causing the increases. In the following statistical study, all the albino rats involved in the experiments with carbon tetrachloride, olive oil and liquid paraffin were divided into two groups, (A) within two days of interference and (B) untreated, or more than two days after interference.

The preliminary analysis of differences between the two groups is as follows:

Group	No.	Phenyl units of Glucuronidase Activity. (100s)
A	12	26.3, 55.6, 27.5, 55.4, 47.5, 30.3, 29.8, 52.4, 45.6, 23.6, 24.2, 26.
B	21	34, 29.7, 36.8, 28.3, 25.6, 28.8, 34.9, 29.5, 37.9, 42.6, 44.2, 24.3, 28.1, 21.8, 22, 32.6, 25.2, 31.2, 31.0, 39.6, 32.8.

Group	No.	d.f.	Mean	Sum of Squares	s <sup>2</sup>	s	C
A	12	11	37.3	1895.4	172.3	13.1	35.1 %
B	21	20	31.1	883.5	44.2	6.65	21.37%
		31	$\bar{x} = 6.2$	$Sx^2 = 2778.9$			

Pooled variance (s<sup>2</sup>) =  $2778.9:9/31 = 89.5$

$Sx = \sqrt{s^2(n_1 + n_2)/n_1n_2} = 3.42$

$t = 1.81$                        $P = 0.08$

This analysis indicates that there is a 1/12 chance of these results being fortuitous, and this is not generally regarded as significant. However, it is obvious that there is a much greater variation in Group A. than there is in the control Group B., the coefficient of variation (C) being more than half as great again. The significance of these differences can be determined by the F. test. Before applying this test, however, it is necessary to observe that, since the above standard type of analysis assumes equal variation about the means in the two groups, it is inaccurate, and the following analysis gives a more accurate interpretation of the findings in a case of this type:

Group	No.	d.f.	Mean	$Sx^2$	$s^2 = Sx^2/d.f.$	$Sx^2 = s^2/n$	Tabular $t_{0.05}$
A	12	11	37.3	1895.4	190.5	15.88	2.207
B	21	20	31.1	883.5	44.2	2.105	2.086
		31	$\bar{x} = 6.2$			17.985	
$S\bar{x} = \sqrt{17.985} = 4.24 \quad \bar{x}/Sx = 6.2/4.24 = 1.462$							
$5\% \text{ level of } \bar{x}/S\bar{x} = (15.88 \times 2.207) + (2.105 \times 2.086)/15.88$ $+ 2.105 = 2.109.$							

Since the value 1.462 is considerably less than 2.109, it follows that no significance can be placed on the difference

between the means of the two groups. However, we may now apply the F. test.

$$\text{Variance ration (F)} = 190.5/44.2 = 4.31 \text{ (n}_1=11, \text{ n}_2= 20)$$

This exceeds the 1% tabular value of 3.3 considerably, and indicates that the amount of variation about the mean in Group A. is significantly greater than the amount of variation about the mean in Group B.

Thus although the actual absolute difference between the means is not significant, there is a recognisable difference between the two groups. There may be a number of explanations for this, but the most obvious one is that the alteration in glucuronidase activity occurred soon after injection, and that in most cases, the levels had returned to normal by the time the animals were killed, but a few remained high enough to influence the analysis. Considering the profound physiological and biochemical alterations that have been described by Selye (1946) as part of the 'general adaptation syndrome' it might not be at all surprising to find more or less non-specific alterations in enzymic activity following interference. Considerable variations might be expected from species to species and some difficulty might be found in explaining the results. The results so far obtained could be explained by this theory, but they could be equally well

explained by a great many more, and therefore such a hypothesis would require a great deal more investigation.

The claims made by other workers, namely Fishman and Anlyan (1947; 1947a); Fishman, Anlyan and Gordon, (1947); Anlyan and Fishman, (1947), that glucuronidase activity is greater in tumour tissues than in unaffected tissues must also receive critical investigation. Their figures convincingly demonstrate that in many tissues normal levels are exceeded by the levels found in malignant tumours arising from them. However, it must be pointed out that some of their control investigations do not survive a superficial scrutiny. For instance, (Fishman, Anlyan and Gordon, 1947), in a case of metastatic ovarian carcinoma, the control tissues consisted of uninvolved rectal mucosa and uninvolved vaginal mucosa. There is only the remotest resemblance between these control tissues and the type of tissue from which the tumour arose. Other examples which may be quoted are the use of myometrium as a control tissue for carcinoma of the uterus and of uninvolved lymph glands for metastasising breast tumours. It is noteworthy that in one set of experiments on carcinomata of the colon where the control tissues were rather more fortunately chosen, no significant differences were observed between affected and unaffected tissues.

The point at issue in this particular problem is whether high glucuronidase activity is a characteristic of malignant cells. The use of unrelated control tissues discussed above, gives a clue to the fallacy inherent in the experiments performed by these workers. In the first place, it has been observed that, in general, tissues of mesodermal origin (with the exception of kidney mesothelium) and in particular muscle, fibrous tissue and blood cells, have a lower glucuronidase activity than those of ectodermal or particularly entodermal origin. Now breast tissue as a rule contains a large amount of connective tissue, and particularly adipose tissue (which is practically devoid of glucuronidase activity). On the other hand, carcinoma of the breast contains practically no adipose tissue, a varying amount of fibrous tissue, and frequently consists almost entirely of malignant cells derived from the glandular cells of the ducts, which are probably responsible for most of the glucuronidase activity of normal breast tissue. There is, indeed, rarely much resemblance between the cellular composition of a breast carcinoma and of a normal piece of breast. Thus the differences in glucuronidase activity probably represent nothing more than changes in the relative proportions of the cellular elements of the tissue, changes which can be

recognised more simply and with more certainty by the microscope. That this interpretation of these findings is justified is borne out by figures quoted by Fishman et al, (1947), for cases of chronic cystic mastitis, which were very high. In one case the specimen consisted of a cyst wall, and the other contained cystic fluid, which would be expected to contain the secretions of the cells lining it. In the face of these considerations, one must have grave doubts about the significance of these authors' startling claims, and there is little doubt that many more carefully controlled experiments will be required before it is possible to correlate glucuronidase activity with malignancy.

#### The Function of Glucuronic Acid and Glucuronidase.

Having considered these various arguments, it must be concluded that we do not yet have any definite knowledge to indicate the possible function of glucuronides and glucuronidase in the animal body. However, the fact that the body can synthesise glucuronic acid suggests that it has a normal function. It may be that it is synthesised in order to be incorporated in mucopolysaccharides and mucoproteins, (which, incidentally, it would be expected to endow with the properties of cation-exchange resins, i.e. strong buffering properties which may be important in the case of mucin). If this

is the case, then we would expect almost every tissue to be capable of synthesising it and this may well be true, only the liver possessing significant quantities of the conjugating enzyme. On the other hand, it might be synthesised in the liver and transported to other parts of the body, possibly in the form of mucoproteins.

Again, it has been a point of discussion since the days of Salkowski and Neuberg (indeed since Schiedeberg and Meyer) whether glucuronic acid might not prove to be an intermediate in the formation of other carbohydrate derivatives, and, in particular, pentoses.

One may also consider whether glucuronides are possibly not formed normally in the tissues, though never excreted in the urine. For instance, the structure of cholesterol is such that it might not be surprising if it were capable of conjugation as a glucuronide. Fatty acids, too, might well form ester glucuronides since Dzewiatkowski and Lewis (1945) demonstrated that trimethylacetate and tertiary butylacetic acids are excreted as glucuronides.

If this is so, the glucuronides would have to be destroyed locally, since we should expect much higher concentrations in the blood than have been reported. In this connection, it may be recalled that in the in vitro liver

slice experiments recorded in section 3 above, there was a suggestion of the formation of an ethyl acetate soluble glucuronide other than menthylglucuronide, and paper chromatography of the material showed a spot in addition to menthylglucuronide. It should be pointed out, however, that too much emphasis should not be placed on these very preliminary studies.

Even greater than the mystery surrounding the possible function of glucuronic acid is that surrounding glucuronidase. Its very wide distribution and analogies with phosphatase suggest that it may be involved in the metabolism of the mucopolysaccharides of connective tissues. Indeed, Meyer, Chaffee, Hobby and Dawson, (1941) have reported observing the hydrolysis of hyaluronic acid by glucuronidase on one occasion. It would be extremely interesting if glucuronidase proved to be associated with mucopolysaccharase. On the face of it, it seems unlikely that glucuronidase would attack aldobiuronides, since these do not have the structure of glucuronides. On the other hand, it is possible, though not likely, that glucuronidase could hydrolyse aldobiuronides from the parent molecule. Such points, however, can only be determined by experiment.

At present, it must be confessed that we know little more about the function of glucuronidase than did



Röhman in 1908, and little more about the function of glucuronic acid than did Schmiedeberg in 1891. A great deal of information has accumulated, much of it unfortunately misleading, and it is to be hoped that the application of reliable techniques and methods may result in the solution of many of the problems confronting us.

SUMMARY.

Chemistry of Glucuronic Acid. Section 2.

- 1) An observation on the behaviour of glucurone in strong alkali is reported, and its significance discussed.
- 2) An attempt to prepare glucuronic acid from the hydrolysis of starch oxidised with nitrogen dioxide is reported.
- 3) The naphthoresorcinol reaction for the estimation of glucuronic acid has been reinvestigated.

a) The tolerance has been markedly extended by the addition of an oxidising agent to the reaction mixture.

b) The use of sulphuric acid has been found superior to hydrochloric acid and permits the use of higher concentrations of naphthoresorcinol.

c) Owing to the achievement of more reliable blank readings by these modifications, accuracy has been increased.

d) Owing to the use of a higher naphthoresorcinol concentration than commonly employed, sensitivity has been increased.

e) By the use of more critical solvents than usually employed, specificity has been made almost absolute in relation to the true sugars, and has been greatly increased otherwise.

f) By a systematic investigation of the mechanism of

the reaction and of the effect of interfering substances, methods have been devised to circumvent their influence.

4) A technique employing ion-exchange resins has been developed for the concentration of glucuronides from very dilute solutions. It is not yet directly applicable to urine.

5) Methods of partition chromatography have been developed for the separation and identification of glucuronides, employing both filter-paper and column chromatograms.

### Natural Occurrence and Synthesis of Glucuronides. Section 3.

1) The naphthoresorcinol reaction, as modified in the present work, has been found to be directly applicable to human urine but not to rabbit urine, in a series of pilot experiments.

2) By neutralising the reducing effect of the glucose in blood by means of an oxidising agent, it has been found possible to estimate directly the glucuronic acid in deproteinised blood by means of the naphthoresorcinol reaction.

3) After the removal of mucin, evidence has been obtained for the presence of glucuronic acid in bile.

4) A preliminary series of experiments on the in vitro synthesis of glucuronides in liver slices is reported.

a) The process was inhibited by cyanide, fluoride and iodoacetate.

b) Synthesis in breis could be achieved by the use of boiled liver extracts but not by the addition of saccharate, cytochrome C, adenosine triphosphate or combinations of these.

c) It was not increased by the addition of glucose, glucuronic acid, or pyruvic acid to the medium, except on an occasion when magnesium was omitted, activity then being increased by all three.

d) Synthesis was greatly increased by the use of a bicarbonate saline in place of a phosphate medium.

e) Synthesis was only found to occur to a significant degree in liver slices, to a smaller degree in kidney, and to a very small and dubious degree in intestinal mucosa.

f) Inhibition by iodoacetate was reversed by the addition of glucurone to the medium. This observation is considered highly important and is discussed.

5) No significant difference in synthetic ability was found between the livers of normal rats and the livers of rats after hepatectomy, rats with fatty livers and pregnant rats.

#### Biological Degradation of Glucuronides. Section 4.

1) The results of investigations into the physical properties

of glucuronidase are reported.

a) Evidence for the existence of at least three, and probably four different enzymes with glucuronidase activity has been obtained.

b) These enzymes have different pH optima, substrate-enzyme dissociation constants and activation energies and their behaviour towards inhibitors differs widely. Reasons for this behaviour are discussed.

c) Attempts have been made to separate these by the application of a number of techniques, some of which are novel. Only partial success has been attained to date.

2) Biological experiments have been conducted with a view to establishing or refuting theories advanced by other workers.

a) During growth, the glucuronidase activity of rat liver increased, whereas in mouse liver, the opposite was true.

b) Inconstant increases of glucuronidase activity in regenerating liver were found, but such increases did not correspond with maximum cell proliferation.

c) Statistical analysis of the liver glucuronidase activities in rats which had been subjected to various forms of trauma or interference, suggest that these factors themselves may have an effect on enzymatic activity.

d) Marked quantitative differences were noted between different species and also between different varieties of rats. There is evidence that the response of different animals to various forms of trauma may also be affected by species differences.

# A P P E N D I C E S

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## Appendix A - Chromatography Apparatus.

### 1. Fraction Collector.

The fraction collector consisted of a circular test-tube rack. Each time electrical contact was made between a stationary brush and contact studs on a disc driven by an electric-clock motor, the test-tube rack was driven round to bring an empty tube under the chromatogram column. The rate of flow from the column being kept constant by maintaining a constant pressure by means of a pressure bottle, equal fractions were collected in the tubes in even spaces of time. A switch enabled the selection of 1, 2, 4, or 8 fractions per hour. The apparatus was very cheap to build and has proved reliable and accurate in operation, and superior to syphon systems. The circuit is illustrated in fig. A.1 and was based on one originally devised by Dr G. Leaf.

### 2. Pressure Bottle

The apparatus used for maintaining pressure consisted simply of a rubber-stoppered Winchester bottle, fitted with a rubber-bulb hand-pump, a mercury manometer and an outlet to the column. After pumping up the required pressure the rubber bulb was isolated by a screw clip. The apparatus was capable of maintaining a pressure of up to 15 cms of mercury for two days with negligible error.

3./



Driving  
Motor

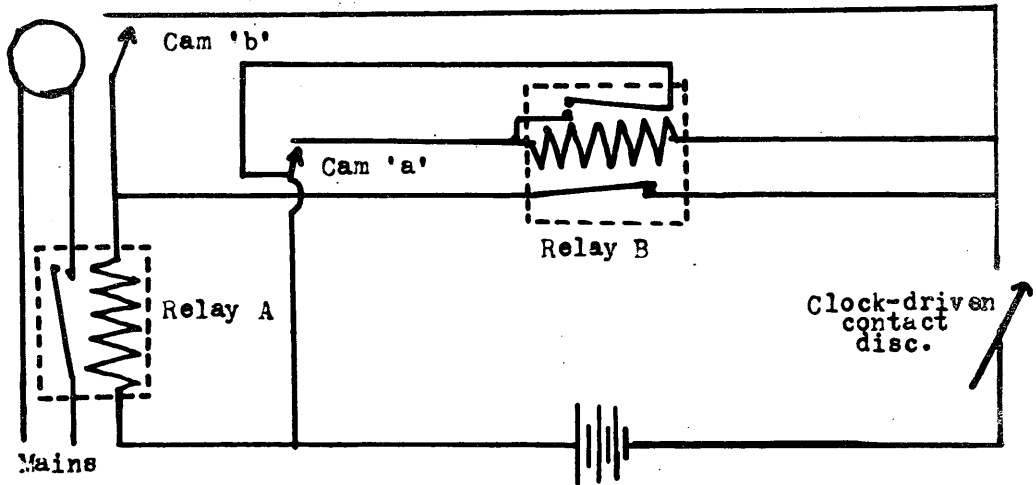


Figure A.1 Circuit for fraction-collector

When the clock-driven contact makes, the relay A is actuated and closes the mains circuit to drive the motor which turns the rack. This also turns cams 'a' and 'b' which complete their circuits simultaneously. Cam 'a' closes the circuit to relay B and this breaks the circuit to relay A and closes a self-retaining circuit. Cam 'b' keeps the circuit to relay A intact until contact is broken. At this moment the motor shuts off and remains off owing to relay B remaining in operation. Finally, when the clock-driven contact breaks, the current to relay B is cut off and the circuit is thus reset.

### 3. Automatic Pipette or Dispenser.

For mass analyses the simple dispenser illustrated in fig A.2 was found both convenient and surprisingly accurate. Liquid to be dispensed is delivered to the three-way tap under pressure. When this is turned to allow communication with the barrel of the syringe the pressure forces the plunger up till it is halted by the adjustable stop. By turning the tap the liquid is then allowed to escape by the outlet, being assisted by the weight of the plunger. Where large syringes are used it has been found advisable to weight the plunger.

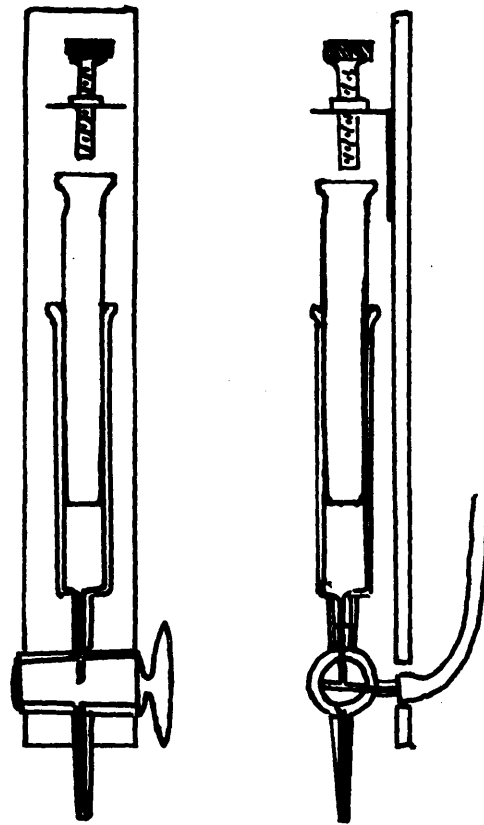


Figure A.2 Automatic Pipette.

Appendix B -Enzyme Kinetics.

(General reference - Lineweaver and Burk (1934)).

Analysis of enzyme kinetics was made by the methods proposed by Lineweaver and Burk. Based on the Michaelis-Menten (1913) equation ( $v = V_{max} (S)/(K_s + (S))$ ) these authors deduced linear equations

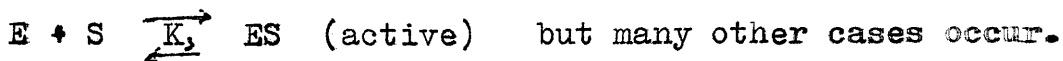
(i)  $1/v = K_s/V_{max} (S) + 1/V_{max}$  and

(ii)  $(S)/v = (S)/V_{max} + K_s/V_{max}$

((S) - substrate concentration; v - observed velocity; V<sub>max</sub> - theoretical maximum velocity; K<sub>s</sub> - substrate-enzyme dissociation constant)

Thus by plotting 1/v against 1/(S) in case (i) and (S)/v against (S) in case (ii) values may be obtained from the slope and intercept from which the unknowns can be calculated readily.

Such mathematical interpretations are based on the assumption



In particular there is the case ( $2E + S + I \rightleftharpoons ES$  (active) + EI (inactive)) representing the action of inhibitors.

Lineweaver and Burk also deduced for this the linear equation,

$1/v = (1/V_{max})(K_s + K_s(I)/K_1) (1/(S)) + 1/V_{max}$  (iii)

Again/

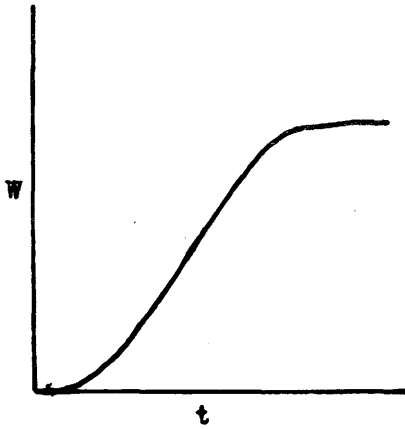
Again  $1/v$  may be plotted against  $1/V_{max}$  to give a straight line. If a velocity curve in the absence of inhibitor is analysed simultaneously in a similar manner, (I) being known then it is possible to calculate  $K_1$ , the enzyme inhibitor dissociation constant. Competitive inhibition is recognised by a line of different slope which has the same intercept on the ordinate as the line deduced from the findings in the absence of inhibitor. In non-competitive inhibition on the other hand, the two lines do not meet at the ordinate, but the line due to inhibitor has a higher ordinate intercept.

The third case considered in this study was the case of substrate inhibition,  $E + S \xrightleftharpoons{k_s} ES$  (active),  $ES + (n-1)S \xrightleftharpoons{k_2} ES_n$  (inactive) and Lineweaver and Burk have also deduced linear equations which may be applied to determine the values of  $n$  and  $k_2$  in this case. All that need be said is that  $k_s$  values may be calculated as before by using the values obtained at low substrate concentrations where the effect of substrate inhibition is negligible.

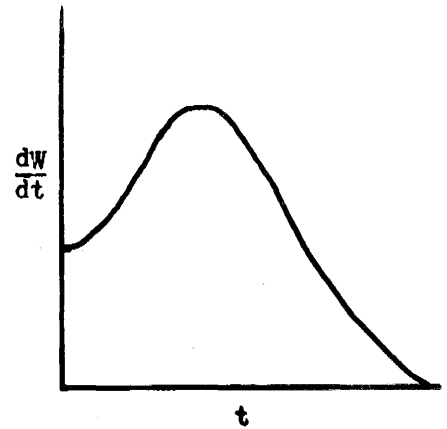
Appendix C - Interpretation of Growth

(General reference - Medawar-In-Essays on growth and form. Oxford, 1945).

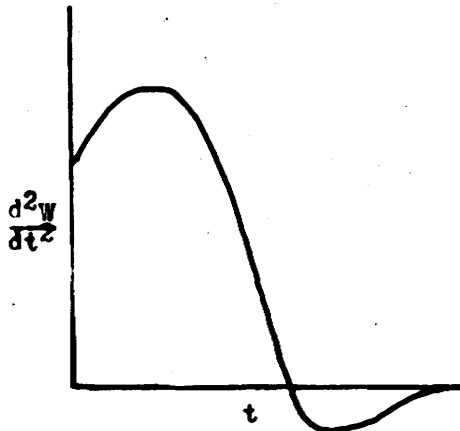
Growth, may, of course, refer to any increment but in this discussion it will be convenient to refer to increments of weight. When body weight is plotted against time in a growing animal the curve A (fig A.3) is normally produced. From this there have been deduced the two curves B and C to represent the growth rate and acceleration of the tissue respectively. However, these curves convey little evidence about the state of proliferation of unit mass of the organ or, in other words, the rate of proliferation of the individual cell. Such interpretations must be derived from the increment of unit mass of the tissue. Minot (1908) suggested plotting  $DW/WDt$ , against 't' where DW and Dt represent increases in weight and time respectively. (curve F). Minot's formula has been objected to on the grounds that, particularly in the early stages of growth, it is not possible to measure DW as a small enough increment. However, if we consider the increments of weight and time to be infinitely small we can express this formula in the differential form  $dW/W \cdot dt$ . By integration and redifferentiation this can be shown to be the same as  $d \log W / dt$  which is/



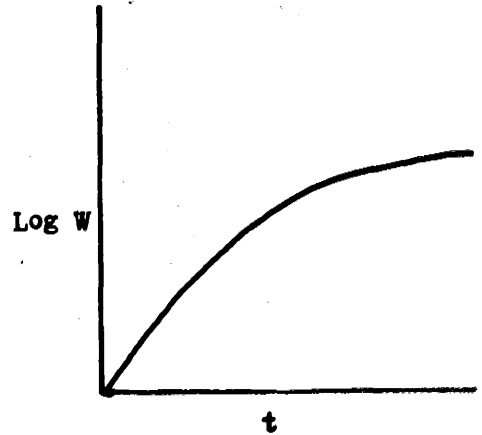
A. Growth Curve



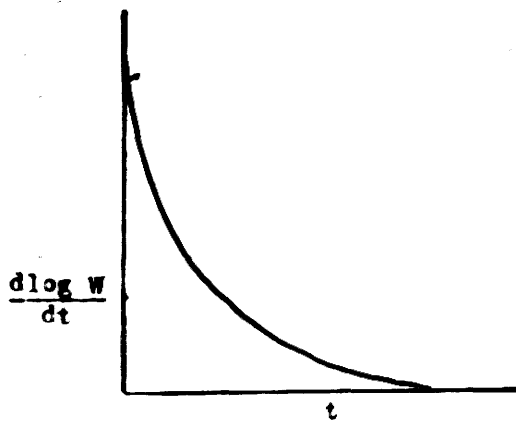
B. Growth Rate



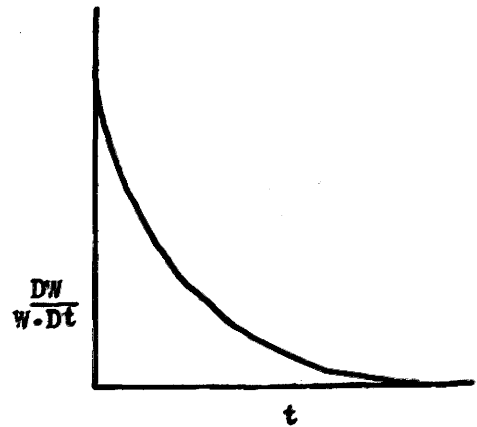
C. Acceleration



D. Specific Growth



E. Specific Growth Rate



F. Minot's Formula

Figure A.3

Methods of Growth Analysis

is the same as the specific growth rate (E) which itself was originally derived empirically from the specific growth rate (D). This method of plotting (E) is an expression of the rate of proliferation of a unit number of cells. The curve formed is of the familiar "die-away" type indicating an exponential relationship between  $d \log W / dt$  and 't'. It was a point for discussion among biologists for many years whether there was any justification in applying the yardstick of man-made time to such measurements. Arithmetical time is, of course, only a convention. In actual fact if time is plotted on a logarithmic scale many growth formulae may be made linear, which is another way of saying they involve exponential relationships.

In order to circumvent the necessity of considering time Huxley (1924) proposed the concept of allometry in relation to growth. This assumes that two organs growing in the same organism are related by the equation  $y = bx^a$  (y and x representing the two organs. The formula can be derived from two exponential equations involving 't'.) This formula may also be expressed in the form  $\log y = \log b + a \log x$  and by plotting  $\log y$  against  $\log x$  one obviously obtains a straight line from which 'a' may be derived/



derived as the slope of the line. When 'a' = 1 the conditions of isometry are fulfilled, i.e. the growth of the two organs is directly related. If 'a' is greater than 1 it indicates a more rapid accumulation of 'x' than 'y', and vice versa.

These formulae are very convenient for obtaining a rapid impression of the relationship between two different factors but they are in many cases liable to be affected by subjective factors in fitting curves to points. It is more satisfactory to invoke the admittedly more tedious methods of modern statistics by which the covariance of two variables can be estimated without subjective errors by calculating the regression of one factor on another. In this way, for instance, it is possible to apply the allometric method with some degree of reliability.

Appendix D - Statistics.

(General reference - Snedecor, 1946)

For convenience a short account of the conventions used and their meaning and significance is presented here.

m - expected results.       $\bar{X}$  - mean.      n - total number  
s - sum of.      d.f. - degrees of freedom.      x - deviation from  $\bar{x}$   
(e.g.  $x = X - \bar{x}$ )       $s^2$  - mean square or variance. (=  $Sx^2/n-1$ ). s -  
standard deviation.       $S\bar{x}$  - standard error ( $s/ n$ ). C - coefficient  
of variation (=  $s/\bar{x}$  %)

Most statistical calculations are based on the principle of testing the validity of the null hypothesis that there is no significant difference between the results obtained and the results expected.

(i.e. in most cases between tests and controls.)

t - a ratio defined by the formula =  $\frac{(\bar{x} - m)}{S\bar{x}}$ . Comparison of the experimental value of t with those in a normal distribution table of t values gives a value for P.

P - probability, expressed as a ratio or as %. A null hypothesis is usually rejected at the 5% level but if a very exacting estimate of significance is required it may only be rejected at the 1% (i.e. 0.01) level.

F - variance ratio ( $\frac{\text{Mean square of group means}}{\text{Mean square of individual means}}$ ).

This/

This ratio provides a very convenient comparison of the degree of variation among the individuals of different groups. By reference to normal distribution tables of F the significance of such variations may be estimated.

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