β- GLUCURONIDASE.

by

Evelyn Elizabeth Brown Smith B.Sc.

A Thesis presented for the Degree of Doctor of Philosophy in the University of Glasgow.

Department of Biochemistry

February 1952.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Acknowledgements.

It is a pleasure to record my appreciation to all who have assisted me in the course of the present research. In particular I wish to express my thanks to Professor Davidson for all the facilities offered in the course of this study. While there are few people in this Department to whom I am not in some manner indebted, I also wish to express my thanks to Dr. Lominski for advice on problems of bacteriological origin, to Dr. Leaf for his chromatographic analysis of the purified β-glucuronidase, and to Dr. Reid, Mr. Watson and Dr. Ongley for the provision of a number of organic compounds for inhibition studies.

Most of all I am indebted to Dr. Mills for introducing me to an interesting and stimulating problem, while his constant advice and encouragement throughout the course of this research has proved invaluable.

The major part of the present work was rendered possible through the generosity of the Carnegie Trust for the Universities of Scotland, to which Trust I also wish to express my appreciation.

CONTENTS.

		Page
Section I.	Introduction.	1
Section II.	General Methods.	28
Section III.	Physical Properties of β-Glucuronidase.	
	A. Purification of β-Glucuronidase.	36
	B. Properties of Purified β-Glucuronidase.	46
	C. Fractionation of Ox Liver and Spleen β-Glucuronidases.	64
	D. Kinetic Studies on β-Glucuronidase.	79
Section IV.	Physiological Rôle of β-Glucuronidase.	91
Section V.	Bacterial Glucuronidase.	103
Section VI.	Discussion	124
	Summary	161
	References	163

β-Glucuronidase may be defined as an enzyme capable of hydrolysing β-glucuronides into glucuronic acid and an aglycone. It is an enzyme which is widespread throughout the body tissues, and to which, as yet, no specific function has been assigned. Indirect evidence has contributed much to the field of speculation in this respect, but while in vitro experiments demonstrate the hydrolytic function of the enzyme, our knowledge of its function in vivo is slight. Nevertheless a wealth of experimental detail has accumulated providing the basis of an interesting and stimulating problem.

The earliest reference to the enzymic splitting of glucuronides is that of Neuberg and Niemann (1905), who observed the hydrolysis of euxanthic acid (euxanthone glucuronide) and phenylglucuronide by plant emulsin. Other preparations of emulsin were shown to split vanillin glucuronide, (Hildebrandt 1905), l-camphoglucuronide (Hämäläinen 1901), phloroglucin glucuronide (Cremer and Seuffert 1912), campho- and menthyl-glucuronides (Ishidate 1929), and the pigment baicalin (5,6,7, trihydroxy flavone glucuronide),(Miwa 1936). Inasmuch as emulsin was specific for the glucosidic linkage, it was further suggested by Hildebrandt (1906), that the glucuronides tested were in effect β-glycosides.

The splitting of menthylglucuronide by an enzyme present in dog liver was first discovered by Röhmann (1908). This observation was pursued by Bass (1911) who showed that, while hydroquinone glucuronide was split by liver and kidney prepar-

ations from dog or rabbit, arbutin, the glucoside of hydroquinone was unaffected. He therefore concluded that the hydrolysis of conjugated glucosides and glucuronides was catalysed by two specific enzymes. This first reference to the specificity of the glucuronide-splitting enzyme was substantiated in later years by other workers. Sera (1913) failed to obtain the hydrolysis of vanillin glucuronide with emulsin after a prolonged period of incubation. He further observed (Sera 1914a), that while phloroglucin glucoside was hydrolysed rapidly by purified enulsin, the corresponding glucuronide remained unaffected. The latter however, was readily split by extracts of liver, kidney and spleen, from either ox, rabbit or dog (Sera 1914b).

Bergmann (1933), observed that β-naphthyl β-glucuronide was not split by either emulsin or takadiastase, while Helferich and Sparmberg in the same year, (Helferich and Sparmberg 1933), showed that the splitting of menthyl-β-D-glucuronide by purified emulsin was not paralleled by the hydrolysis of menthyl-β-D-glucoside.

The differentiation between conjugates of glucose and conjugates of glucuronic acid thus appeared to be reasonably well established at this period, and it remained for Masamune (1934), to confirm that enzyme extracts of ox kidney, while catalysing the hydrolysis of phloroglucinol, menthyl-, bornyl- and β-naphthyl- glucuronides, did not attack a number of related α or β glucosides. He concluded that the enzyme was different from β-glucosidase, and inasmuch as α-menthylglucuronide remained

intact when incubated with the enzyme solution, suggested the name "β-glucuronosidase" for the enzyme. The latter term has now been abbreviated to β- glucuronidase, and while no further investigations on the optical specificity of the enzyme have been reported, the term "β- glucuronidase" is employed in view of lack of evidence to the contrary. Moreover the existence of naturally occurring α glucuronides is unknown.

Properties of β - Glucuronidase.

I. Occurrence.

Miwa (1935,1936), and Shibata (1923,1930), isolated from the roots of Scutellaria baicalensis, an enzyme hydrolysing baicalin (5,6,7, trihydroxy flavone glucuronide) and scutellarin (4',5,6,7, tetrahydroxy flavone glucuronide), which they called baicalinase. Various emulsin preparations also exhibited glucuronide splitting properties, (Neuberg and Niemann 1905; Hildebrandt 1905; Hämäläinen 1910; Cremer and Seuffert 1912; Ishidate 1929).

The distribution of the enzyme in animal tissues is widespread, the first comparative study being reported by Oshima (1934). Using chloroform-water extracts of ox and dog tissues, and menthylglucuronide as substrate, he showed that spleen and such endocrine organs as suprarenals and thyroid were particularly rich in the enzyme, liver, kidney and lung moderately so, while brain, uterus and pancreas were poorly represented. Little or no glucuronidase activity was detected in blood. In recent years however, Fishman, Springer and Brunetti (1948), have shown that in man, the enzyme is present in blood mainly in the leucocytes

and lymphocytes with little or no glucuronidase activity in the erythrocytes and platelets. Rossiter and Wong (1950), in a study of rabbit blood have confirmed this finding, and have shown that the mean activity of the white cell is some 2,000 times greater than that of plasma.

Few details on the distribution of the enzyme in animal tissues have since been published, but the original work of Oshima (1934) has been amply confirmed in this laboratory, while spleen is well known to be the richest source of the enzyme.

The production of a glucuronide decomposing enzyme by bacteria has also blaimed considerable attention in recent years, a subject which it is proposed to discuss in detail under a separate heading.

2. Substrates.

A convenient biosynthetic method for the preparation of substrates for the assay of β- glucuronidase is provided by the so-called "detoxication reactions" of the body. The term in its more general sense implies those chemical changes which foreign organic compounds undergo in the body, in being converted to derivatives which are excreted in the urine. Frequently we find that substances of an alcoholic or phenolic nature are conjugated in β- glycosidic linkage with glucuronic acid and eliminated in this manner. Examples of endogenous origin are found in the sex hormones, (e.g. oestriol glucuronide), while exogenous materials such as phenol and borneol may also be eliminated through the intervention of glucuronic acid. Compounds which form glucuronides

are those which possess, or can form by oxidation or reduction, the following groups, (Williams 1947):

(I) OH in almost any type of compound.

(2) COOH group in certain types of aromatic compounds.

We have therefore, conjugation according to two well defined methods. (a). In conjugated glucuronides of the ether type, the OH group of an alcohol or phenol is conjugated with the HO of the hemi-acetal group of β- glucuronic acid, the substances thus formed being analagous to the β- glucosides. (b). Conjugation also occurs through an ester linkage, where we have esterification of the COOH group of an aromatic acid with the hemi-acetal group of β- glucuronic acid.

The number of possible conjugates of glucuronic acid is obviously considerable, and while a large number of the latter have been isolated from the urine of experimental animals following oral or parenteral administration of the requisite aglycone, certain limitations are indicated in a choice of suitable substrate. Development of assay procedures favours the selection of phenolphthalein glucuronide (prepared by the method of Talalay, Fishman and Huggins 1946), and phenylglucuronide (prepared by the method of Masamune 1933 as modified by Garton, Robinson and Williams 1949) for a study of enzymatic activity.

3. Methods of Assay.

Hydrolysis of conjugated glucuronides results in liberation of the aldehydic group of glucuronic acid with subsequent increase

in reducing power of the digest. Masamune (1934) and Oshima (1934) measured the activity of β-glucuronidase on the basis of this reaction, using the Hagedorn and Jensen method for estimation of reducing power. The difference between control tubes containing inactivated enzyme and experimental tubes, was taken as a measure of the liberation of free glucuronic acid due to enzymic hydrolysis of the β-glucosidic linkage. Masamune (1934) also estimated the free menthol and glucuronic acid liberated from menthylglucuronide and found that they were equivalent, thus eliminating the possibility of non-hydrolytic decomposition of the substrate.

For bornyl- and menthylglucuronides, the reductimetric method can be applied directly to the protein free filtrates. When β-napthyl- and phenylglucuronides are used however, the ferricyanide is reduced not only by the free glucuronic acid, but also by the free phenols, and these must be removed by extraction before the reducing power of the residual solutions is measured.

In a re-examination of the assay procedure, Fishman (1939a) substituted a modification of the ceric sulphate titration of Miller and van Slyke (1936), and using sodium menthylglucuronide and bornylglucuronide as substrates, the reducing powers were compared by means of a standard curve of pure glucurone. Levvy (1946), described a method for the cerimetric determination of glucuronic acid in tissue preparations for values ranging from 10 - 300 µg., using the Conway micro-burette.

To remove protein from the enzyme digests, Levvy favoured the use of alkaline copper tungstate as precipitant. Mills (1948), however, found the method employed by Levvy (1946) to be unsatisfactory if the pH of the enzyme digest was less than 4.6, and recommended the retention of Fishman's (1939a) trichloracetic acid deproteinisation method. In the latter, the neutralisation of excess trichloracetic acid after removal of protein was accomplished by N. sodium hydroxide in the presence of phenolphthalein. This method gives rise to large blank values in the subsequent ferricyanide reduction, and Mills (1948), suggested as the most satisfactory procedure, a trichloracetic acid deproteinisation followed by the addition of excess sodium carbonate, thus eliminating the use of an acid-base indicator.

All assay procedures dependent on the measure of increase in reducing power of the enzyme digests are necessarily subject to certain limitations. Where tissue extracts or biological fluids are involved in assay, reducing substances other than glucuronic acid may well be present in relatively high amounts. To ensure reliability of results, the increment in reducing power produced by enzymatic action per se must be large compared to the control. This necessitates the use of very long incubation times or of large amounts of enzyme. Moreover the production of nonglucuronic acid reducing substances in the reaction mixture due to the presence of enzymes other than β-glucuronidase in tissue extracts cannot be excluded; this will not occur in the controls if these contain heat inactivated enzymes. Again

if the controls are set up without substrate, but containing the active enzyme extract, this may act on preformed natural glucuronides present in the tissue extracts, and thus increase the reducing power of the controls.

Quantitative methods for the estimation of glucuronic acid, such as a spectrophotometric modification of the Tollen's naphthoresorcinol reaction (cf. Hanson, Mills and Williams 1944), cannot be readily applied to the measurement of glucuronidase activity, since, under the conditions of the test, strong hydrochloric acid hydrolyses the glucuronides, and does not distinguish between free and conjugated glucuronic acid.

To quote Talalay, Fishman and Huggins (1946): "the need exists for a method depending either on a specific quantitative reaction distinguishing free and combined glucuronic acid, or upon a satisfactory assay of the non-glucuronic acid part of the substrate molecule, with a minimum of interference from naturally occurring substances. Such a method should be sensitive and eliminate long hours of incubation, with the consequent objections of decay in reaction velocity and destruction of the enzyme, and bacterial action in the mixture".

The above paragraph prefaced the experimental details of a method of estimating β-glucuronidase aglucuronometrically, using phenolphthalein glucuronide as substrate. The method is one of reliability and simplicity of technique. It is difficult to conceive of a more elegant procedure, the principles of which are essentially as follows.

The monoglucuronide of phenolphthalein is yellow in alkaline

solution, while free phenolphthalein exhibits its maximum stable colour intensity at pH 10 - 10.4. The absorption spectra of these two compounds in the visible range were compared in 0.4 M glycine buffer, pH 10.4, and while it was found that the principal maximum of phenolphthalein-mono-β D - glucuronide was 415 - 420 mμ, that of free phenolphthalein was 550 - 555 mμ. The corresponding molecular extinctions of phenolphthalein and its mono-β-D-glucuronide at 552 mμ are respectively 26,000 and 48. Hence at the absorption peak of phenolphthalein, the monoglucuronide has only 0.18% of the absorption of free phenolphthalein for the same concentration of the two compounds. These marked differences in position and degree of absorption maxima made it possible to use phenolphthalein-mono-β-glucuronide as substrate by measuring free phenolphthalein colorometrically near its absorption maximum. The removal of absorption due to the monoglucuronide, and its replacement by free phenolphthalein will only introduce an error of 0.2% compared to the control if measured at 550 mμ.

The use of phenylglucuronide in the assay of β-glucuronidase was detailed by Kerr, Graham and Levvy (1948), and Mills (1948), in a method based upon the determination of phosphatase using phenyl phosphate (King and Armstrong 1934; Folley and Kay 1935). Phenylglucuronide is incubated with the enzyme preparation in a buffer solution. After a standard time the reaction is stopped, and the free phenol estimated using the reagent of Folin and Ciocalteau (1927). It should be mentioned in this

respect that Kerr et al (1948) employ an Ilford 602 Blue filter for the photometric estimation of the blue colour developed under the conditions of reaction, a fundamental error in photometric principles.

From a comparative point of view, the use of phenolphthalein glucuronide as substrate provides the more simple and direct method of assay. The necessity for blanks is obviated, while the enzyme reaction can be conveniently stopped by the addition of glycine buffer, pH 10.4, to the reaction mixture, the colour so developed being read directly after removal of protein by centrifugation.

In the case of phenolphthalein glucuronide, the colour of the aglycone is measured in the visible part of the spectrum. More recently, Spencer and Williams (1951), have introduced a spectrophotometric method of assay using p - chlorphenyl-glucuronide as substrate. In alkali, p-chlorphenol has absorption bands at 245 and 298 mμ., where the absorption of glucuronide is negligible, and this sharp difference in the ultra-violet absorption spectra between the anion and the glucuronide provides the basis of a new assay technique. The comparative ease with which pure crystalline p-chlorphenylglucuronide can be obtained biosynthetically is an asset, but a consideration of the technicalities involved in accurate determinations of enzyme activity using the latter as substrate, inevitably leads to the selection of the more rapid and efficient method of assay as outlined by Talalay et al. (1946).

4. Preparation and Purification of the Enzyme.

Preparation of the enzyme for assay varies according to the nature of the experiment, ranging from straight tissue homogenates in water to highly purified material. As the study of the properties and kinetics of the enzyme system advanced, so also it became necessary to obtain the relevant data from more pure material.

The first attempt to purify the β-glucuronidase of animal tissues was that of Masamune (1934). Briefly the method consisted of liberation of the enzyme from a 0.85% sodium chloride homogenate of ox kidney by autolysis at 38° C. for three days. Following centrifugation, three volumes of 95% ethanol were added to the supernatant, the precipitate removed, dried with filter paper and extracted overnight with water at 0°C. Inactive protein was then removed by the addition of H_2SO_4, the solution centrifuged, and the enzyme precipitated from the supernatant with three volumes of absolute ethanol. The precipitated material was partially dried in vacuo, resuspended in water, and denatured protein removed by filtration. Oshima (1936), carried the purification procedure of Masamune (1934) a step further by adsorption of the enzyme on kaolin at acid pH, with subsequent elution in an alkaline medium. In neither instance is any data given on the purity of the final material.

Fishman (1939b), however realised little success in obtaining a potent purified enzyme extract by the procedure outlined by Masamune (1934), Hardly a surprising fact in view

of the rather harsh treatment employed by the latter worker. A method of purification of ox spleen β-glucuronidase was accordingly devised by Fishman based on :

(a) An initial extraction of the minced tissue with water.

(b) Acetone precipitation of the crude extract and subsequent extraction of the precipitate so obtained with water.

(c) Acidification of the aqueous extract with acetic acid to pH 4.8 - 5.0, and autolysis at 37.5°C. for twenty minutes.

(d) Evaporation to small volume in a current of air.

(e) The application of ammonium sulphate fractionation to the concentrate.

140 fold purifications were achieved in this manner, with however, an overall loss in activity of 85%.

A much improved method of purification of ox spleen glucuronidase was introduced by Graham (1946), in which a 315 fold purification of the enzyme was achieved with an overall loss in activity of 50%. Precipitation by organic solvents was avoided, although an initial extraction of minced spleen with acetone was found to be distinctly advantageous, reducing the total protein extracted to one third with no loss in enzyme activity. Autolysis at 37°C., pH 5.0, of an aqueous extract of acetone treated spleen powder was followed by ammonium sulphate fractionation. The variation in solubility of the enzyme at pH's between 4.3 and 7.3 was found to be different from that of much of the inactive protein, and fractionation with ammonium sulphate was carried out at three different pH's between these limits.

In 1925, Cohn showed that the solubility of proteins in salt solutions obeys the equation :

$$\log S = \beta - kI,$$

where S = solubility, I = ionic strength and β and k are constants. While k is independant of temperature and pH, β is markedly influenced by both these factors. The importance of temperature and pH control was emphasised by Mills (1948), in a more critical evaluation of the factors affecting the fractionation of ox spleen β-glucuronidase. Graham (1946), while taking into consideration the effects of pH during ammonium sulphate fractionation did not indicate how the limits employed were derived, and in order to obtain more precise data on this point, Mills (1948), applied the variable solvent solubility test as defined by Falconer and Taylor (1946) to crude glucuronidase solutions at two different pH values. The data obtained at pH 5.0 and 7.0 revealed the existence in spleen extracts of two proteins having glucuronidase activity. Interpretation of the limits of solubility as indicated by the solubility test permitted the separation and purification, by fractional precipitation with ammonium sulphate, of two enzyme fractions having different pH optima. It is apparent from the work of Masamune (1934), Oshima (1936), Fishman (1939 a & b) and Talalay et al (1946), that these authors considered that they were dealing with a single enzyme, nor does the data of Graham (1946) provide any evidence which could indicate that there was more than one enzyme present in his extracts. As pointed out by Mills (1948) however : "at pH 5.0 the two proteins having glucuronidase activity are precipitated by ammonium sulphate

within the limits 31.5 - 38.5% and 38.5 - 44% saturation..... Fishman (1939b) used the limits 37 - 50% saturation and Graham (1946) 36 - 46% saturation with ammonium sulphate in the preparation of their enzyme extracts. It is obvious therefore that these workers eliminated the major portion of one of the enzymes.."

5. Factors Influencing Enzyme Activity.

(a). Hydrogen ion concentration.

The original investigations of Masamune (1934 a & b), Hofmann (1935) and Oshima (1936) on the β-glucuronidase of various tissues indicated that enzyme activity was maximal in the pH range 5.0 - 5.6 for the hydrolysis of menthylglucuronide, phenylglucuronide and bornylglucuronide in both acetate and citrate buffers. Fishman (1939 b), using the limits 37 - 50% saturation with ammonium sulphate for precipitation, confirmed that fractions so obtained from ox spleen had a pH optimum for the hydrolysis of laevo-menthylglucuronide of 5.2. In a study of the velocity of hydrolysis of glucuronides by ox spleen extracts (Fishman 1940), optima were given for the hydrolysis in acetate buffer of bornylglucuronide and oestriolglucuronide of 4.4 and 4.3 respectively.

In later work Talalay et al (1946) pooled a preparation from a mixture of livers, spleens and kidneys of mice and obtained an optimum for the hydrolysis of phenolphthalein glucuronide of pH 4.5. The word "optimum" is interpreted by Talalay et al (1946) in its widest sense however, the curve presented showing no sharp peak of activity, but exhibiting a well defined plateau over the pH range 3.4 - 5.4 in acetate buffers.

That this variability in opinion is due to the presence of two separate enzymes was shown by Mills (1948). The two enzymes were separated and purified by fractional precipitation with ammonium sulphate, and were shown to have the following optima: with laevo-menthylglucuronide 4.5 and 5.0; phenylglucuronide 4.5 and 5.2; phenolphthalein glucuronide 4.5 and 5.2. Up to this point no indication had been given by previous workers of the dual nature of the enzyme system. An activity curve of the type presented by Talalay et al (1946) is suggestive of the presence of more than one enzyme, but where we are dealing with impure enzyme material, the interpretation of results is obscured by a variety of other factors. Kerr, Campbell and Levvy (1949), in a study of the β-glucuronidase of uterus and other organs considered that the shapes of the curves for the hydrolysis of phenylglucuronide by mouse liver or kidney extracts indicated the presence of the two glucuronidase fractions found by Mills (1948) in ox spleen, and his technique was applied to their separation. Between the limits 31.5 - 38.5% saturation with ammonium sulphate an enzyme fraction was precipitated with optimal activity at pH 4.5 for the hydrolysis of phenylglucuronide, while precipitation between 38.5 - 44% permitted the separation of the enzyme with a pH optimum of 5.2, all assays being determined in citrate buffers. Both β-glucuronidase fractions found by Mills (1948) in ox spleen were thus shown to be present in mouse liver and kidney. In the case of the uterus however, the activity curve was symmetrical about pH 4.5

in the initial homogenate, and subsequent fractionation of the latter with ammonium sulphate in the limits defined failed to demonstrate the presence of the 5.2 enzyme.

(b) Substrate Concentration.

Fishman (1939b) provided the first published data on the effect of substrate concentration on the reaction velocity of β-glucuronidase. In a study of factors affecting the rate of hydrolysis of the sodium salts of oestriol, bornyl- and menthyl-glucuronides by ox spleen extracts, Km values calculated according to the method of Michaelis and Menten (1913) were found to be substantially different for the three substrates studied, as summarised in the following table:

pH (Acetate Buffers)	Substrate	Km
4.3	Na oestriolglucuronidate	0.0005
4.4	Na bornylglucuronidase	0.01
5.3	Na menthylglucuronidase	0.04

The enzyme is seen to have a much greater affinity for oestriol glucuronide than for the other two substrates, where affinity is expressed by I/Km.

Talalay et al (1946), introducing the use of phenolphthalein glucuronide for β-glucuronidase assays, studied the effect of varying substrate concentration on enzyme activity, using as enzyme source a collective extract of mouse liver, kidney and spleen. On plotting reaction velocity against substrate concentration in the classical manner, a slight inhibitory effect was

observed at high concentrations of the latter, amounting to 15% for a five fold increase in substrate concentration above that at which the maximum velocity was obtained. The maximum velocity was observed at 0.0005 M phenolphthalein glucuronide, and a substrate concentration of 0. 001 M.was chosen for assay, at which point the velocity is close to maximal, while changes in substrate concentration of ± 10% produce a change in velocity of only ± 0.5%. Calculation of the Michaelis-Menten constant for phenolphthalein glucuronide gave a value of 0.000053 M. (acetate buffer pH 4.5); this is considerably lower than the constants obtained for oestriol, bornyl- and menthylglucuronides, (Fishman 1939b)

The validity of reaction kinetics on relatively impure enzyme preparations is open to criticism, particularly in view of the apparent duality of the system. The date presented by Fishman (1939b) for the hydrolysis of menthylglucuronide may, however, be substantially correct for one of the enzymes, that of optimum pH 5.2, as his method of purification would theoretically eliminate a major portion of the enzyme with optimum at pH 4.5. Talalay et al (1946), however, give no indication of the multiplicity of the system, but from a study of the pH activity curve of their enzyme preparation, we may reasonably assume that the Km value quoted is obtained from an enzyme mixture. An additional factor is also apparent from the work of Kerr, Graham and Levvy (1948). Talalay et al (1946) assumed that the conditions for optimum hydrolysis by β-glucuronidase are the same no matter

the source of the enzyme; Kerr et al (1948) observed however, that the substrate optimum for spleen enzyme in the case of phenylglucuronide was greater than that of liver, the values being 0.025 M and 0.01 M respectively. Inhibition of β-glucuronidase by excess substrate was also noted by the above workers to be much more pronounced with phenylglucuronide than with phenolphthalein glucuronide.

Little further data is available on the kinetics of the enzyme system. Activity has been shown to be linear with enzyme concentration for bornylglucuronide (Fishman 1939 b), phenolphthalein glucuronide (Talalay et al 1946) and phenylglucuronide (Kerr et al 1948). A consideration of the effect of temperature on the enzyme system recalls the earlier work of Oshima (1936), who records a Q_{10} value for ox spleen β-glucuronidase of 2.66 for a temperature range of 20°-50°C. Application of the Van't Hoff equation:

$$\frac{d.\ln.k}{dT} = \frac{q}{RT^2} \quad \text{where } q = \text{heat of activation,}$$

gave a value of 36,340 calories for the heat of activation of the enzyme.

The need obviously exists for a more comprehensive characterisation of the β-glucuronidase system. The relevant data is very incomplete and in many aspects conflicting.

III. Physiological Rôle.

When one considers the function of the enzyme system in vivo, the situation is equally confusing. While many suggestions as

to the physiological role of β-glucuronidase have been advanced, the explanation of the function of the enzyme is not yet apparent. Nevertheless the accumulation of both experimental detail and circumstantial evidence is diverse.

The fact that β-glucuronidase is capable of hydrolysing conjugated glucuronides into glucuronic acid and the relevant aglycone was established by Levvy (1948). Until this time it was generally assumed that the hydrolytic action of the enzyme was confined to rupture of the glycosidic linkage. Evidence for this however was indirect, and restricted to experiments by Nasamune (1934), who determined colorometrically the free menthol in enzyme hydrolysates of menthylglucuronide, and found it to correspond to the reducing material produced when the latter was calculated as glucuronic acid. The method of Lohmar, Dimler, Moore and Link (1942) was applied by Levvy (1948) to isolate and identify the uronic acid liberated from menthylglucuronide by ox spleen β-glucuronidase as the dibenziminazole derivative of D-glucosaccharic acid, thus confirming previous suppositions.

Whether or not the hydrolytic function of the enzyme in vitro could be reversed thereby effecting synthesis of glucuronides in vivo received early consideration by various workers. In 1934, Oshima suggested that β-glucuronidase effected detoxication of alcohols in the body, basing his postulate on the observation that carnivorous animals are less capable of eliminating alcohols in conjugation with glucuronic acid than are herbivorous animals. In relation to this effect, the liver and kidney of dog were shown

to have less glucuronidase than the corresponding organs in ox.

There is little evidence however to show that the reverse synthesis can occur. Houet, Duchateau and Florkin (1941) demonstrated a synthesis of conjugated glucuronide from glucuronic acid and borneol by β-glucuronidase, but the extent of the synthesis was extremely small, being 2.5% of theoretical, and in the words of the authors: "quant à savoir si la mode de synthèse... intervient dans la glucuronoconjugaison in vitro, c'est évidemment un autre problème". While the work of De Meio and Arnolt (1944) suggests that phenol may combine directly with glucuronic acid in liver slices, Lipshitz and Bueding (1939) demonstrated that the synthesis of glucuronides by liver slices is stimulated, not by glucuronic acid but by certain C_3 compounds, while glucuronic acid cannot be utilised by the liver slice for the synthesis of glucuronides. In view of this observation, and inasmuch as the hydrolytic action of the enzyme is confined to splitting of the glucosidic link of conjugates (Levvy 1948), it is difficult to accept the postulate that β-glucuronidase is responsible for the production of conjugated glucuronides in the body by a reversal of the hydrolytic action seen in vitro.

More recently Karunairatnam, Kerr and Levvy (1949) have shown that the ability of β-glucuronidase to catalyse condensation is negligible, and the ability to synthesise glucuronides does not seem to be related to enzyme activity. The use of an inhibitor of glucuronidase is an obvious step towards the elucidation of the physiological function of the enzyme. In this respect,

Karunairetnam et al (1949) have shown that saccharic acid, an effective inhibitor of the enzyme, does not inhibit glucuronide formation. Almost complete inhibition of the hydrolysis of phenylglucuronide by mouse liver β-glucuronidase was obtained with 5×10^{-3} M saccharate, while concentrations up to 10^{-2} M saccharic acid had no appreciable effect on glucuronide synthesis by mouse liver slices.

On the whole we may say that evidence for the participation of β-glucuronidase in the synthesis of glucuronides is far from convincing. Mills (1946) suggested that the hydrolytic action of the enzyme in vitro may also be effective invivo. Glucuronides when fed to animals are, according to Quick (1928), subject to hydrolysis in the body, as on feeding benzylglucuronide to dogs about 80% of its benzoic acid is excreted conjugated with glycine. The work of Mitsuba (1927) also suggests that the spleen may play a part in the destruction of glucuronides. After splenectomy, the amount of glucuronic acid excreted by rabbits and dogs in response to anthranilic acid was greater than in the intact animal. Mitsuba's method for the estimation of glucuronic acid is now considered unreliable however. As pointed out by Mills (1946), if the interpretation of Oshima (1936) is correct, one would expect that removal of the spleen would be followed by a decrease in the conjugation of menthol with glucuronic acid. Rabbit was chosen by Mills (1946) as experimental animal. In this species the total spleen glucuronidase is small compared with liver glucuronidase, 95% of the enzyme

being accounted for by the latter, and consequently any alteration in amount of conjugation should be highly significant. Two weeks following splenectomy an increase was observed firstly in the normal output of urinary glucuronic acid, and secondly in the percentage conjugation of menthol; by the fourth to fifth week, both values had declined to their original levels. After splenectomy certain functions of the spleen are taken on by other organs, a fact which may also apply to the destructive action of the spleen on glucuronides. The above results are more readily explicable in terms of the assumption that spleen is concerned with the hydrolysis of glucuronides in the body rather than with their synthesis.

Reverting to the original postulate that β-glucuronidase is concerned with the synthesis of glucuronides in vivo, Fishman (1940) designed experiments to show whether or not the oral administration of large amounts of glucuronidogenic substances would induce an increase in the enzyme content of animal tissues. After repeated feeding of menthol to mice, he obtained results which, on statistical examination, showed an increase in β-glucuronidase activity in liver, spleen and kidney, as compared with organs from untreated animals. Similar results were obtained in dogs fed with borneol. Glucuronidase in uterus and other sex organs was unaffected in both series of experiments. In Fishman's own interpretations of these experiments, β-glucuronidase is assumed to be responsible for glucuronide synthesis in the body, the enzyme increase being a measure of adaptation by

glucuronidase in response to the presence of excess substrate for its hypothetical synthetic action. The main criticism at issue is the purely circumstantial nature of the evidence, and it would seen at least equally plausible to suppose that β-glucuronidase acts purely in a hydrolytic manner, increase in the amount of enzyme occurring simply in response to the presence of excess glucuronide formed from the aglycone administered. Later Fishman extended his theory to explain the elevation in uterine glucuronidase observed after administration of oestrogens to ovariectomised mice (Fishman and Fishman 1944; Fishman 1947a). Oestrogens did not affect the enzyme in liver, spleen and kidney, and the additional assumption was required and made, that the enzyme is specific in its synthetic action, according to its source, for different groups of substrate. No such specificity is, however, observed in the in vitro hydrolysis of glucuronides by glucuronidase from varying sources.

This correlation of enzymic activity with steroid hormone conjugation was investigated further by Fishman (1947b). A relationship was found to exist between the increased amounts of steroid excreted during pregnancy and blood glucuronidase activity, and it was concluded that oestrogen is at least one of the factors influencing the β-glucuronidase level in the serum.

In 1947, Odell and McDonald observed that serum β glucuronidase activity increased progressively during pregnancy with increase in serum oestrogens and pregnanediol of urine, thus

confirming Fishman's (1947b) observation. It is worthy of note however (Odell and McDonald 1948), that during pre-eclampsia of pregnancy, the anomaly apparently exists of high serum β-glucuronidase levels coincident with low serum, tissue and urine substrate.

In 1948, it was observed by Odell, Fishman and Hepner that infant umbilical cord blood possessed less β-glucuronidase activity than maternal venous blood. Within ten days from birth however, the activity of infant venous blood had increased ten fold to a value much greater than that of maternal blood. A fortuitously wide interpretation was applied to these results and it was suggested that inasmuch as the pre-eclamptic state of pregnancy was associated with oedema, difficulty in excreting sodium chloride, and increased β-glucuronidase of blood serum, the increased enzymic activity of the blood of new-born infants was probably associated with the susceptibility of the latter to oedema.

This apparent correlation between β-glucuronidase and the "metabolic conjugation" of oestrogenic hormones, and the possible role of the latter in the production of malignant growth, led to a study of the glucuronidase activity of cancerous tissue. Fishman and Anlyan (1947 a&b), and Fishman, Anlyan and Gordon (1947), reported increased glucuronidase activity in tissues excised from malignant neoplasms of various organs, including breast, uterus, stomach, mesentery, abdominal wall and oesophagus. Metastases in lymph nodes from cancers originating

in such organs likewise contained increased β-glucuronidase values relative to the uninvolved lymph nodes. The original concept of Fishman (1947a), where the equilibrium in reactions supposedly catalysed by β-glucuronidase in vivo lies in the direction of synthesis was considerable modified by Fishman, Anlyan and Gordon (1947). The latter authors suggested that it was equally conceivable to assume the occurrence of glucuronide hydrolysis to an extent greater than normal in the presence of increased enzyme activity, the conjugate producing, in such an event, a potent cell-growth stimulating substance, the free steroid hormone. Fishman and Anlyan (1947a) also reported that the activity of the enzyme was closely correlated with the clinical malignancy of the tumour. While benign tumours exhibited low activity, adenocarcinoma and medullary carcinoma had high enzyme values. The major difficulty in cancer studies however, is to obtain the corresponding normal tissue unless an organ like the liver is involved, and as pointed out by Campbell (1949), while an organ may be a complex of tissues, only one of these is usually involved in neoplasia, leading to a serious criticism of the normal tissue controls used by Fishman and Anlyan (1947a).

The original experiments of Fishman (1940), designed to show an increased β-glucuronidase activity in liver, kidney and spleen after repeated feeding of menthol to mice were confirmed by Kerr, Levvy and Campbell (1947), Kerr and Levvy (1947), and Levvy, Kerr and Campbell (1948). Within twenty four hours of a single intraperitoneal injection of laevo-

menthol into mice, there was a marked rise in glucuronidase activity in liver, but not in spleen and kidney. Liver damage was confirmed histologically, and it was subsequently shown that a rise in enzyme activity in liver or kidney, depending on the organ or organs attacked, followed administration of a variety of toxic agents to mice. In addition to the effect on liver, delayed damage to the kidney was observed, followed by an increase in glucuronidase activity in this organ also.

On the basis of these experiments it was suggested that the effects of menthol on β-glucuronidase activity <u>in vivo</u> bear no relation to its glucuronidogenic property, but are secondary to its toxic action on liver and kidney. In general the increase in glucuronidase activity in an organ was found to be associated with active cell proliferation produced by injury, rather than with injury itself. As further confirmation, high values were quoted for enzyme activity in the livers of adult mice after sub-total hepatectomy, and in the liver, spleen and kidneys of infant mice. The rise in enzyme level occasionally slightly preceded the first appearance of cell division which was definitely in excess of normal, and a close parallelism was drawn between the glucuronidase activity in an organ and the amount of tissue growth in progress in that organ.

Extending these observations further, Kerr, Campbell and Levvy (1949) cite the usefulness of β-glucuronidase as a biochemical index of growth. A comparative study was again made of the effects on the glucuronidase activities of mouse

liver, kidney and spleen, of various measures causing proliferative changes in one or more of these organs. In uterus as in other organs examined, an increase in enzyme activity appeared to be associated with mitotic activity. Furthermore, one week after injection of ovariectomised mice with chloroform or carbon tetrachloride, increases in uterine weight and glucuronidase activity were observed. That this effect was secondary to liver regeneration provoked by the toxic agent was shown by further experiments in which ovariectomised mice were submitted to partial hepatectomy, with similar results. These results were explained on the assumption that the body is capable of producing an extra-ovarian growth hormone.

The present work was undertaken in the hope of co-ordinating some of the more conflicting aspects of the chemical, physical and biological properties of the enzyme. Two approaches have been adopted, one concerned with the purely physical aspects and reaction characteristics of β-glucuronidase, and the other concerned with the rôle of the enzyme in vivo. While these two methods of approach constitute the major part of the work here presented, a subsidiary study has also been made of the glucuronide decomposing enzyme found, under certain conditions, in various strains of bacteria.

SECTION II.

GENERAL METHODS

Preparation of Enzyme Extracts for Assay.

Where no specific fractionation techniques were required, as in all biological experiments, the enzyme preparation used was a 1/100 (w/v) water homogenate of the requisite tissue obtained by grinding the latter for 2 minutes in a glass homogeniser (Potter and Elvehjem 1936). While it has been shown by Kerr and Levvy (1951) and Walker and Levvy (1951) that a partition of glucuronidase exists between the sedimented cytoplasmic granules and the supernatant of mouse liver homogenates, the use of the whole homogenate for assay obviates any difficulties which might be anticipated in this respect. That the total glucuronidase content of a tissue is measurable by such means has been made apparent by the above authors, and a straight water homogenate was accordingly used throughout for assay.

Experiments involving fractionation and purification of the enzyme necessitated the use of specific methods of preparation, details of which will be included in later data.

Assay of β-Glucuronidase.

Phenolphthalein-β-D-glucuronide, prepared biosynthetically by the method of Talalay *et al* (1946) was used as substrate, and the method of assay was that described by Mills, Paul and Smith (1952a).

Enzyme digests consisting of 0.5 ml. buffer, 0.2 ml. 0.005 M

phenolphthalein glucuronide (Na salt) and 0.3 ml. enzyme solution were uncubated for a specific time, and the reaction terminated by the addition of 2 ml. glycine - NaOH buffer of pH 10.4, followed by centrifugation to remove any precipitated protein. 2 ml. aliquots of the supernatant were added to a further 6 ml. glycine - NaOH buffer, and the resultant colour of the free phenolphthalein determined in the Spekker photoelectric absorptiometer using an Ilford 605 yellow-green filter (absorption maximum for phenolphthalein = 553 mμ). A calibration curve was prepared using phenolphthalein solutions carried through the above procedure.

The unit of activity under such conditions is defined as: I phenolphthalein unit = I μg. phenolphthalein liberated per hour per ml. enzyme.

Unless otherwise stated the following buffers were employed: For the pH range 3.4 - 6.0, 0.2 M. acetate buffers. For the pH range 6.0 - 7.0, the veronal - acetate buffers of Michaelis (1931).

Estimation of Deoxyribonucleic Acid Phosphorus (DNAP) and Ribonucleic Acid Phosphorus (RNAP).

DNAP and RNAP were freed from acid soluble phosphorus and lipid phosphorus by the method of Schmidt and Thannhauser (1945). Phosphorus was estimated by the method of Allen (1940).

2.5 ml. of a 1/5 (w/v) water homogenate of the tissue were introduced into a 15 ml. centrifuge tube. 1.25 ml. ice cold 30% trichloracetic acid (TCA) were then added, the mixture ground with a glass rod and the precipitate centrifuged at 1,000 r.p.m.

for 4 minutes. These conditions gave a fine granular precipitate which facilitated the later stages of extraction.

The supernatant was discarded and the remaining acid soluble phosphorus extracted from the precipitate by grinding twice with 5 ml. volumes of 10% ice cold TCA, centrifuging between extractions; the whole process was completed within 30 minutes from the initial addition of TCA.

Removal of lipid phosphorus from the solid residue was effected by treating the latter successively with 5 ml. portions of the following:

a). 80% ethanol

b). absolute alcohol.

c). three extractions with a 1:3 chloroform-ethanol mixture for half-an-hour at 70°-80°C.

d). a final extraction with redistilled ether, coupled with centrifugation after each extraction.

5 ml. silica free N NaOH solution were then added to the residue, and the tube incubated at 37°C for 15-20 hours. At the end of this time the volume of the solution was made up to 10 ml., from which 0.4 ml. was withdrawn for the estimation of protein nitrogen. RNAP and DNAP separations were carried out on 5 ml. aliquots of the digest by adding 1.25 ml. 2.5 N HCl, followed by 3.15 ml. 30% TCA. The DNAP thus precipitated was centrifuged at 3,000 r.p.m. for 7 minutes, washed twice with 1 ml. portions of 5% TCA, dissolved in 1 ml. NaOH and transferred with washing

to a microkjeldhal flask. The combined supernatant and washings representing the total RNAP was adjusted to standard volume (20 ml.) and 5 ml. of the above likewise transferred to a digestion flask.

To each flask was added 1.2 ml. 10 N. H_2SO_4 with the further addition of a few drops 100 vols. hydrogen peroxide to facilitate digestion. Heating was continued for 15 minutes after clearing to ensure complete removal of hydrogen peroxide; at the end of this period the flask was allowed to cool and 6.4 ml. water added, followed by 2 ml. amidol reagent (1 g. 2:4 diaminodiphenylhydrochloride in 100 ml. of a 20% solution of potassium metabisulphite made up freshly every 5 days), 1 ml. ammonium molybdate (8.3% (w/v) solution of ammonium molybdate in N/100 H_2SO_4), and a further 15 ml. of water. The final concentration of H_2SO_4 so obtained was 0.5 N, with a total volume of 25 ml. 10 minutes were allowed for stabilisation of the blue colour so developed, and with the added precaution of carrying out all readings within 30 minutes of colour development, the intensity of the latter was estimated photometrically in the Spekker absorptiometer using an Ilford 608 red filter. The results so obtained are expressed in terms of μg. phosphorus per 100 mg. tissue.

Estimation of Protein Nitrogen.

The method employed for the estimation of protein nitrogen was based on that of Ma and Zuazaga (1942). Requisite samples of material were transferred to a microkjeldhal flask and digested with 1 ml. concentrated H_2SO_4, using as catalyst a

a knife point of the following mixture:

Powdered selenium	:	1 part
K_2SO_4	:	1 part
$CuSO_4.5H_2O$	:	3 parts

Digestion was continued for a standard 30 minutes after clearing, followed by distillation in the apparatus of Markham (1942). The NH_3 liberated with excess of 10 N NaOH was distilled into a 2% boric acid solution, and titrated directly with standard 0.01 N HCl, using a mixed indicator consisting of 1 vol. methyl red and 5 vols. bromocresol green, both in the form of 0.1% solutions in 95% ethanol.

Determination of Total Fat in Liver.

The total fat in liver was estimated according to the method of Channon and Wilkinson (1935). The liver tissue was dissolved in 20 ml. 40% NaOH by boiling, 75 ml. industrial alcohol added and the mixture slowly evaporated on a hot plate until most of the alcohol was removed (2-3 hours). The residual solution was then washed into a separating funnel, acidified with 30 ml. concentrated HCl and extracted five times with ether. The ethereal extract was washed twice with water, evaporated down, dried *in vacuo* and dissolved in 50 ml. light petroleum ether (B.P.40°-60°). After standing overnight the solution was filtered into a weighed flask, the petrol distilled off with removal of the last traces *in vacuo*, and the flask cooled and weighed. Material obtained in this manner represented total fatty acids and unsaponifiable matter.

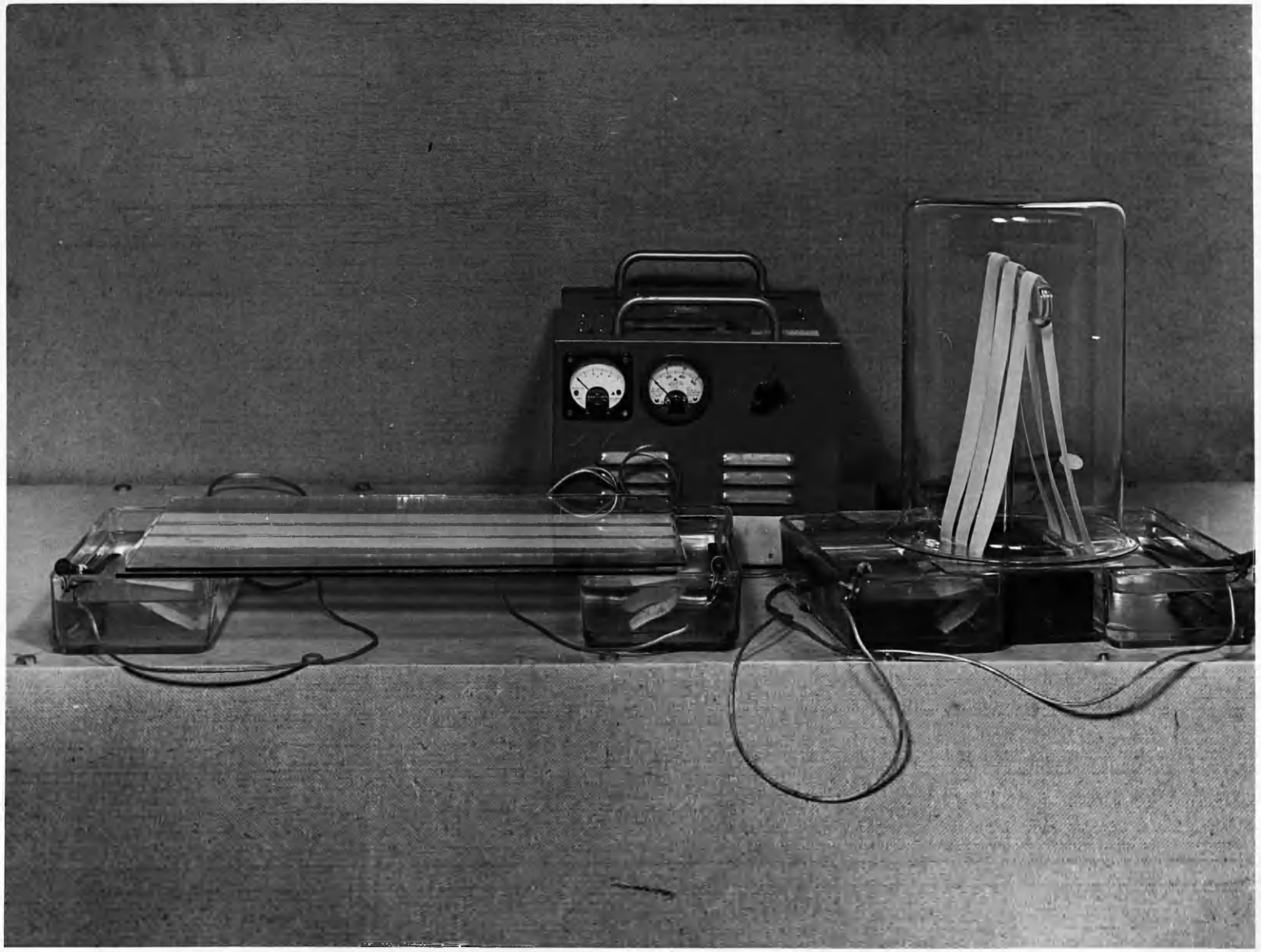
Electrophoretic Analysis

In performing electrophoretic separations use was made of the conventional methods of free electrophoresis and also of electrophoresis on filter paper.

In the former instance analyses were carried out using the Tiselius (1937) apparatus employing the Philpot-Svensson (Svensson 1939) optical system (Adam Hilger & Co. London). Illumination was provided by a mercury vapour lamp and a green (546 mμ.) filter, photographic records being made on Ilford Halftone panchromatic plates. The cells used were either the double section of II ml. capacity or the double section of 3 ml. capacity.

In the latter instance, filter paper electrophoresis was carried out by the method of Mills and Smith (1951) which was based on that of Durrum (1950). The type of apparatus devised by the former authors is shown in Plate A. While less elaborate than that employed by Durrum (1950), Cremer and Tiselius (1950), Turba and Enenkel (1950) or Flynn and de Mayo (1951), satisfactory electrophoretic separations were obtained without recourse to the special cooling arrangements introduced by the above authors. Filter paper strips were supported either by laying across a flat glass plate superimposed by another covering plate to minimise evaporation, or by suspension over a glass rod in the manner shown, using in this instance an inverted beaker for control of evaporation. The hanging method was probably the more satisfactory, a certain amount of protein adsorption on the glass plates occurring in the former instance. The ends of the

PLATE A



paper strips dipped into electrode vessels containing a suitable buffer and direct current was supplied from a rectifier with adjustable output.

Reagents Used.

1). Phosphate buffer, pH 8.0 : 116.1 g. $Na_2HPO_4.2H_2O$ and 5.96 g. KH_2PO_4 per 2 litres of distilled water. An ionic concentration of 0.1 was generally employed necessitating a 1/10 (v/v) dilution of the above solution.

2). Staining reagent : a saturated solution of mercuric chloride in 95% alcohol containing 0.1 g./100 ml. of bromophenol blue (tetrabromophenolsulphonphthalein).

Procedure.

Protein solutions for analysis (30-40 µl.) were applied to the middle of Whatman 3 MM filter paper strips, 60 cm. long and 3 cm. wide, by means of a micro-pipette, care being taken to avoid undue spreading of the material on application. The strips were soaked in buffer solution and arranged in either of the manners illustrated. A suitable potential (as specified for each individual experiment) was applied across the carbon electrodes for a specific time period. At the end of the run, the strips were removed, dried for 5 minutes in an oven at 100°C., immersed in the staining reagent for 10 minutes and washed in running water until all traces of blue colour had been removed from the background. Distinct blue zones comparable to the migration of the separated entities were localised in this manner. Displacement of the entire protein pattern towards the negative

pole as a result of an electro-endosmotic flow in this direction was determined by the movement of a spot of glucose applied to a control strip; localisation of glucose was effected by spraying the dried strip with aniline hydrogen oxalate solution and heating in the oven at 100°C. for five minutes.

The migration of β - glucuronidase per se was determined by virtue of its hydrolytic action on a chromogenic substrate. After completion of the run, a further strip of paper soaked in 0.2 N acetate buffer pH 4.5 and containing phenolphthalein glucuronide (final concentration 0.001 M), was superimposed on the original strip containing the enzyme. The two were firmly clamped between glass plates, incubated at 37°C. for 30 minutes, and the liberated phenolphthalein detected by holding either strip over concentrated NH_4OH. This method was particularly useful in instances where the low concentration of protein present in purified enzyme preparations was insensitive to the staining method employed above.

SECTION III

PHYSICAL PROPERTIES OF β-GLUCURONIDASE

A. Purification of β-Glucuronidase.

An investigation of the mechanism of enzyme action and the characterisation of any enzyme by means of reaction kinetics, is in-part dependent on the purity of the material employed, and it was thus considered desirable to effect the maximum possible purification of the enzyme under study before attempting to determine its characteristics. Ox liver was chosen as the source of enzyme material. Liver and spleen are the two richest sources of β-glucuronidase, but whereas a fairly extensive characterisation of the preparation and properties of the spleen enzyme have been recorded, (Fishman 1939 a&b; Graham 1946; Mills 1948; Mills, Paul and Smith 1949; Bernfeld and Fishman 1950a; Mills, Paul and Smith 1952 a&b), the available data on liver glucuronidase is confined to the work of Sarkar and Sumner (1950).

The separation of protein fractions from complex mixtures of proteins can be achieved in several ways. Advantage can be taken of the salting out effect of neutral salts. The dependance of such precipitation on the four variables - salt concentration, protein concentration, pH and temperature - was considered by Mills (1948), who achieved considerable success in the fractionation of ox spleen β-glucuronidase by this method. Edsall (1947) indicated the advantage to be gained by introducing

another variable into the system. The presence of the latter, in this instance a water-miscible organic solvent, enormously increases the range of possible conditions which can be chosen for the separation of any given component. In 1950, Bernfeld and Fishman, by combining alkaline ammonium sulphate fractionation with methanol fractionation, obtained a glucuronidase preparation from calf spleen with a purity of 7,000 phenolphthalein units of activity (as previously defined), per mg. of enzyme, the initial material containing 23 phenolphthalein units of activity per mg. enzyme. Sarkar and Sumner (1950) utilised low temperature dioxane fractionation for the purification of ox liver β-glucuronidase, achieving in the process a 6,000fold purification of the enzyme. Their method of expressing purity however, is based on an initial expression of activity per gram of fresh tissue, this being compared with a final activity in terms of mg. dry weight of enzyme. It is undoubtedly more accurate to enumerate both values in terms of a common factor, namely protein nitrogen, in which case a 900 fold purification was achieved.

The most recent publication of Cohn, Gurd, Surgenor, Barnes, Brown, Derouaux, Gillespie, Kahnt, Lever, Liu, Mittelman, Mouton, Schmidt and Uroma (1950) on the separation of the protein components of human plasma utilising the interactions of different proteins with bivalent cations, suggested the possibility of using such metallo-protein complexes in the purification of β-glucuronidase. Here again the introduction of a new solid phase greatly increases the complexity of the system, particularly if

heavy metal precipitation is carried out in the presence of varying protein, salt and solvent concentration, and varying temperature and pH. The number of possible permutations and combinations is infinite, and the quotation of Edsall (1947) to the effect that: "the separation of more or less homogeneous protein fractions from the complex mixtures of proteins found in nature is still in many respects an art rather than a science" needs little comment.

By a combination of metallo-protein interactions, organic solvent precipitation and ammonium sulphate fractionation, it has been found possible to achieve a purification of ox liver β-glucuronidase equivalent to that obtained by Sarkar and Sumner (1950). While it has not been possible, as yet, to improve upon their ultimate purification, the method here presented is advantageous in many ways. The above authors employ dioxane as protein precipitant, the enzyme being left to equilibrate in the presence of dioxane over a period of ten hours at three separate stages in the purification. While rigorous precautions were observed with respect to temperature control, it is the experience of the present author that prolonged contact of β-glucuronidase with organic solvents, with temperature as low as -5°C., leads to marked denaturation of the enzyme. It is of particular interest that purification by dioxane precipitation and by the method to be outlined results in totally different pH activity curves. The dissimilarity in the latter is attributed to the denaturing effects of

dioxane, a point which will be further discussed when the properties of the enzyme are considered.

As previously indicated, the permutations and combinations involved in protein purification are illimitable, and it would hardly be profitable at this point to outline the large number of initial experiments carried out to determine the most suitable conditions for purification of the enzyme. Broadly speaking, advantage has been taken of the interactions of proteins with zinc and barium ions in combination with acetone precipitation. The lower solubility of the protein salts formed allows precipitation of the enzyme at a much lower acetone concentration than normally employed in the absence of metallic ions, thus minimising protein denaturation.

The starting material employed is the Stage C fraction described by Mills (1948) in studies on ox spleen β-glucuronidase.

Finely minced liver was extracted twice with 2 vols. acetone, and the filter cake dried in air at room temperature. The powder so obtained was then extracted with water, adjusted to pH 5.0 with acetic acid, buffered with acetate buffer, and autolysed at 38°C. for four hours. The voluminous precipitate so formed was removed in the Sharples centrifuge, and solid $(NH_4)_2SO_4$ stirred into the solution to 60% saturation, followed by equilibration at 0°C. for 18 hours. The precipitate was then removed by filtration using Standard Super-Cel (519 A), extracted with water and dialysed, the extract constituting the Stage C of Mills's (1948) original purification.

Further purification can be conveniently divided into three

stages:

Stage I.

In principle, initial treatment of the Stage C solution with zinc acetate solution resulted in precipitation of a large percentage of inactive protein. If this was removed by centrifugation, glucuronidase could then be precipitated completely from the supernatant by the addition of acetone to a final concentration of 20%. This was in contrast to the higher value of 45% acetone required for precipitation of the enzyme in the absence of zinc ions. Elution of the precipitated material with a 15% acetone solution containing barium ions resulted in a selective extraction of β-glucuronidase, yielding the so-called Barium Eluate. The procedure is represented schematically in Figure 1.

The percentage distribution of the enzyme obtained by the above fractionation is shown in the following table:

	% Distribution of Enzyme
Barium Eluate	60
Precipitate (A)	15
Supernatant (B)	0
Residue (C)	17

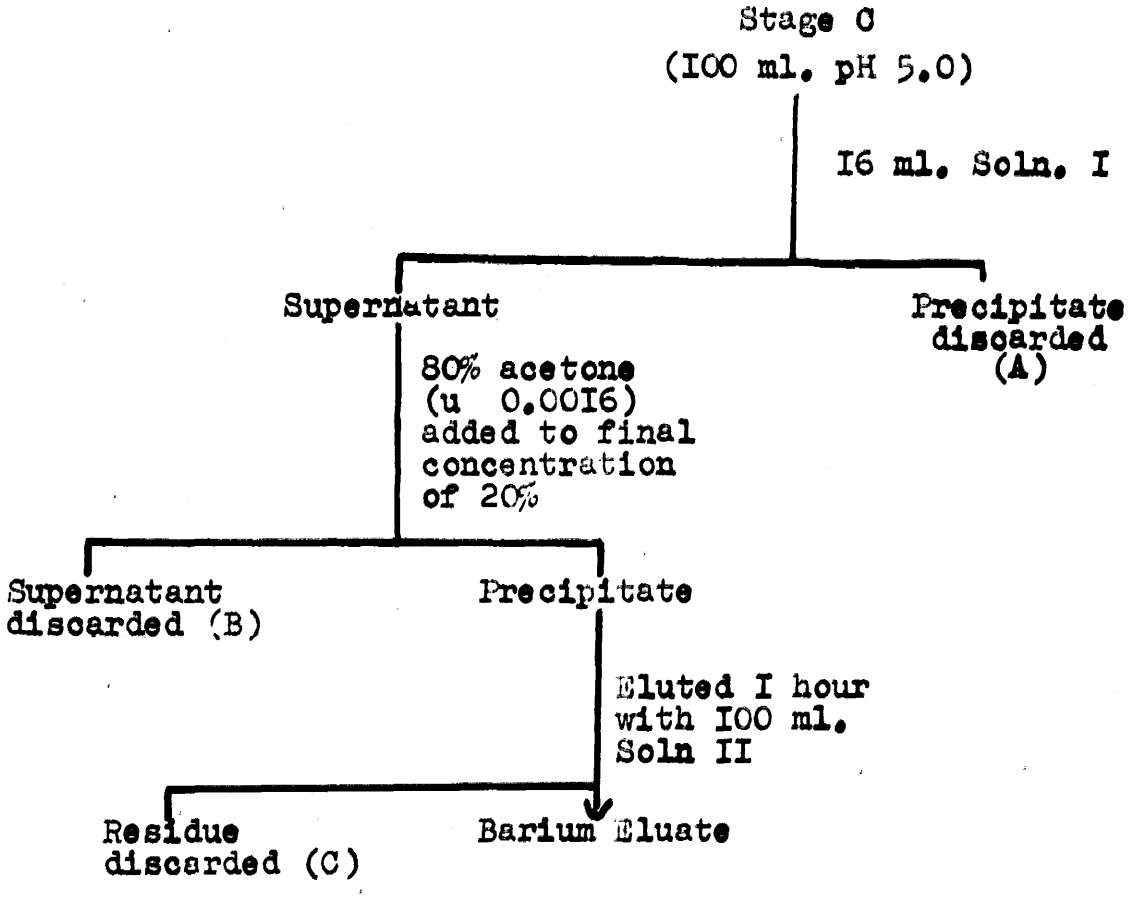
The 8% of activity unaccounted for may be considered as loss due to denaturation. The relative purities of the Stage C solution and Barium Eluate are 2,500 units per mg. protein N and 14,000 units per mg. protein N respectively.

The success of Stage I fractionation was dependent on a

Figure I.

Purification of Ox Liver β - Glucuronidase

Stage I



Solution I : 5.48 g. Zinc acetate dihydrate to I00 ml. with water

Solution II: 0.26 g. Barium acetate
2.0 ml. 0.8 M. Sodium acetate
0.73 ml. M. Acetic acid
I5 ml. Acetone
] to I00 ml. with water pH 5.5

number of critical factors, control of which was important for reproducibility of results.

(1). The initial ratio of enzyme activity to protein concentration in the Stage C solution was an important controlling factor, and a value of 2,000 - 4,000 enzyme units of activity per mg. protein N appeared to be essential to avoid undue loss of enzyme in the initial zinc precipitation. Provided the method outlined by Mills (1948) for obtaining this particular preparation was adhered to, no difficulty was apparent in this respect, as remarkably constant activity/protein ratios have invariably been obtained from a variety of different preparations.

(2). Strict temperature control was essential throughout. The denaturing effect of organic solvents on proteins is less marked at low temperatures, while small variations in temperature may lead to critical disturbances in the complexity of the phase system. This effect was particularly noticeable in the precipitation of inactive protein by zinc ions. At a temperature of $0^{\circ}C.$ loss of enzyme was minimal (15%). Any decrease of temperature was attended by a correspondingly greater loss of enzyme rising to as much as 40% at $-5^{\circ}C.$ A temperature of $0^{\circ}C$ was maintained throughout Stage I of purification.

(3). It will be noted that the presence of barium ions allowed the precipitated enzyme to be re-eluted over a 5% acetone range, suggesting the possibility of the formation of a readily soluble barium salt of the enzyme. A complete recovery of glucuronidase was not effected, some 16% remaining in the residue. This was dependent on the time period of elution. Extraction for

one hour allowed the maximum separation of enzyme relative to the amount of inactive protein simultaneously eluted. With longer extraction periods almost all of the enzyme could be recovered at the expense of decreasing purity.

(4). Initially a pH of 5.0 was chosen for fractionation purposes, this pH approximating closely to the isoionic point of the enzyme. Whether or not a more effective purification could be achieved with marked alterations in pH has yet to be investigated. A few preliminary experiments in this direction over a pH range of 5.0 - 8.0 indicated that the initial choice of pH was probably the most satisfactory.

Stage II.

Following dialysis of the Barium Eluate at 0°C, glucuronidase was precipitated from solution with $(NH_4)_2SO_4$ at a concentration of 60% saturation. 2% Celite (519 A) was added to adsorb the enzyme, which was then successively eluted from the adsorbing material with 100 ml. portions of 1.8M, 1.6M, 1.4M and 1.2M $(NH_4)_2SO_4$ solutions. A final concentration of 3 M.$(NH_4)_2SO_4$ was used to reprecipitate the enzyme from each of the above eluates, the enzyme precipitate spun off at 0°C. and dissolved in the minimum volume of water, (generally 3 ml.).

No particular precautions need be observed at this stage, and fractionation can be carried out at room temperature. In view of the length of time required to spin down the enzyme precipitate however, (40 mins; 5000 r.p.m.), it was essential to centrifuge the latter at 0°C.

90% of the total glucuronidase present in the Barium Eluate can be recovered by the above procedure with the following percentage distribution:

1.8 M. $(NH_4)_2SO_4$ eluate - 43%

1.6 M. $(NH_4)_2SO_4$ eluate - 30%

1.4 M. $(NH_4)_2SO_4$ eluate - 15%

1.2 M. $(NH_4)_2SO_4$ eluate - 2%

The major portion of enzyme activity is associated with the 1.8 M. eluate, but for further purification this was selectively discarded in view of its high content of inactive protein in favour of the 1.6 M. and 1.4 M. fractions. The latter were highly active enzyme preparations (80,000 - 100,000 units activity per ml.) associated with low protein content, yielding on dialysis (Stage III) the finallypurified product.

Stage III.

On dialysis of the combined 1.6 M. and 1.4 M. $(NH_4)_2SO_4$ elution fractions so obtained, it was found that a large percentage of β-glucuronidase was precipitated from solution. An investigation of the effect of controlled dialysis on purification of the enzyme was thus considered, and two methods of approach were found feasible.

(a). It was apparent that a fairly exhaustive dialysis was required to precipitate the enzyme from solution, and inactive material could be removed initially by a short dialysis period (24 hours). A more prolonged dialysis of the supernatant, (3 - 4 days), against several changes of distilled water pre-

cipitated the enzyme, which, on elution with 3 ml. 1% NaCl solution yielded a glucuronidase preparation containing 200,000 units of activity per mg. protein N.

(b). Alternatively the glucuronidase was first precipitated from solution by a 3 - 4 day dialysis against distilled water, followed by extraction of the precipitate with 6 - 8 ml. of a 1% NaCl solution. Dialysis of the extract reprecipitated the enzyme, which on elution with 3 ml. of the salt solution gave an enzyme preparation equivalent in purity to that obtained by method (a).

The control of dialysis while expressed above in well defined terms, on occasion becomes more empirical, and preparations were sometimes obtained which failed to yield the enzyme of desired purity with a single dialysis and NaCl extraction. In such instances, the latter procedures were repeated a number of times. Methods (a) and (b) as outlined are thus necessarily subject to a certain amount of latitude, but the general principles are essentially as indicated, and with care an enzyme preparation containing 200,000 units of activity per mg. protein N was invariably obtained.

Several controlling factors can however be emphasised.

(1). A temperature of $0^{\circ}C$ or less must be maintained throughout Stage III of purification. When dialysis was carried out against running water ($12^{\circ}C$) the rapidity of denaturation was pronounced, an overall loss in enzyme activity of 35% occurring over a period of 8 hours; losses appeared to be negligible at $0^{\circ}C$.

(2). Extraction of the enzyme from the precipitate obtained on

dialysis was effected with a 1% NaCl solution. The solubility of β-glucuronidase in salt solution is extremely high, and it was apparent that a short extraction period would preferentially elute the enzyme from the precipitate. The latter was accordingly stirred at 0°C with 1% NaCl for 15 minutes. With longer extraction periods, higher yields of enzyme were obtained at the expense of decreasing purity.

Quantitative details of a typical purification are shown in Table 1. From an initial homogenate of ox liver containing 240 units of activity per mg. protein N, an overall purification of 800 fold is apparent. While the recovery of enzyme is 5% for this degree of purification, it should be emphasised that a considerable part of enzyme loss was selective, the most suitable fractions having been chosen for further purification, the least suitable discarded.

Table 1.

Purification of Ox Liver β-Glucuronidase

	Units/ml.	Volume	Total Units	Purity	% Yield
Liver Homogenate	6,000	-	-	240	-
Stage C.	30,000	100	2×10^6	2,500	100
Ba.Eluate	12,000	100	1.2×10^6	14,000	60
1.6M Fraction	80,000	4	32×10^4		16
1.4M Fraction	60,000	3	18×10^4		9
Stage III Purified Material	20,000	5	10×10^4	200,000	5

Purity expressed in terms of activity per mg. protein N.

Unit of activity = 1 µ.g. phenolphthalein liberated per hour per ml. enzyme.

B. Properties of Purified β-Glucuronidase

The purified β-glucuronidase so obtained was a pale straw-coloured solution of marked instability. While it was apparently impossible to keep such an enzyme solution longer than two days at 0°C without appreciable loss in activity, preparations of the order of 150,000 units of activity per mg. protein N were found to be relatively stable and could be stored at 0°C for several weeks with little deterioration in activity. Accordingly all studies on the properties of the purified material have been conducted on the latter.

pH Activity Curve.

The first interesting feature to be observed is the effect of hydrogen ion concentration on enzyme activity. A typical pH activity curve for the purified enzyme is shown in Figure 2. While the stability of the enzyme over a wide pH range appears to be marked, the presence of well defined optima at pH 3.8, 4.5, 5.2 and 6.3 in a preparation of such high purity is suggestive of the complexity of the enzyme system *per se*. Although the activity curve presented may be considered representative of the purified enzyme, different preparations showed individual variations in the proportionality of activity at the four pH maxima.

Coenzyme of β-Glucuronidase.

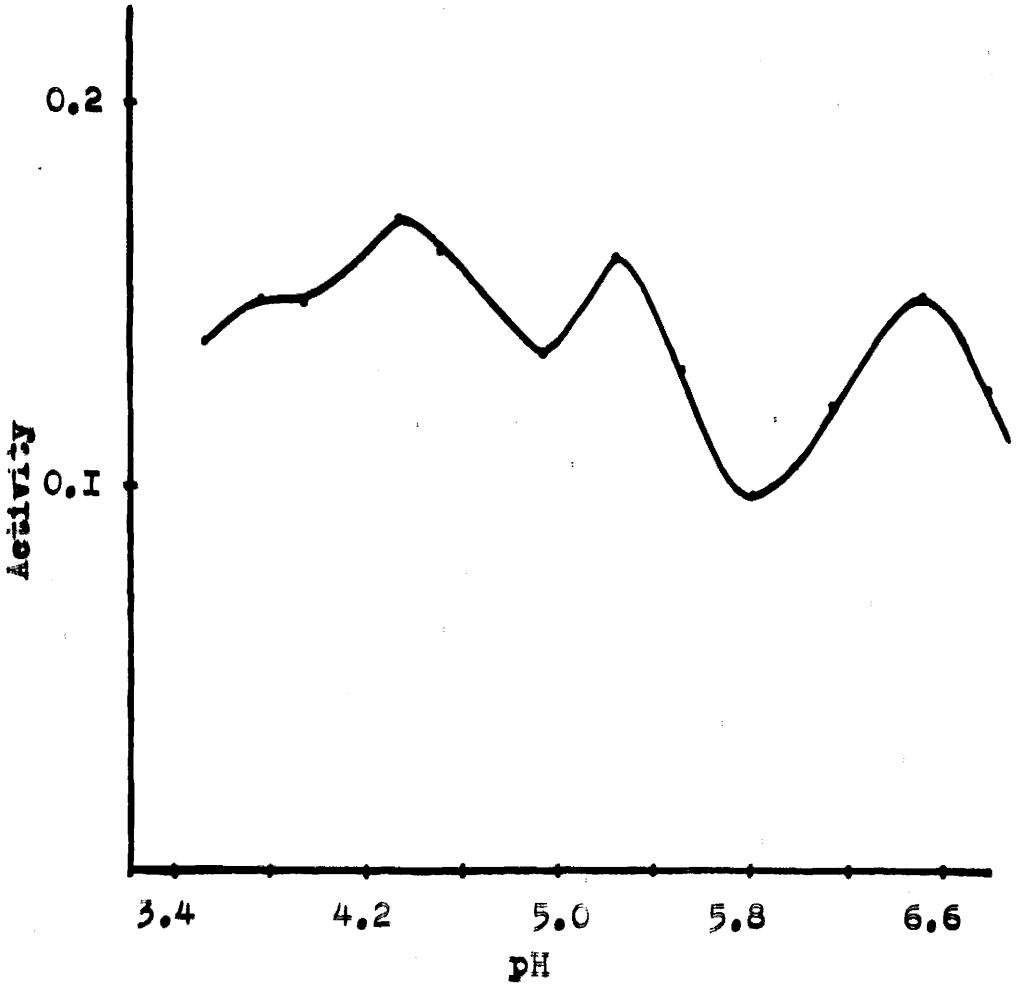
Bernfeld and Fishman (1950b) published evidence to show that calf spleen β-glucuronidase preparations contained a

Figure 2

pH Activity Curve of Purified Ox Liver

β- Glucuronidase

(I60,000 units per mg. P N.)



pH 3.4 - 5.8 Acetate Buffers

pH 5.8 - 6.4 Veronal-acetate Buffers.

and Figure 4 for spleen glucuronidase.

thermostable coenzyme which dissociated from the parent molecule on dilution. Studies indicated that deoxyribonucleic acid (DNA) fulfilled the necessary requisites of the coenzyme, and it was correspondingly suggested that DNA was in effect an essential complement to glucuronidase activity.

Studies on purified liver glucuronidase however do not permit the conclusion that DNA in any way functions as coenzyme of the former. The possibility inevitably arises that the liver enzyme may be intrinsically different from that of spleen. While this seemed highly improbable, it was nevertheless considered essential in this respect to compare the enzyme from both sources.

Enzyme activity was determined at varying dilutions in the presence and absence of DNA (final concentration 0.06%; prepared from calf thymus by the method of Mirsky and Pollister (1946)). If glucuronidase activity is dependent on a coenzyme dissociating on dilution, the ratio of enzyme activity to protein concentration (E/P) will drop greatly with dilution, (as observed by Bernfeld and Fishman 1950b) and a plot of the E/P ratio against that of protein concentration should take the form of an S - shaped curve in accordance with the dissociation expression:

$$\frac{(P - E)\ (C - E)}{E} = K$$

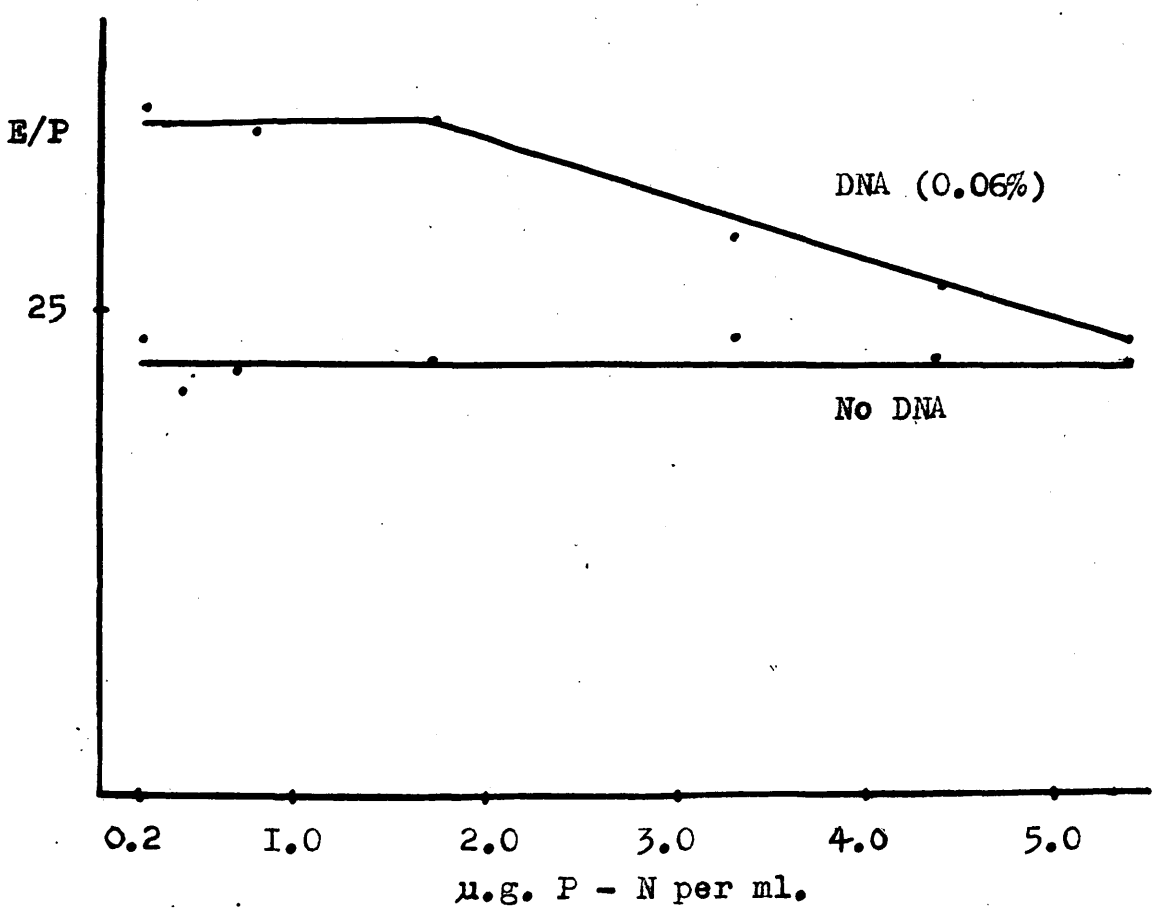
Where P = total protein, C = total coenzyme, E = active enzyme, P/C constant, and K a constant. Furthermore in the presence of DNA, the activity of β-glucuronidase should be proportional to the protein concentration.

Such a plot is presented in Figure 3 for liver glucuronidase and Figure 4 for spleen glucuronidase. While the spleen preparat-

Figure 3.

Dilution Effect on Ox Liver β-Glucuronidase

(160,000 units per mg. P.N.)



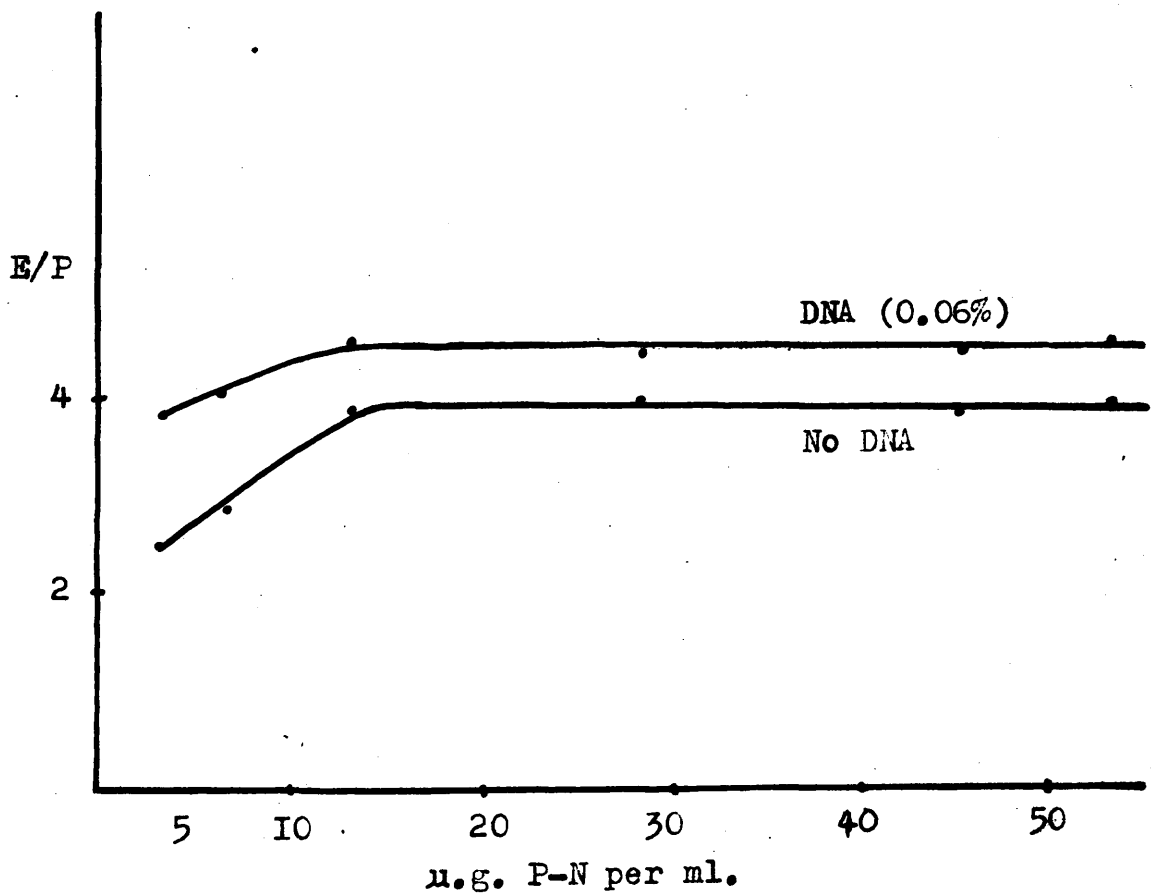
E/P ratio = Enzyme activity/µ.g.P-N

Figure 4.

Dilution Effect on Ox Spleen β-Glucuronidase

I.4 M $(NH_4)_2SO_4$ Fraction of Stage C Solution

I.3I5 mg.P-N per ml.



E/P Ratio = Enzyme activity / µ.g.P-N

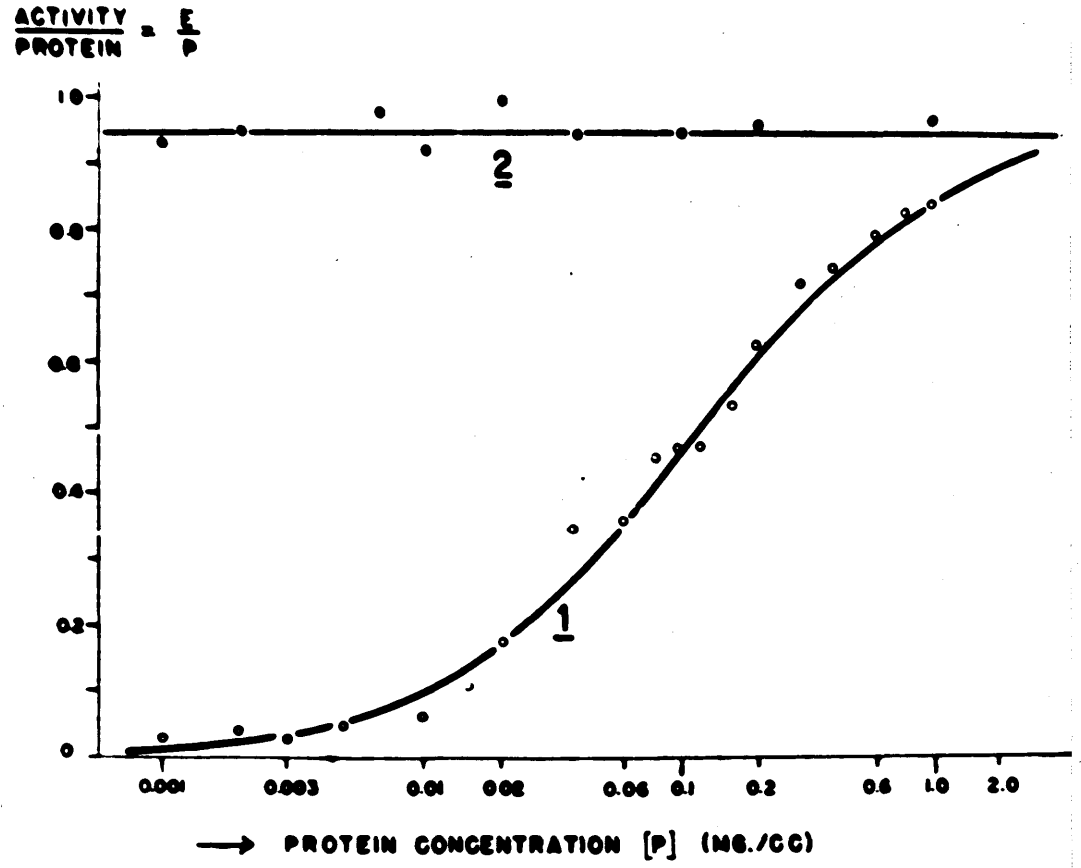
ion in no way approaches the liver enzyme in terms of purity, it being a Stage C preparation obtained by the method of Mills (1948) subjected to $(NH_4)_2SO_4$ fractionation using Celite as adsorbing agent as previously described, the dilutions of protein employed in this case are sufficiently comparable with those employed by Bernfeld and Fishman (1950b) to allow of comparison with the results obtained by these workers. From the point of view of clarity and for comparative purposes, the original graph presented by the above workers is reproduced in Figure 5.

While added DNA failed to exhibit the characteristics of a coenzyme, it was nevertheless an effective activator of both liver and spleen glucuronidase at pH 4.5 in acetate buffer (Figures 3 and 4). It was considered that this activating effect of DNA need not be specific, and it was decided to investigate a number of other high molecular weight compounds in this respect. Albumin, gum acacia and starch were arbitrarily chosen as representing two structural extremes of relatively high molecular weight, and the effect of the above compounds on the assay of purified liver glucuronidase of varying dilution is shown in Figure 6. Employing the plot of E/P against protein concentration, an interesting parallelism is evident when considering the specific effects of such compounds on glucuronidase activity.

These preliminary experiments, carried out at a single pH (pH 4.5 acetate buffer), led to a study of the activation and inhibition of purified β-glucuronidase at varying pH by a variety of different compounds. While activation and inhibition are two opposing principles, the agents responsible cannot be

Figure 5.

(Reproduced from Bernfeld P. & Fishman W.H.,(I950), Science, II2, 653)

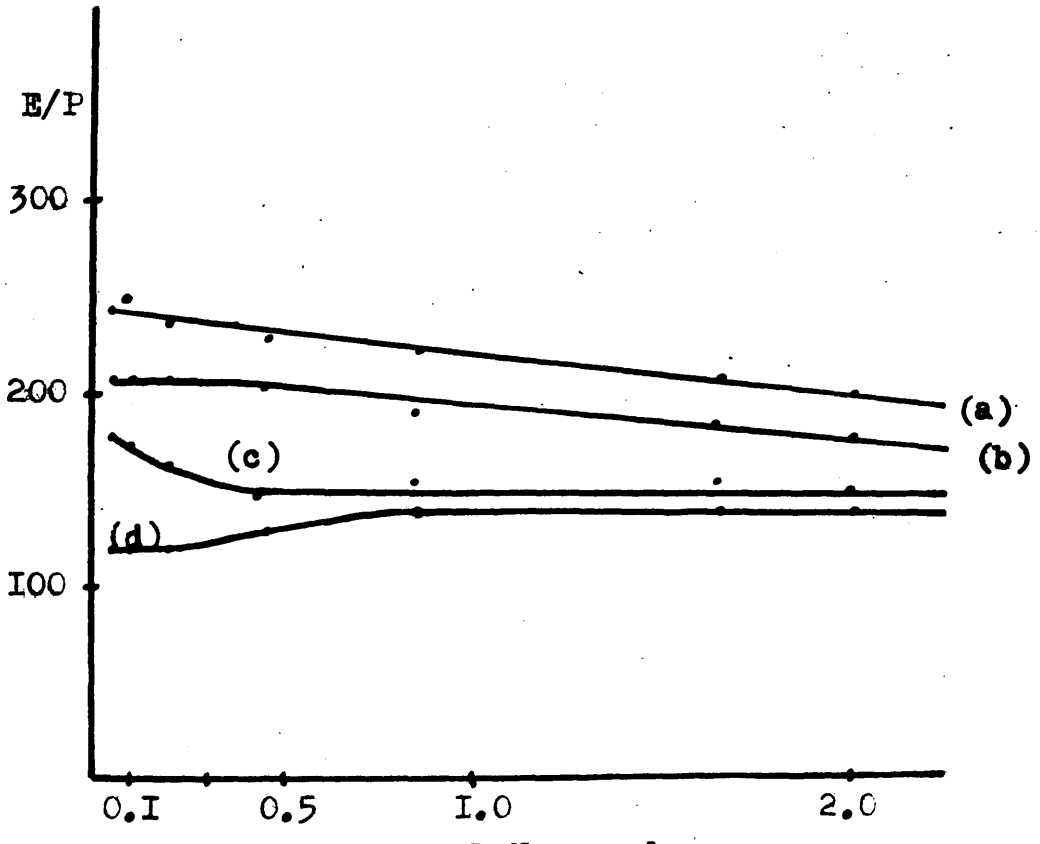


1 = Activity of purified spleen β- glucuronidase relative to protein concentration in the absence of DNA.

2 = Activity of purified spleen β - glucuronidase relative to protein concentration in the presence of 0.3% DNA

Figure 6.

Dilution Effect on Ox Liver β-Glucuronidase in the Presence of Albumin, Gum Acacia & Starch (I70,000 units per mg. P-N)



(a) - Dilution Effect in Presence of 0.05% Albumin

(b) - Dilution Effect in Presence of 0.I% Gum Acacia

(c) - Dilution Effect in Presence of 0.2% Starch

(d) - Dilution Effect with no Additives

E/P Ratio = Enzyme Activity / μ.g. P-N

classified with any convenience into two specific groups in their effect on β-glucuronidase. A substance causing enzyme activation at one specific pH was frequently found to inhibit at another. Although a considerable overlap must be anticipated, an attempt has been made to rationalise the available data here presented.

Activation and Inhibition Studies.

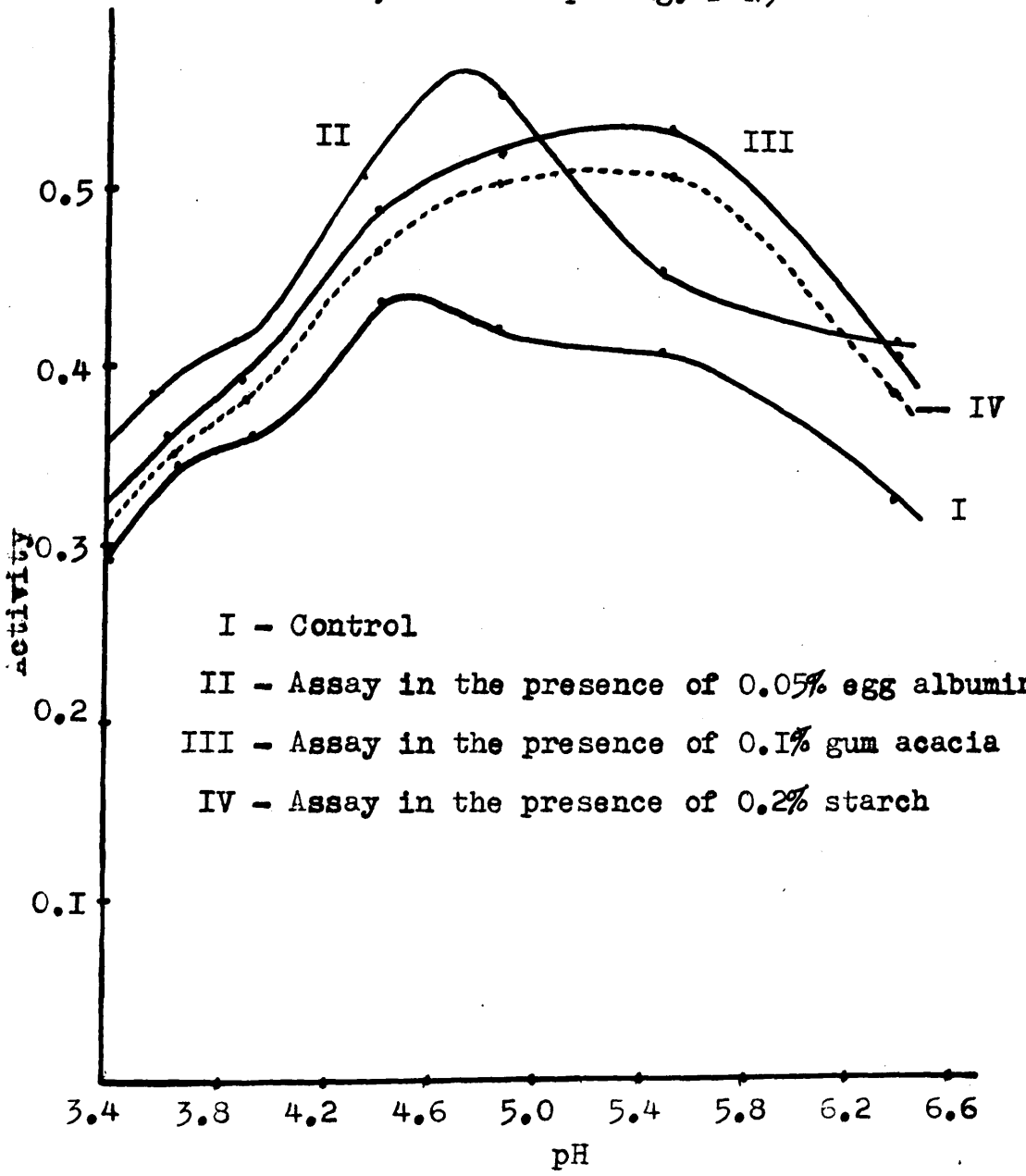
DNA, albumin, gum acacia and starch were primarily investigated for their activating effect on purified liver glucuronidase over the pH range 3.4 - 6.4. As the addition of DNA to enzyme assays resulted in a more interesting and specific effect than that observed in the latter instances it will be considered ex parte.

Figure 7 shows the results obtained with the above mentioned additives. Egg albumin, gum acacia and starch were added to a final concentration of 0.05%, 0.1% and 0.2% respectively; while activation varied in degree with the material, increase in enzyme activity was apparent over the entire pH range in all instances. Further experiments showed that an increase in concentration of all three constituents appeared to have little overall effect on the percentage activation obtained.

The principles underlying this mode of activation appeared to be relatively unspecific. To qualify this assumption, the protein components of human serum were separated electrophoretically on filter paper strips, eluted with 1% NaCl solution and the individual fractions assayed for their possible activating effect on the purified enzyme.

Figure 7

Effect of Egg Albumin, Gum Acacia and Starch on the Assay of Purified Liver β - Glucuronidase (170,000 units per mg. P N)



I - Control

II - Assay in the presence of 0.05% egg albumin

III - Assay in the presence of 0.1% gum acacia

IV - Assay in the presence of 0.2% starch

pH 3.4 - 5.8 Acetate Buffers

pH 5.8 - 6.4 Veronal-acetate Buffers

60 µl. of serum were separately applied to two filter paper strips in the manner previously described and a potential of 4 v./cm. applied for 18 hours; veronal buffer pH 8.5 of ionic strength 0.05 was employed. On completion of the run, protein was localised on one of the strips by the method of Durrum (1950), a reproduction of which is shown in Figure 8. The corresponding fractions representing albumin, α, β and γ globulins were eluted from the other strip by soaking overnight in 1 ml. 1% NaCl solution.

The results obtained are shown in the following table:

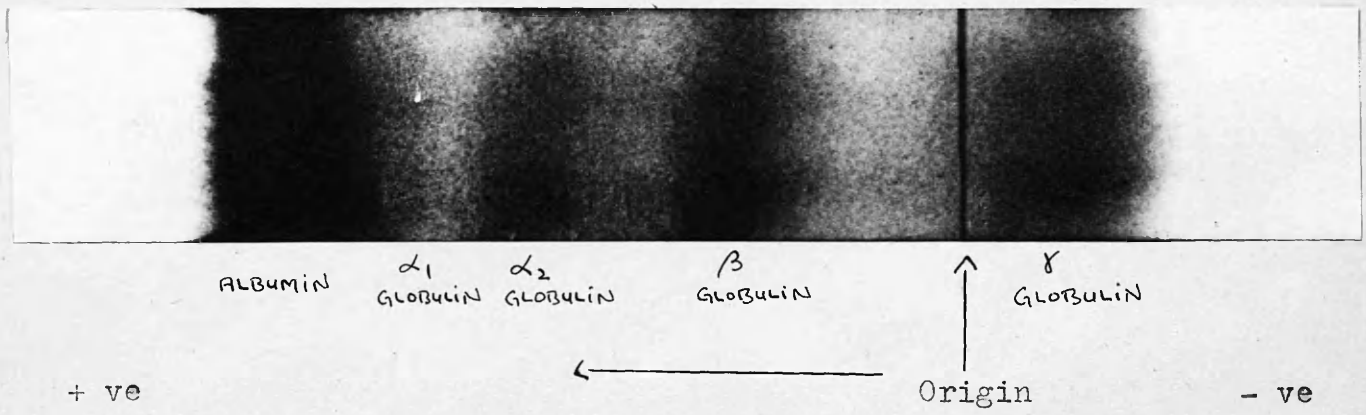
	pH (0.2 N Acetate Buffer)		
	3.4	4.5	5.2
Control	120	145	195
Albumin	240	283	290
α Globulin	201	280	282
β Globulin	202	280	284
γ Globulin	220	250	251

Results expressed as direct Spekker readings.

Assuming a total protein concentration of 7% in serum, with a distribution of albumin, α, β and γ globulins of 60%, 10%, 15% and 15% respectively, the corresponding assay tubes will each contain 0.25, 0.04, 0.06 and 0.06 mg. protein. The activating effect of the individual fractions thus appeared to be unspecific and independent of protein concentration within the limits employed.

Figure 8.

Paper Electrophoresis of Human Serum



60 μ.litres serum applied
Veronal buffer ; pH 8.5 ; μ = 0.05
4 v./cm. I8 hours.

The latter observation was also substantiated by a study of activation by whole serum. At a final dilution of 1/50, where the protein concentration was 1.2 mg. comparable results were obtained as shown:

	pH (0.2 N Acetate Buffer)		
	3.4	4.5	5.2
Control	120	145	195
Whole Serum (F.C. 1/50)	245	315	315

Results expressed as direct Spekker readings.

DNA Effect.

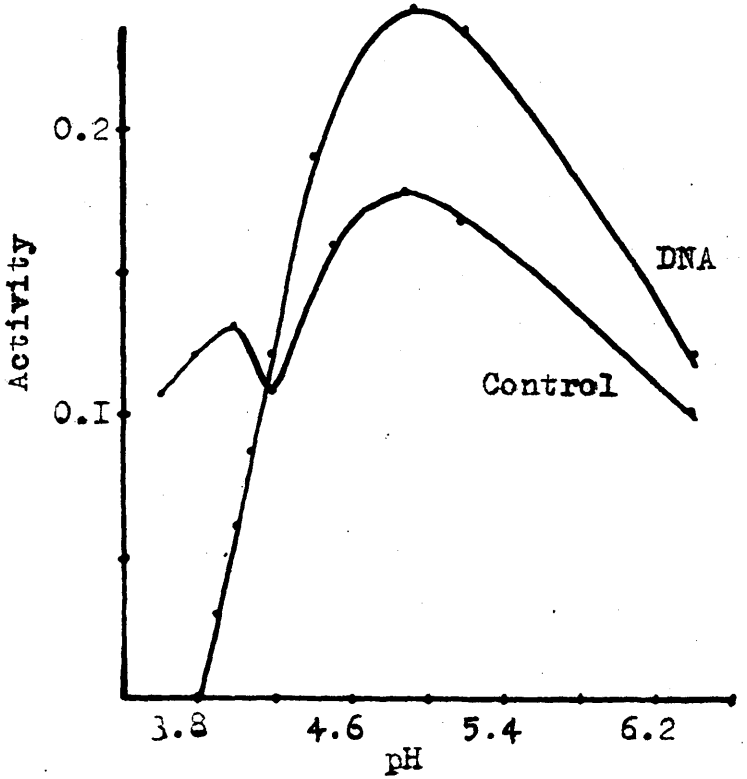
The activating effect of added DNA on purified liver β-glucuronidase at pH 4.5 has already been illustrated. Of even greater interest however, is the inhibitory action of DNA at lower pH values. A typical activity curve in the presence of 0.06% DNA is shown in Figure 9, where sharp inhibition was invariably observed over a narrow pH range (0.2 pH units).

An interesting comparison may be drawn between the effect of added DNA and the action of suramin on certain enzymes. Wills and Wormall (1950) have shown that suramin, a polysulphonic acid, inhibits many enzymes on the acid side of their isoelectric points. When percentage inhibition was plotted against pH in such instances, a sharp drop was evident in the inhibition - pH curve, 50% inhibition occurring at or about the isoelectric point of the enzyme, an effect which is also applicable to studies

Figure 9

Effect of DNA on the Assay of Purified Liver β - Glucuronidase

DNA - Final Concentration 0.06%



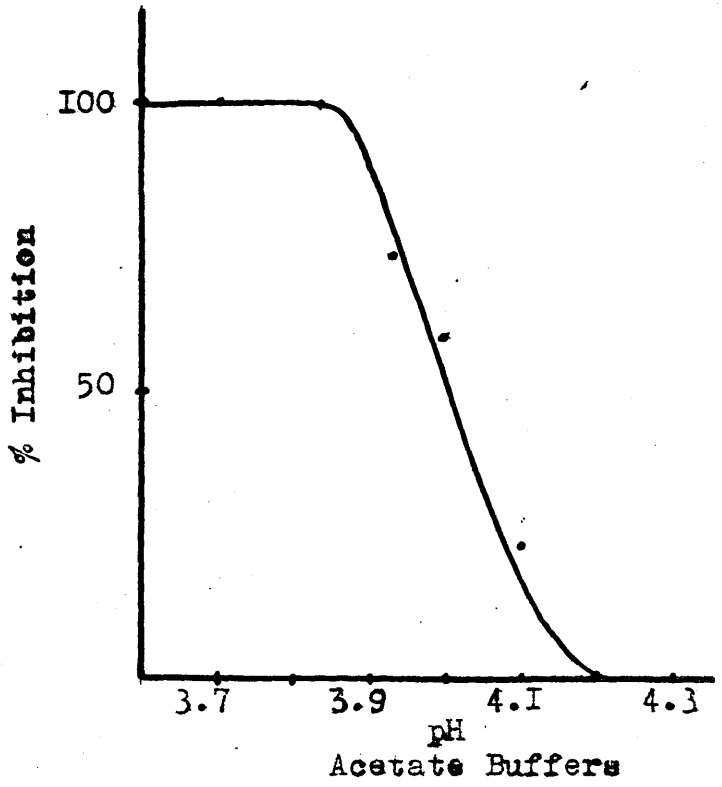
pH 3.4 - 5.8 Acetate Buffers

pH 5.8 - 6.6 Veronal-acetate Buffers

Figure IO

Inhibition - pH Curve of Purified Liver β - Glucuronidase in the Presence of DNA (I70,000 units per mg. P N)

DNA - Final Concentration 0.06%



on β-glucuronidase (vide Suramin Inhibition).

A similar sharp drop was evident in the inhibition - pH curve of liver glucuronidase in the presence of DNA (Figure 10). To extend the correlation further, the sulphonic acid groups of suramin are essential for inhibition of enzyme activity (Wills and Wormall 1950), although inhibition is not solely due to this factor but depends to a very considerable extent on the size of the molecule. The polyphosphoric acid groups of DNA also appeared to be essential for inhibition. When a solution of DNA was allowed to stand for two weeks at $0^{\circ}C.$, partial depolymerisation to nucleotides and nucleosides was apparent ionophoretically, (from an analysis kindly determined by Mr. Wm. McIndoe of this department), concurrent with a complete loss of inhibitory power. Adenylic acid per se (final concentration $10^{-3}M$) had no effect on enzyme activity.

Although DNA and suramin are dissimilar in the pH range over which inhibition occurs, their effects are none the less comparable in many respects. As will be seen, suramin also exerts a similar activating effect to that of DNA in the higher pH range.

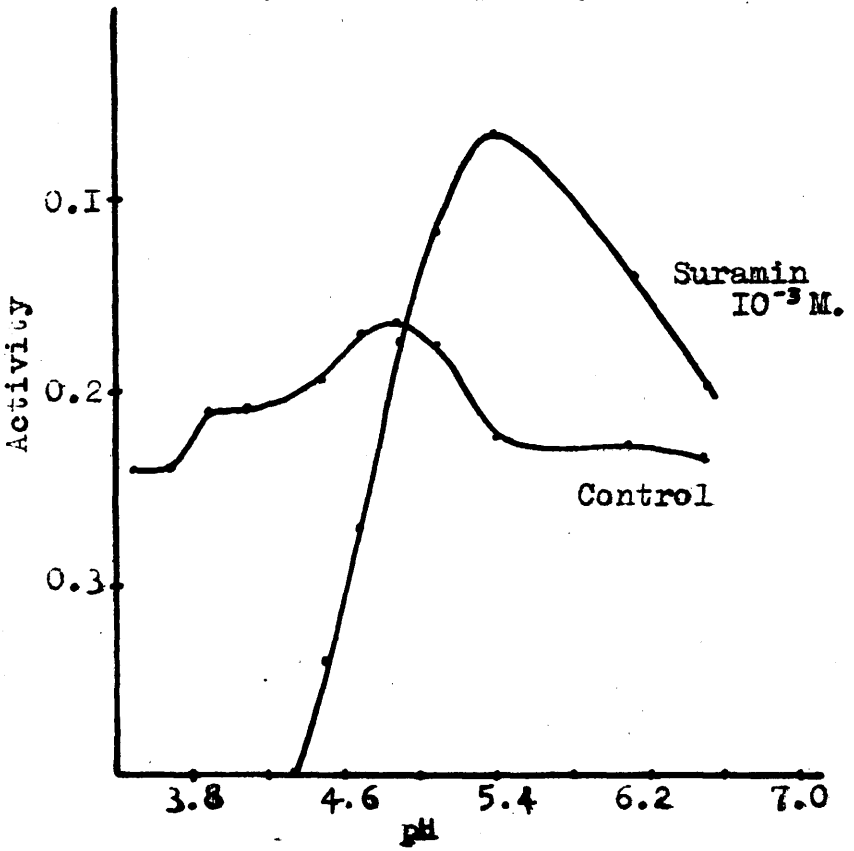
Suramin Inhibition.

The main features of inhibition of certain enzymes by suramin have already been described. Figure 11 shows an activity curve of purified liver β-glucuronidase in the presence of $10^{-3}M$ suramin, while Figure 12, expresses the results in terms of an inhibition - pH graph.

Figure II

Activity Curve of Purified Liver β - Glucuronidase in the Presence of Suramin (10^{-3} M)

(150,000 units per mg. P N)



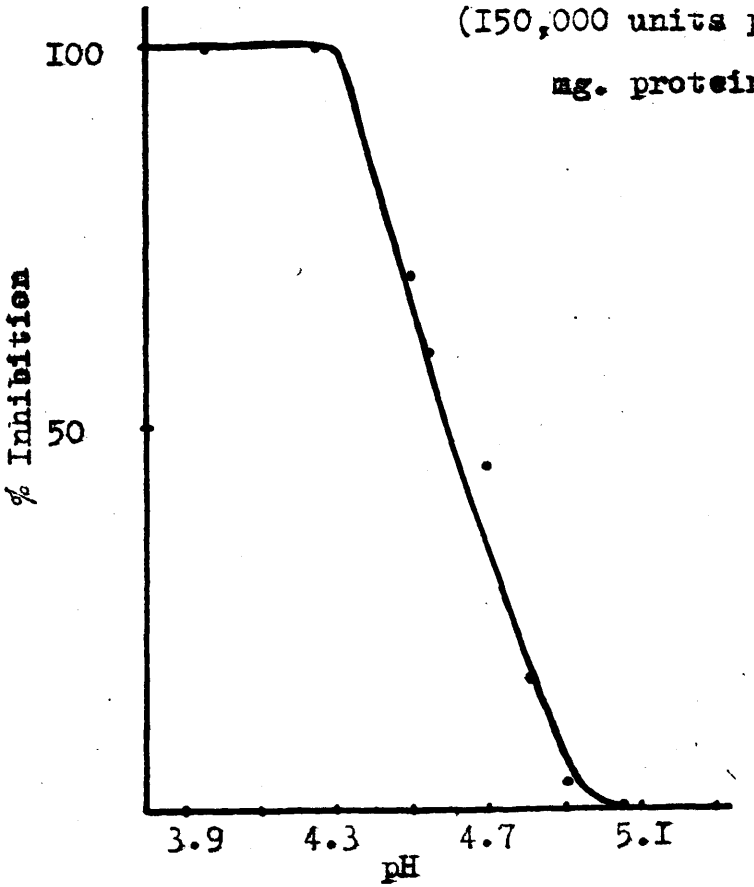
pH 3.4 - 5.8 Acetate Buffers

pH 5.8 - 7.0 Veronal-acetate Buffers

Figure I2

Inhibition pH Curve of Purified Liver β - Glucuronidase in the Presence of Suramin (10^{-2} M.)

(150,000 units per mg. protein N



Acetate Buffers

Wills and Wormall (1950) have shown that the duration of contact of drug and enzyme before addition of the substrate is an important factor, maximum inhibition being attained when the suramin - enzyme mixture is kept for 20-60 minutes before addition of the substrate. Accordingly the enzyme, buffer and suramin mixture was allowed to stand at room temperature for at least 45 minutes prior to assay. Control tubes containing water instead of suramin were similarly treated.

On the interpretation of Wills and Wormall (1950), reference to the inhibition - pH curve of purified liver glucuronidase gives a value of 4.6-4.7 for the isoelectric point of the enzyme (pH of 50% inhibition). The value so obtained compares favourably with that obtained electrophoretically.

Serum Inhibitor.

Fishman, Altman and Springer (1948) reported the presence in serum and plasma of a powerful inhibitor of glucuronidase activity. To quote the above authors: "β-glucuronidase activity is strongly inhibited in the presence of untreated or boiled mammalian plasma or serum. Only a slight decline in inhibitory activity is observed with plasma dilutions of 1 to 5 or 10. With greater plasma dilutions the inhibitory effect diminishes more markedly, although demonstrable effects are still evident at 1 to 100 dilutions....."

A study of this inhibitor was considered desirable, but the issue became complicated when no inhibition could be achieved with

either serum or plasma under a variety of circumstances. As previously indicated, activation of the purified liver enzyme was obtained with human serum and the various components separated therefrom. It was considered possible that the enzyme in a high state of purity need not necessarily act in a comparable manner to less pure preparations, and a variety of other preparations were assayed in the presence of whole serum. The former varied from straight liver homogenates in water to celited $(NH_4)_2SO_4$ fractions of the Stage C preparation described by Mills (1948), but in no instance could inhibition be achieved, the general effect being that of activation rather than the converse. Variation of the serum source likewise produced negative results. These conflicting observations are further exaggerated by experiments on the serum inhibitor conducted some months previously by Dr. Mills and Dr. Paul. The above workers experienced no difficulty in reproducing the results of Fishman et al. (1948) on either liver or spleen glucuronidase, although the degree of inhibition appeared to be dependent on the physical state of the enzyme itself, different enzyme preparations showing different degrees of inhibition towards the same serum. As yet no explanation has been found for the opposing results expressed.

Specific Inhibitors of β-Glucuronidase Activity.

Morrow, Carrol and Greenspan (1951) in a study of the glucuronidase activity of inbred mice made reference to the single pH optimum obtained in the assay of mouse kidney homogenates. While the curve presented showed an optimum at pH 4.5,

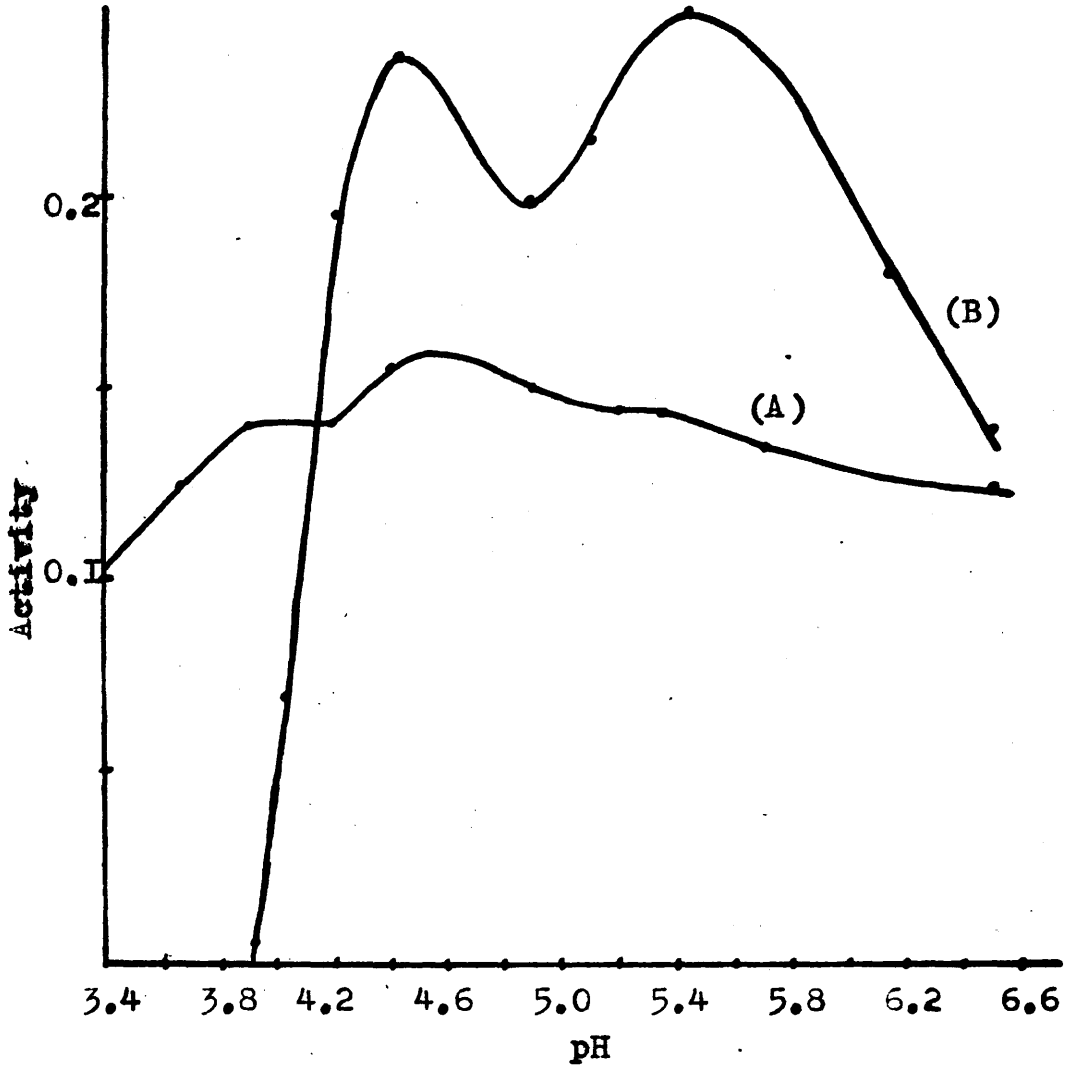
considerable activity was nevertheless present at pH 5.2-5.6. The most surprising feature however, was the total absence of activity below pH 3.8, the curve rising sharply from zero activity at this pH to the optimum around pH 4.5. This has never been the experience of the present author under normal assay circumstances. It was noted however that phthalate - HCl and phthalate - NaOH buffers were used by Morrow et al. (1951), and it was considered that the lack of activity in the lower pH range was probably due to specific inhibition of enzyme activity by phthalic acid. That this was indeed the case was demonstrated by assaying the enzyme in both phthalate and acetate buffers. Reference to Figure 13, shows the marked and specific inhibitory effect of phthalate in the lower pH range coincident with considerable activation above pH 4.0. A further consistent feature of phthalate activation was the emphasis in activity at the known pH optima of the enzyme (pH 4.5 and 5.2).

These detached observations preceded a study of inhibition of liver β-glucuronidase activity by a series of structurally related organic compounds. Inhibition was determined at the three pH optima of the enzyme (pH 3.4, 4.5 and 5.2; acetate buffers), and the results obtained are summarised in Table ..,2. While the results quoted are mainly confined to the effect of aromatic analogues on liver β-glucuronidase activity, a fairly extensive investigation of the effect of certain aliphatic compounds on the activity of the spleen enzyme has recently been determined by Mills, Paul and Smith (1952b) to which further reference will

Figure I3

Assay of Ox Liver β - Glucuronidase in Phthalate and Acetate Buffers

(I70,000 units per mg. protein N)



(A) - pH 3.4 - 5.8 Acetate Buffers

pH 5.8 - 6.6 Veronal-acetate Buffers

(B) - pH 3.4 - 3.8 Phthalate-HCl Buffers

pH 3.8 - 6.6 Phthalate-NaOH Buffers

Table 2.

The Effect of Structurally Related Aromatic Compounds on Liver β-Glucuronidase Activity

All compounds assayed at a final concentration of 10^{-2}M.

\+ indicates percentage activation at the pH specified.

\- indicates percentage inhibition at the pH specified.

Activity determined at pH 3.4 4.5 and 5.2 (0.2 N acetate buffers).

Enzyme purity - 170,000 units activity per mg. protein N.

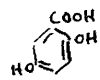
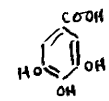
Compound	Formula	pH of Assay 3.4	4.5	5.2
Benzoic acid		+ 37	+ 6	+17
2(OH) benzoic acid		-100	-4	+ 9
3:5$(OH)_2$benzoic acid †		-9	0	0
2:6$(OH)_2$benzoic acid †		-100	+5	+26
3:4$(OH)_2$benzoic acid †		0	0	0
2:5$(OH)_2$benzoic acid †		-100	-85	-76
3:4:5$(OH)_3$benzoic acid		-29	-40	-19
2:3:5:6$(OH)_4$ cyclohexane carboxylic acid		-23	+ 6	+10
Phthalic acid		-25	+50	+47
Isophthalic acid *		-23	+50	+45

Table 2 (Contd.)

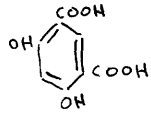
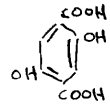
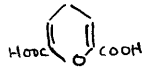
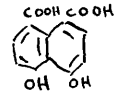
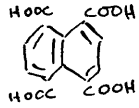
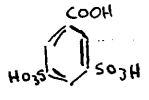
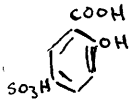
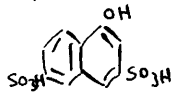
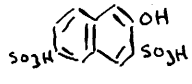
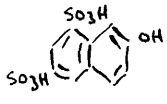
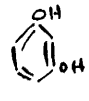
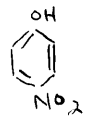
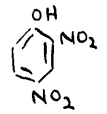
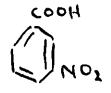
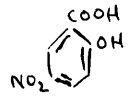
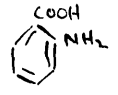
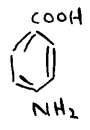
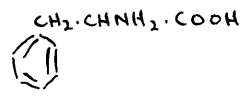
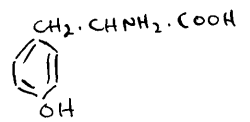
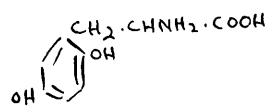
Compound	Formula	pH of Assay 3.4	4.5	5.2
4:6(OH)$_2$isophthalic acid *		-89	+48	+40
2:5(OH)$_2$terephthalic acid *		-100	+57	+41
Chelidonic acid		-100	-40	-26
4:5(OH)$_2$naphthalene 1:8(COOH)$_2$acid		-100	-77	-30
1:4:5:8 naphthalene tetra carboxylic acid		-82	-73	-70
Benzene sulphonic acid		+56	+25	+28
Benzoic acid 3:5 disulphonic acid †		-100	+55	+93
Salicylsulphonic acid		-100	-4	+8
1 naphthol-3:6 disulphonic acid		-100	-45	0
2 naphthol 3:6 disulphonic acid		-100	+32	+16
2 naphthol 6:8 disulphonic acid		-100	+23	+30
Resorcinol		0	0	0

Table 3 (Contd.)

Compound	Formula	pH of Assay 3.4	4.5	5.2
p-nitro phenol		-7	-12	-32
2:4$(NO_2)_2$ phenol		0	0	33
m - nitro benzoic acid		+7	+37	+25
5 nitro salicylic acid		-100	+41	+46
O - amino benzoic acid		-28	-20	0
p - amino benzoic acid		+17	+8	+3
Alanine	$CH_3 \cdot CHNH_2 \cdot COOH$	0	0	0
Phenylalanine		+62	+14	-70
Tyrosine		+47	+10	-100
2:5$(OH)_2$phenyl alanine		-100	-13	+19

* Acids were kindly provided by Dr. Ongley, Chemistry Department, University of Glasgow.

† Acids were kindly provided by Dr. Reid and Mr. R. Watson, Gardiner Institute of Medicine, The Western Infirmary, Glasgow.

be made.

In the present study, it will be noted that inhibition of enzyme activity at one pH was frequently associated with activation at another pH. While certain anomalies are obvious, a few guiding principles were apparent, which may be expressed tentatively as follows:

(1). The presence of one acid group on the benzene ring caused activation at all pH's studied. The sulphonic acid group appeared to be more effective in this respect than the less strongly ionised carboxyl group.

(2). Introduction of another acid group into the molecule invariably resulted in inhibition of enzyme activity at pH 3.4 concurrent with activation at pH 4.5 and 5.2. Reference to inhibition by phthalic acid and iso-phthalic acid showed that, in this instance, the relative positions of the COOH groups bore little relation to the overall effect.

(3). Introduction of OH groups into the monoacidic aromatic molecule was specific in effect. Provided the position α to the acid group was not substituted, the activating effect of the latter decreased progressively with OH substitution. 3,4 dihydroxy benzoic acid was without effect on enzyme activity, while 3,4,5 trihydroxy benzoic acid functioned as an inhibitor at all pH's.

In all instances, introduction of a hydroxyl constituent α to an acidic component resulted in complete inhibition of enzyme activity at pH 3.4 at the concentration of inhibitors studied

(10^{-2}M). Direct attachment of the acid group to the benzene nucleus was not an essential feature of this inhibition, as 2,5 $(OH)_2$ phenylalanine, where the OH group is positioned α to a side chain containing a terminal acidic grouping, acted in a like manner. When the resonating benzene ring was replaced by a substituted saturated cyclic hydrocarbon as in the case of 2,3,5,6 $(OH)_4$ cyclohexane carboxylic acid, this inhibitory effect was considerably reduced.

Inhibition by phenylalanine and tyrosin is of interest in view of their occurrence in vivo. Whether any biological significance can be attached thereto is, however, another matter. The relative concentrations of both amino acids required for inhibition were high, and while tyrosin was the more effective in this respect, a concentration of 10^{-3}M was required to effect 10% inhibition. Of more select interest is the specificity of inhibition at the pH defined, a point which will be considered further when the complex nature of the enzyme system is discussed.

Karuniaratnam and Levvy (1949) showed that saccharic acid was a powerful inhibitor of β-glucuronidase activity. This work has been confirmed by Mills, Paul and Smith (1952b) and a study was made of the inhibition of the spleen enzyme by a series of aliphatic dicarboxylic acids. While this work has not been extended to liver glucuronidase, a summary of the more salient features of the spleen inhibition study is included for future reference in Table 3.

Table 3.

Percentage Inhibition of Ox Spleen β-Glucuronidase by Various Organic Acids.

Substrate = phenylglucuronide (5×10^{-3}M) in acetate buffer at pH 3.4

	Concentration (M)						
Inhibitor	10^{-2}	5×10^{-3}	10^{-3}	2×10^{-4}	10^{-4}	5×10^{-5}	10^{-5}
Citrate	75	65					
L-Tartrate	85						
Tricarballylate	5						
L-Malate	90						
Oxalate	100	90	0				
Malonate	40						
Succinate	25						
Glutarate	5						
Lactate	5						
L-Glutamate	8						
D-Gluconate	25						
D-Galacturonate	50	30	0				
D-Mucate	100	100	100	95	75	20	0
D-Saccharate	100	100	100	100	100	90	25

Synthetic Detergents.

Many observations may be cited indicating that synthetic detergents produce diverse effects on certain enzymes (Putnam 1948). Such effects are well exemplified in the case of purified liver β-glucuronidase. "Triton X 100", (a non ionic alkyl aryl polyether alcohol), was found to activate glucuronidase over the entire pH range in concentrations ranging from 0.01% to 0.1%. "CTAB" (cetyl trimethyl ammonium bromide), a cationic detergent, while inhibiting glucuronidase in high concentrations (0.1%) activated the enzyme at greater dilutions (0.01%). The results obtained are summarised in the following table:

	pH					
	3.4	3.8	4.4	4.9	5.2	6.4
C TAB						
0.01%	+ 20	+ 22	+ 50	+ 52	+ 53	+140
0.05%	- 38	- 46	- 50	- 50	- 56	- 73
0.1%	-100	-100	-100	- 100	-100	- 100
Triton X 100						
0.01%	+ 27	+ 17	+ 22	+ 21	+ 19	+ 59
0.05%	+ 19	+ 14	+ 20	+ 26	+ 32	+100
0.1%	+ 10	+ 8	+ 27	+ 25	+ 23	+120

\+ indicates percentage activation

\- indicates percentage inhibition

Electrophoretic Studies on Purified Liver β-Glucuronidase.

In view of the suggested complexity of the enzyme system (Mills 1948), electrophoresis as a criterion of homogeneity was

envisaged. The method of electrophoresis of enzymes on filter paper (Mills and Smith 1951), and the more orthodox analysis by the Tiselius apparatus were both applied to the study of purified liver β-glucuronidase.

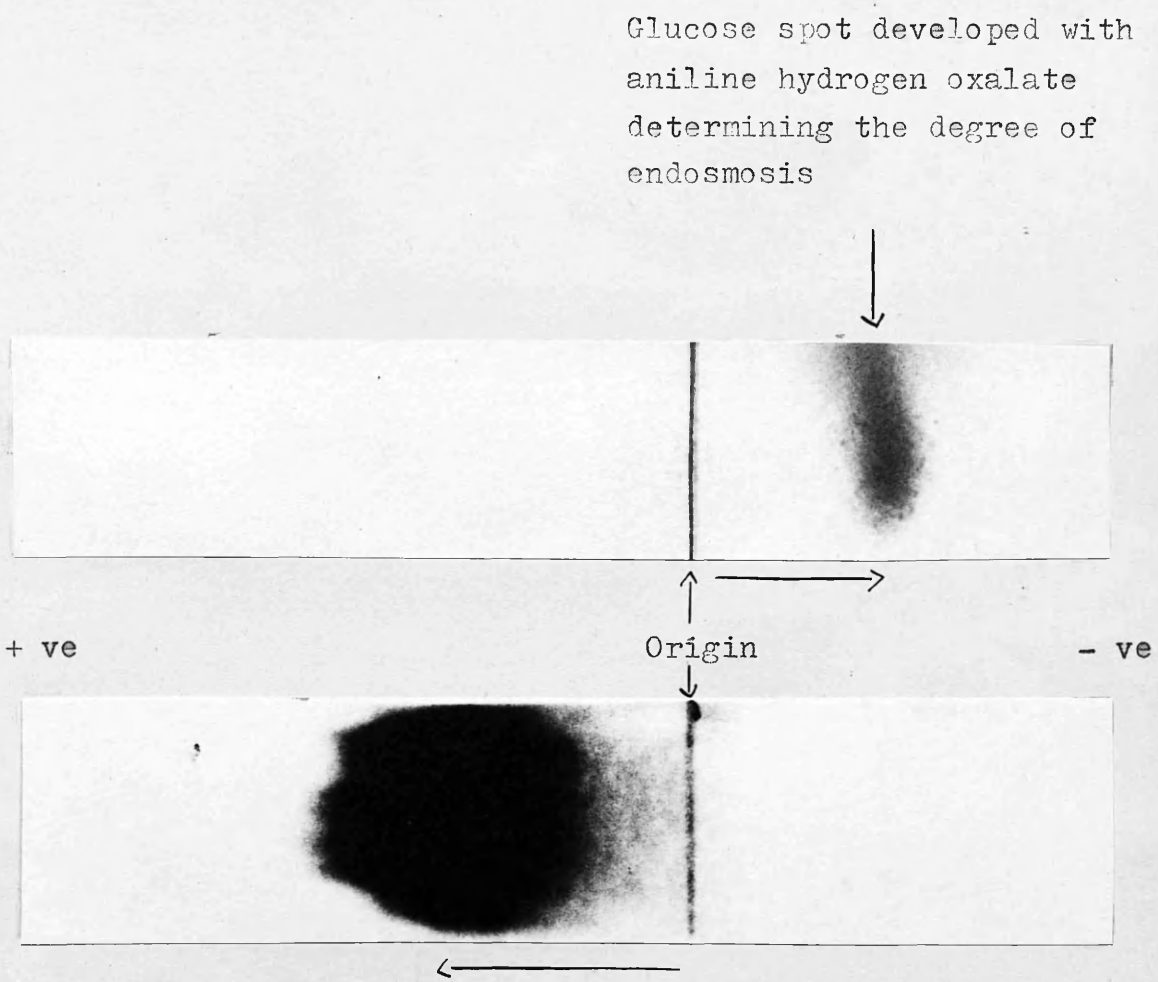
In the former instance, 30μ litres of glucuronidase solution (170,000 units per mg. protein N) were applied to a filter paper strip and electrophoresis carried out for 18 hours (phosphate buffer pH 8.0, $\mu = 0.1$; 5 v./cm.). Localisation of protein by the staining methods of either Durrum (1950) or Turba and Enenkel (1950) was insensitive to the low concentration of protein present, and the enzyme was detected chromogenically in the manner previously described.

A typical electrophoretic run is reproduced in Figure 14. While the area of activity localised is suggestive of the non-homogeneity of the enzyme moiety, the effect of possible adsorption on paper of minute amounts of protein associated with high enzyme activity must be carefully considered. The activity/protein ratio of purified β-glucuronidase is high, and even a slight adsorptive effect would be associated with detectable enzymic activity. This was made more apparent when glucuronidase was electrophoresed in the presence of albumin. The latter experiment was devised to determine whether or not an albumin-glucuronidase complex was formed in the course of enzyme activation by the former.

An enzyme run in the presence of 30μ litres 1% crystalline horse serum albumin is shown in Figure 15, compared with a control

Figure I4.

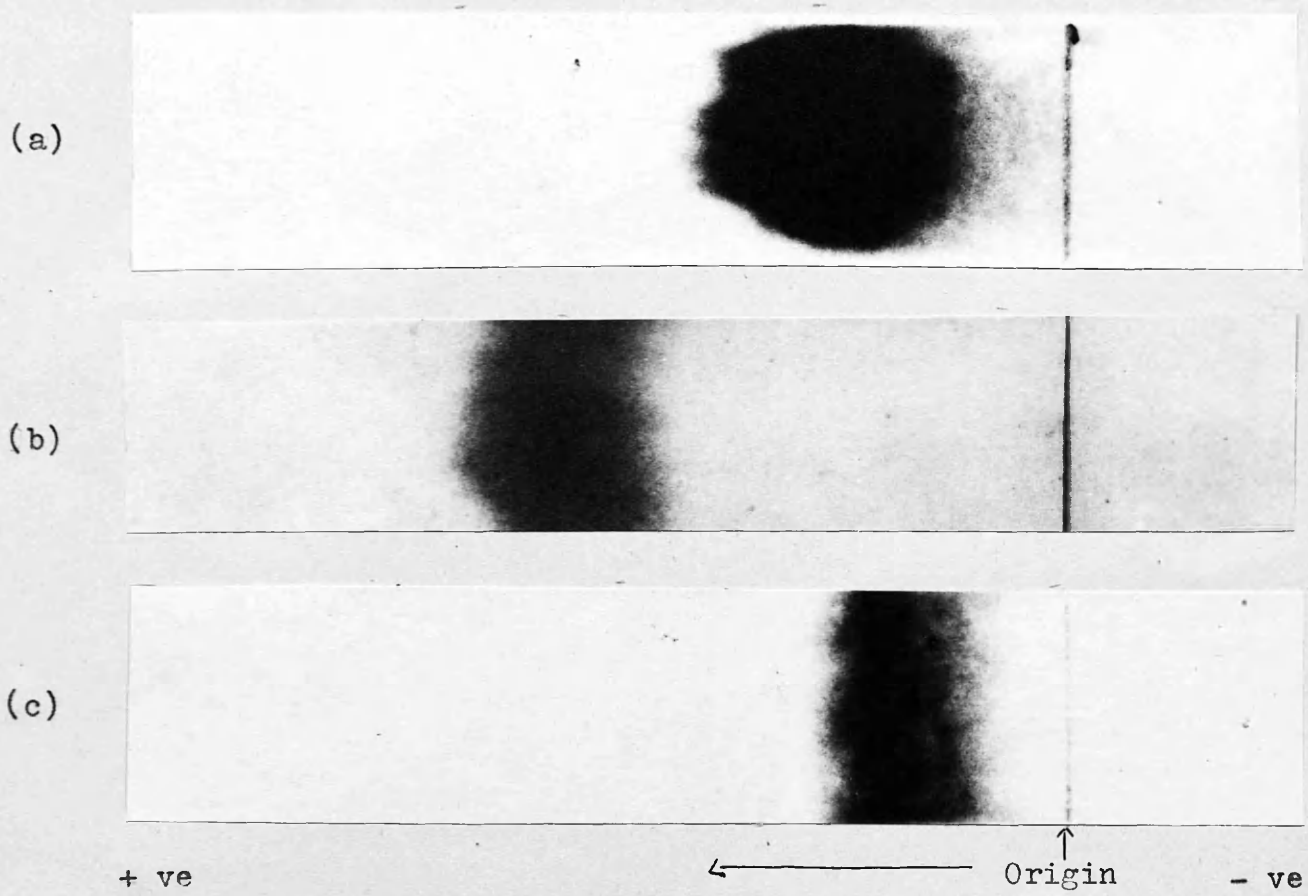
Paper Electrophoresis of Purified Liver β - Glucuronidase.



30 μ.litres β - glucuronidase solution applied
Phosphate buffer ; pH 8.0 ; μ = 0.I
5 v./cm. I8 hours

Figure I5.

Paper Electrophoresis of Purified Liver β- Glucuronidase in the Presence and Absence of Albumin.



(a). Purified liver β - glucuronidase
(b). I% crystalline horse serum albumin
(c). β - Glucuronidase plus albumin

Phosphate buffer ; pH 8.0 ; μ = 0.I
5 v./cm. I8 hours

run of enzyme alone. While no enzyme protein complex was apparent, the albumin migrating ahead of the glucuronidase, a comparison of strip (a) with strip (c) indicates that the area of enzymic activity is more compact in the latter, and more comparable to the migration of a homogeneous protein than otherwise. In the above experiment, the albumin and glucuronidase solutions were mixed prior to application. If however the enzyme was first applied to the paper strip and then superimposed by the albumin solution, adsorption of the former was again apparent.

Figure 14 gives a value for the mobility of purified liver β-glucuronidase of 2.0×10^{-5} $cm^2/v.sec.$, which approximates to the value obtained by the Tiselius electrophoretic technique.

The analysis of purified liver β-glucuronidase by the latter method is necessarily subject to certain limitations. An enzyme preparation of a purity of 170,000 units activity per mg. protein N is associated with 0.05-0.1 mg. protein N per ml., a value far below the limits required for successful electrophoresis. Nevertheless it was considered that by making use of added crystalline albumin to obtain an initial protein buffer boundary of suitable concentration gradient some information could possibly be gained on the electrophoretic migration of a less pure enzyme preparation.

For this purpose, an enzyme solution containing 0.25 mg. protein N per ml., (80,000 units activity per mg. protein N), was obtained. It was found however that prolonged dialysis of the above against phosphate buffer pH 8.0μ = 0.2, previous

to electrophoresis resulted in a gross decrease in enzyme activity. This difficulty was obviated by the addition of crystalline horse serum albumin (final concentration 1%) to the solution before dialysis. Stabilisation of enzyme activity was effected in this manner, and due allowance having been made for the activating effect of albumin on glucuronidase per se, it was found that no loss in enzyme activity had occurred. The results obtained are expressed in the following table:

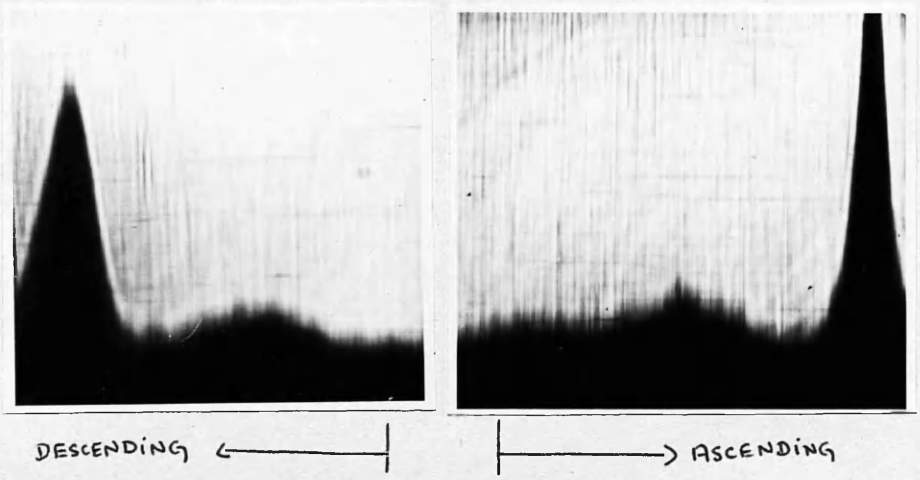
	pH		
	3.6	4.4	5.2
Control	121	130	121
Control + Albumin (Final conc. 1%)	140	153	160
Dialysed control	80	70	68
Dialysed control + Albumin (F.C.1%)	138	156	163

Activity expressed as direct Spekker readings.

The electrophoretic migration of such a glucuronidase preparation in phosphate buffer pH 8.0, $\mu = 0.2$, is shown in Figure 16. The albumin again migrated as a separate entity which, when sectioned off in one of upper limbs of the U - tube, showed no enzyme activity. The slower moving fraction was associated with glucuronidase activity, but whether or not the enzyme migrated as a single or complex protein is obviously exceedingly difficult to interpret; While the breadth of the peak undoubtedly suggests that the protein component is non-homogeneous, the material electro-

Figure I6.

Electrophoretic Analysis (Tiselius Method) of Ox Liver β- Glucuronidase (80,000 units per mg. protein N) in the Presence of I% Crystalline Horse Serum Albumin.



Phosphate buffer pH 8.0 ; $\mu = 0.2$

phoresed was only 40% of ultimate purity, and as such bound to be associated with extraneous protein.

A divergence of opinion must again be expressed between the work of Fishman and his colleagues (Bernfeld and Fishman 1950c) and that of the present author. The former quote a value of pH 7.5-8.0 for the isoelectric point of a purified spleen glucuronidase preparation. While the above experiments were conducted on the liver enzyme, additional electrophoretic studies on material obtained from spleen did not in any way substantiate the claims of Bernfeld et al (1950c) in this respect. In all instances the spleen enzyme behaved in a similar manner to that obtained from liver, the calculated mobilities in each instance being 3.1×10^{-5} cm.^{2}/v.sec. and 2.76×10^{-5} cm.^{2}/v.sec. respectively (phosphate buffer pH 8.0, $\mu = 0.2$.)

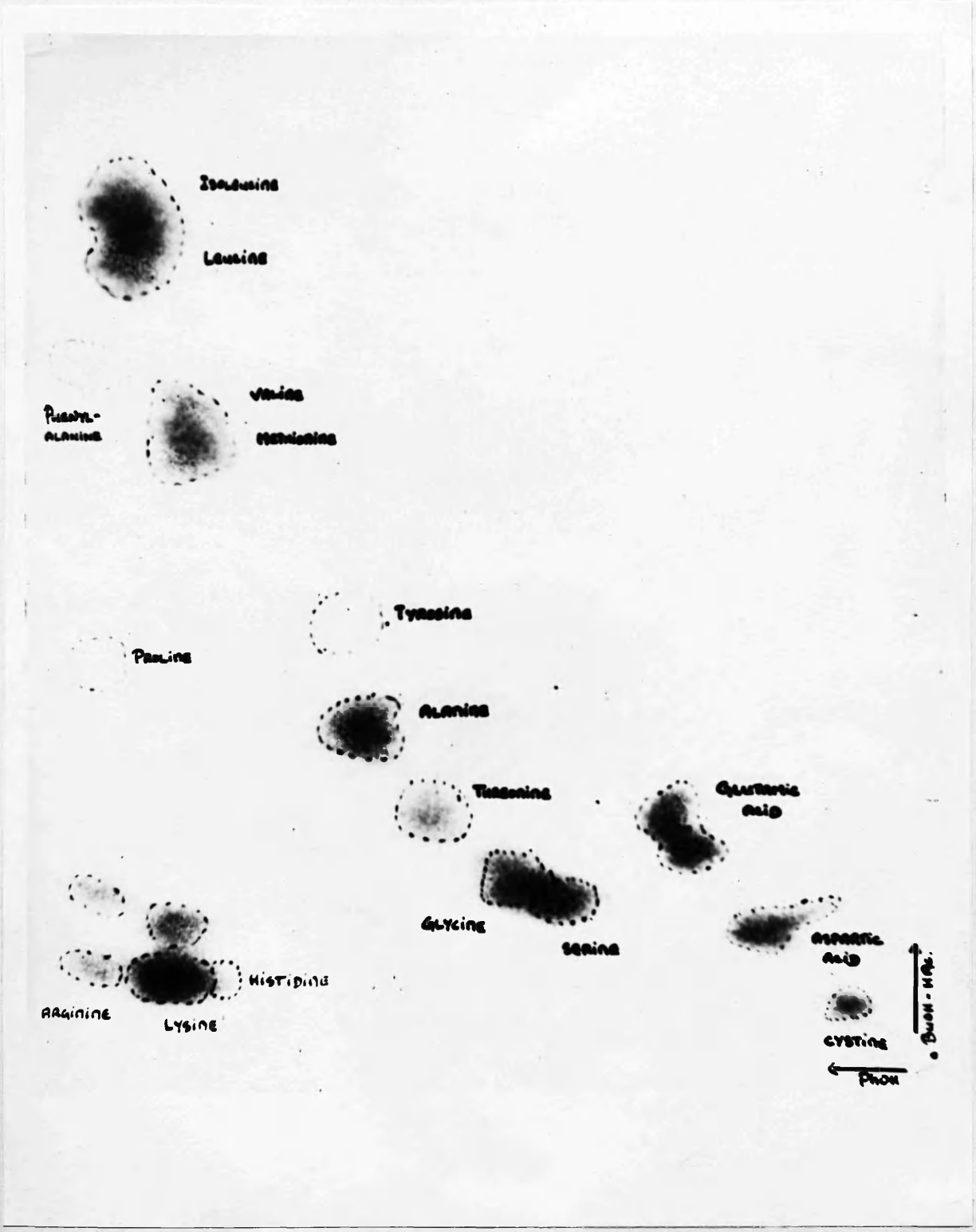
Amino Acid Constitution of Purified Ox Liver β-Glucuronidase.

A qualitative analysis of a purified enzyme preparation containing 150,000 units of activity per mg. protein N was effected chromatographically. The analysis was kindly undertaken by Dr. Leaf of this department and the results obtained from a two dimensional paper chromatogram of the acid hydrolysed material are shown in Figure 17. No unusual feature was apparent in the constitution of the enzyme and a total of 17 amino acids was obtained from the hydrolysate. Whether this is truly representative of the enzyme per se can only be determined by a more comprehensive analysis of the enzyme at varying stages of purity.

Turnover Number. /

Figure I7.

Two Dimensional Chromatogram of an Acid Hydrolysate of Purified Ox Liver β- Glucuronidase (I50,000 units per mg. Protein N).



Solvents : Phenol and butanol - acetic acid.

Turnover Number.

Bernfeld and Fishmann (1950a) quote a value of 40 for the Turnover Number for purified calf spleen β-glucuronidase. A theoretical value of 1.5×10^2 has been obtained for the purified liver enzyme basing the calculation on a series of considerations similar to those employed by the above authors.

The two basic assumptions required are:

(1). Molecular weight of β-glucuronidase = 100,000

(2). Protein/nitrogen conversion factor = 6.5

Application of the above assumptions to a calculation of the Turnover Number gives a value of 1.5×10^2 moles substrate/mole protein/minute for a preparation containing 200,000 units activity per mg. protein N, the ultimate purity so far obtained for the liver enzyme.

C. Fractionation of Ox Liver and Spleen β-Glucuronidase.

Reference has frequently been made to the work of Mills (1948) who separated two enzyme fractions from ox spleen having pH optima at 4.5 and 5.2 for the hydrolysis of phenolphthalein glucuronide. This work has since been extended (Mills and Paul 1949; Mills, Paul and Smith 1949) to include a third enzyme of pH optimum 3.4.

At the outset it should be mentioned that considerable controversy exists over the multiple nature of the glucuronidase system. Apart from the evidence presented by Mills (1948), Mills and Paul (1949), Mills, Paul and Smith (1949; 1952a and b) for the spleen enzyme, few other workers are prepared to accept the theory of an enzyme complex. Kerr, Campbell and Levvy (1949) and Morrow, Greenspan and Carrol (1950), while confirming the presence in mouse liver and kidney of the two β-glucuronidase fractions found by Mills (1948), did not investigate the problem in any great detail; at the other extreme, Bernfeld and Fishman (1950a) and Sarkar and Sumner (1950) failed to adduce any evidence in support of the multiple nature of the enzyme.

While electrophoresis would appear to indicate that β glucuronidase migrates as a single protein, homogeneity in this respect need not necessarily infer simplicity of enzyme action. Furthermore the apparently homogeneous main electrophoretic components of plasma when subjected to the subtle fractionation techniques of Cohn et al (1946; 1950) have been resolved into a considerable number of distinctive protein constituents. While no claim has been made for the existence of separate protein

entities associated with glucuronidase activity, it will be seen nevertheless, that fractionation within fine limits can significantly alter the optimum pH of enzyme activity. Whether this is due to an actual separation of closely related enzyme entities or to less well-defined interactions between a protein carrier molecule and individual prosthetic groups associated with enzymic activity is a matter for speculation and will be considered in the discussion.

In preliminary fractionation experiments on ox liver, no attempt was made to duplicate the carefully controlled conditions outlined by Mills (1948) for the separation of two glucuronidase fractions from ox spleen, having pH optima for the hydrolysis of phenolphthalein glucuronide of 4.5 and 5.2 respectively (acetate buffers). Use was made however of the $(NH_4)_2SO_4$ elution technique devised by Paul (1951) for fractionation purposes. A brief résume of the method which has already been quoted in the purification of ox liver β-glucuronidase, (Stage II) is as follows:

Glucuronidase was precipitated from 100 ml. of the Stage C solution of Mills (1948) with $(NH_4)_2SO_4$ at a concentration of 60% saturation. 2% Celite (519 A) was added to adsorb the enzyme which was successively eluted from the adsorbing material after filtration with 100 ml. portions of 1.8 M.,1.6 M., 1.4 M. and 1.2 M $(NH_4)_2SO_4$ solutions. A final concentration of 3 M. $(NH_4)_2SO_4$ was used to reprecipitate the enzyme from each of the above eluates, the enzyme precipitate spun off at $0^oC.$ and dissolved in the minimum volume of water. A pH of 5.0 was maintained throughout the above procedure.

Initially all assays on such fractionated material were carried out on undialysed preparations, necessitated at the time by the shortage of dialysis tubing. The interesting observation was made by Paul (1951) that this fractionation procedure was invariably attended by a complete loss of enzyme activity below pH 3.4, as shown in Figure 18, where the activity curve of a 1.4 M. fraction is compared with that of the original Stage C solution. This persistent loss of activity was attributed by Paul (1951) to a specific physical effect on the enzyme system dependent on the adsorbing material. When dialysis tubing was again made available however, it was noted by the present author that enzyme activity was regained at the lower pH values in the dialysed material, and the possibility was considered that the loss in activity could more readily be expressed in terms of a general salt effect. A thoroughly dialysed enzyme preparation was accordingly assayed in the presence of varying amounts of $(NH_4)_2SO_4$ with the following results:

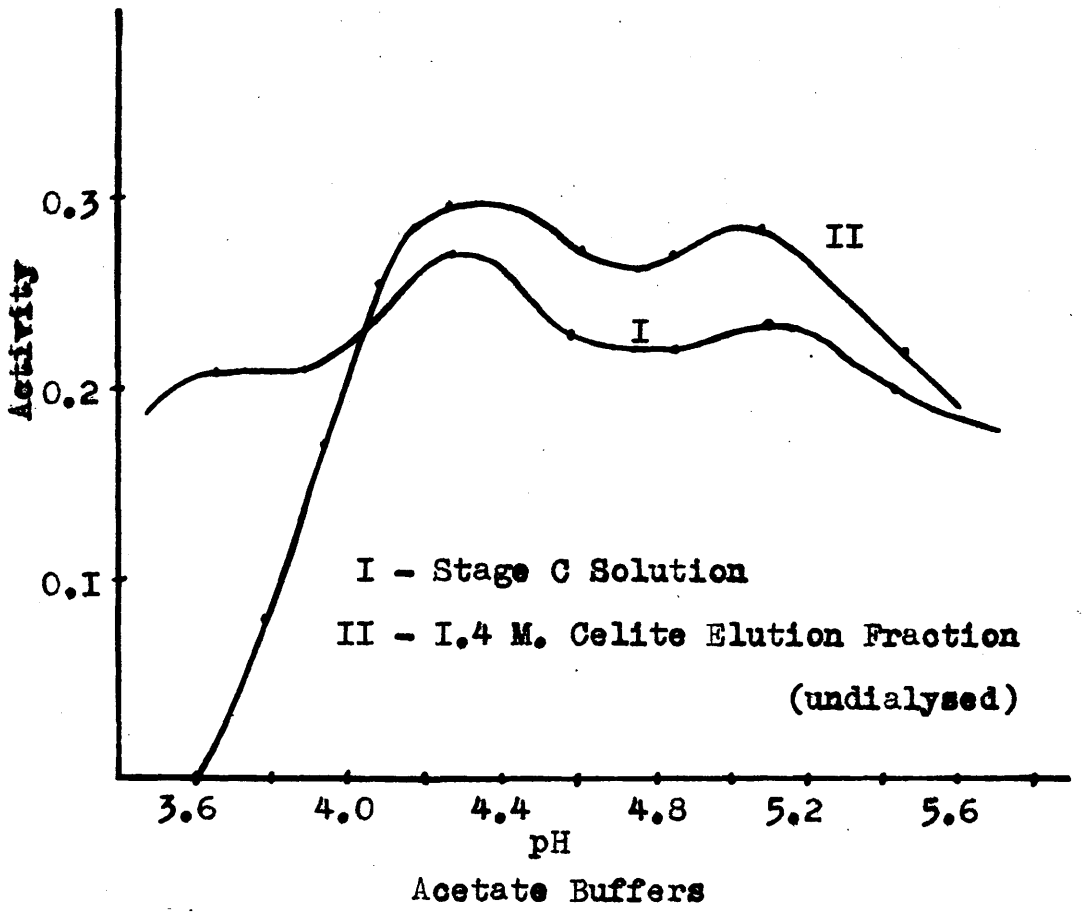
	pH (Acetate Buffers)			
	3.5	3.9	4.6	5.3
Control	130	135	175	163
$(NH_4)_2SO_4$				
0.03 M. μ = 0.09	027	046	185	165
0.01 M. μ = 0.03	060	138	179	166
0.005M. μ =0.015	080	136	175	163
0.001M. μ = 0.003	136	140	173	160

Molarities (M) and Ionic Concentration (μ) expressed as final concentrations.
Results expressed as direct Spekker readings.

Figure I8

Fractional Elution of Ox Liver β- Glucuronidase

(pH 5.0)



While the presence of $(NH_4)_2SO_4$ in the molarities defined undoubtedly depresses enzyme activity specifically at the lower pH values, such results per se do not necessarily invalidate the conclusion derived by Paul (1951). To determine whether loss in enzyme activity was in part dependent on the presence of an adsorbing agent, glucuronidase was fractionated in the presence of Celite 519A, concurrent with an analagous precipitation and elution from centrifuged material without Celite. Equivalent conditions were maintained throughout, the precipitated enzyme dissolved in standard volumes of water and diluted in a comparable manner for assay. In both series of experiments loss of enzyme activity was apparent at low pH values, from which it must be inferred that the conclusions of Paul (1951) are not valid, and that the effect is, ipso facto, dependent on the presence of $(NH_4)_2SO_4$.

This interesting salt effect was further enhanced by the fact that K_2SO_4 acted in an analagous manner to $(NH_4)_2SO_4$. A comparison of ionic strengths however, indicated that the former was less efficient than the latter in supressing enzyme activity at low pH values, the effect being apparent only at ionic strengths above 0.02. KCl and NaCl, while activating at all pH's showed no specific inhibition at concentrations up to 0.05 μ.

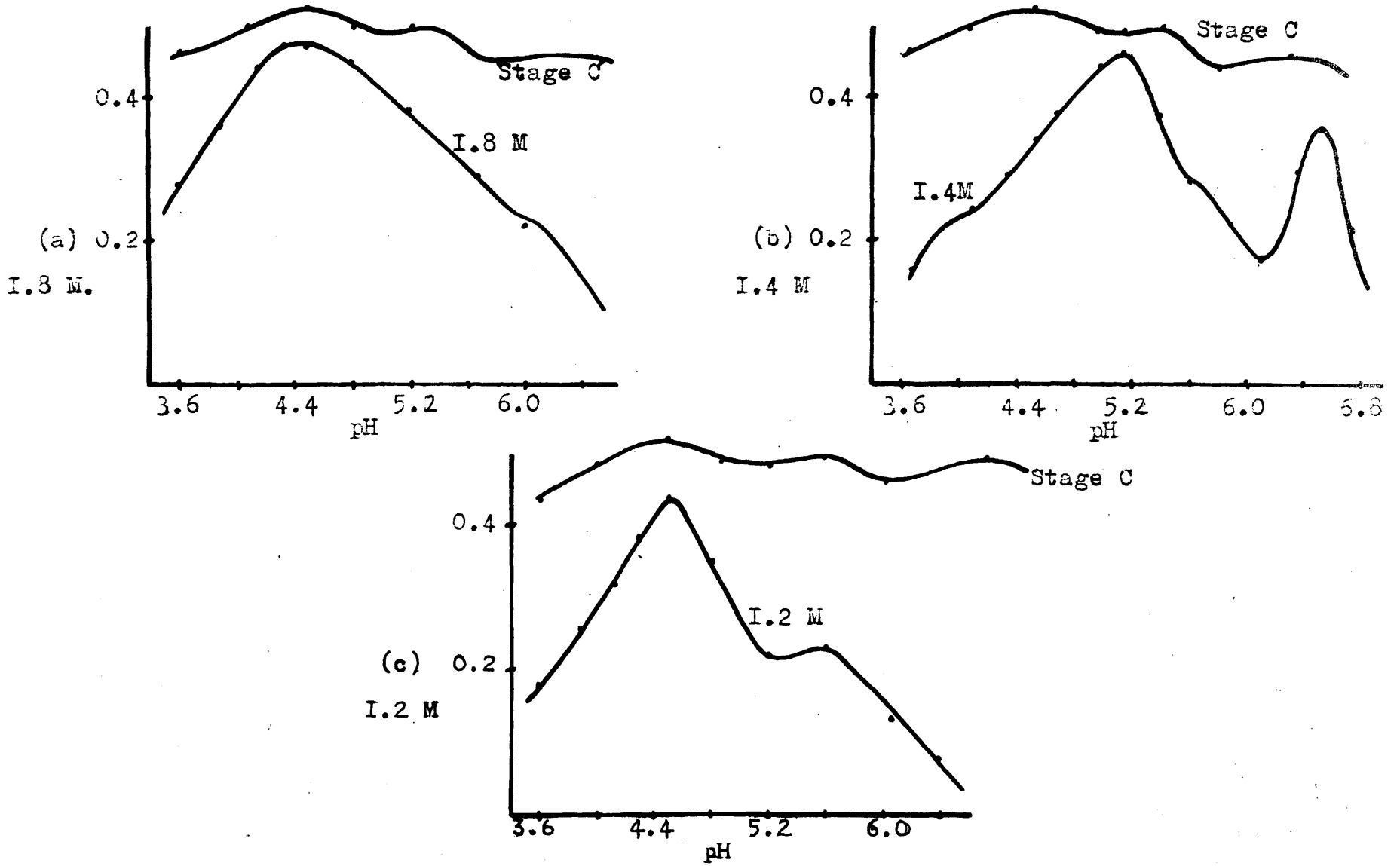
Fractional elution of glucuronidase with $(NH_4)_2SO_4$ solutions in the manner defined, while resulting in variations in the proportionality of activity at the known pH optima of the enzyme (3.4, 4.5 and 5.2, acetate buffers), gave only slight indication

of enzyme separation. Admittedly the method lacks the refinement of the procedure outlined by Mills (1948), where temperature and pH were strictly controlled and the limits more narrowly defined, but further refractionation with $(NH_4)_2SO_4$ was equally discouraging in this respect.

Initially a pH of 5.0 was chosen for separation purposes, a pH approximating closely to the isoionic point of the enzyme system. When the former was adjusted to 8.5 - 9.0 however, and maintained there throughout fractionation, the additive nature of the combined solvent action of salts and alkali resulted in significant alterations in the pH optima of the enzyme fractions so obtained. Figure 19 shows the variations in activity curve attained at varying points in the fractionation. While complete separation of enzyme activity was not achieved, reference to the requisite figures indicates a marked preponderance of activity at both pH 4.5 and 5.2 in different fractions. It should be emphasised that all assays were conducted on completely dialysed preparations to exclude the possibility of variation in activity curves due to the presence of $(NH_4)_2SO_4$.

An interesting feature is also apparent when considering Figure 19(b), where a distinctive pH optima is evident at pH 6.5 (veronal-acetate buffer). If the original Michaelis concept of activity curve can be accepted as a criterion of the multiple nature of an enzyme system, a fourth enzyme of pH optimum as indicated must be included when considering the complexity of the liver system. While subsequent experiments demonstrate more

FIGURE 19. Fractional Elution of Ox Liver β - Glucuronidase pH 8.5 - 9.0



pH 3.4 - 5.8 Acetate Buffers

pH 5.8 - 6.8 Veronal-acetate Buffers

clearly the existence of this optimum of activity, which is also present in the highly purified material (vide Figure 1,2), spleen β-glucuronidase appears to have very little activity in this range, all curves falling off sharply above pH 5.6, whether in fractionated or unfractionated material.

While such results provide a definite indication of the multiplicity of the glucuronidase system, subfractionation of such preparations showing predominant activity at either of the pH optima indicated was frequently ineffectual in improving the degree of separation. Obviously fractionation was dependent on a more critical series of factors than could be encompassed by simple variations in salt concentration, and fractionation was again envisaged in terms of the multi-variable system of Cohn et al (1946) using organic solvents as precipitants with attendant variations in ionic concentration, temperature and pH.

Organic Solvent Fractionation of β-Glucuronidase.

Fractionation of β-glucuronidase was based on the following theoretical considerations. The addition of organic solvents to the water solvent of a solution of protein causes a decrease in the dielectric constant of the solvent, resulting in a great decrease in the solubility of the protein in the absence of salt. If salt is now added to such a solution, the solvent effect of the salt is very marked, providing a method of fractionation more sensitive to the specific chemical characteristics of the protein and less dependent on such comparatively unspecific

factors as size and shape of the molecule as in the case of salting out from highly concentrated solutions. Furthermore the presence of the additional independent variable, namely organic solvent concentration, greatly increases the range of possible conditions which can be chosen for the separation of any given component.

While protein separation based on the above considerations is more advantageous than salting out procedures, certain precautions are of major importance. Although variation in temperature can frequently be used to separate certain components with a large heat of solution, the upper limit of temperature employed in such a system must be under $0^{\circ}C$. in order to avoid denaturation of the protein. Furthermore the mixing of organic solvents and water is attended with the evolution of heat, and it is important that not only the solvent addition should be carried out in cold surroundings, but it is also necessary to ensure additional cooling during the mixing of solvent and water. Even a brief rise of temperature to a few degrees above $0^{\circ}C$ for a few minutes may have deleterious effects on the stability of the end product obtained by fractionation, and to this end a temperature of $0^{\circ}C$ to $-5^{\circ}C$ should be maintained throughout. While the independent variable of temperature is thus necessarily subject to certain limitations, the above precautions are essential to ensure that any variation in the activity curve of fractionated glucuronidase preparations is independent of the effects of denaturation. To further ensure the maximum stability of the protein under study,

fractionation was carried out by elution from the precipitated material in view of the fact that proteins are more stable in the solid state than in solution.

Preliminary experiments to determine the choice of organic solvent for precipitation showed that while dioxane, ether, alcohol and acetone were suitably effective in this respect, the use of dioxane or ether per se resulted in a large loss in enzyme activity with temperature as low as -5°C., which could only be attributed to denaturation of the enzyme. Acetone was accordingly chosen as precipitant although the magnitude of alteration in dielectric constant is theoretically much less with this solvent than with dioxane or ether. Alcohol, like acetone, reduced denaturation to a minimum, but was rejected in favour of the latter in view of the fact that it failed to reproduce the marked variations in activity curve obtained with acetone fractionation.

The first effect studied was that of variation of ionic concentration, the temperature pH and acetone concentration being held constant. A thoroughly dialysed Stage C enzyme solution (25 ml.) was adjusted to an ionic concentration of 0.05 with sodium acetate, and precipitated with 80% acetone also of ionic strength 0.05, at a final concentration of 25%. A temperature of -5°C. was maintained throughout by means of an $(NH_4)_2SO_4$/ice mixture, the solution subjected to constant stirring and the acetone added slowly (1 ml. per minute) by means of a capillary tube. Following centrifugation at 0°C., the precipitate was eluted successively with 10 ml. volumes of a 25% acetone

solution of ionic strengths 0.0075, 0.01, 0.015, 0.02 and 0.25 respectively, a pH of 6.0 being maintained throughout the above procedure. The eluates were dialysed at 0°C. against distilled water for 24 hours and assayed for enzymic activity. The solvent action of salts at low ionic strengths was well exemplified in the above instance and reference to Figure 20 indicates the results obtained. The tendency to separation of glucuronidase activity with changing ionic strength was entirely reproducible, while the type of activity curve obtained at the lower ionic concentrations (0.0075-0.01) can more readily be interpreted in terms of a complex system.

Not only salts but other dipolar ions can increase the solubility of proteins, and the effect of elution at constant ionic strength (0.0025 μ) in the presence of glycine was studied. Surprisingly enough, no significant alterations in activity curve were obtained with glycine concentrations ranging from 0.001 M. to 0.05 M. Glycylglycine with its higher dipole moment also proved negative in this respect, and subsequent fractionation studies considered more fully the combined effect of variable solvent and ionic concentrations.

While a considerable number of experiments were conducted along these lines, no useful purpose can be served by listing all the combinations effected. The issue was further complicated by the fact that, in many instances, separation which could be obtained by one technique could not be repeated when a fresh Stage C solution was utilised as starting material. The

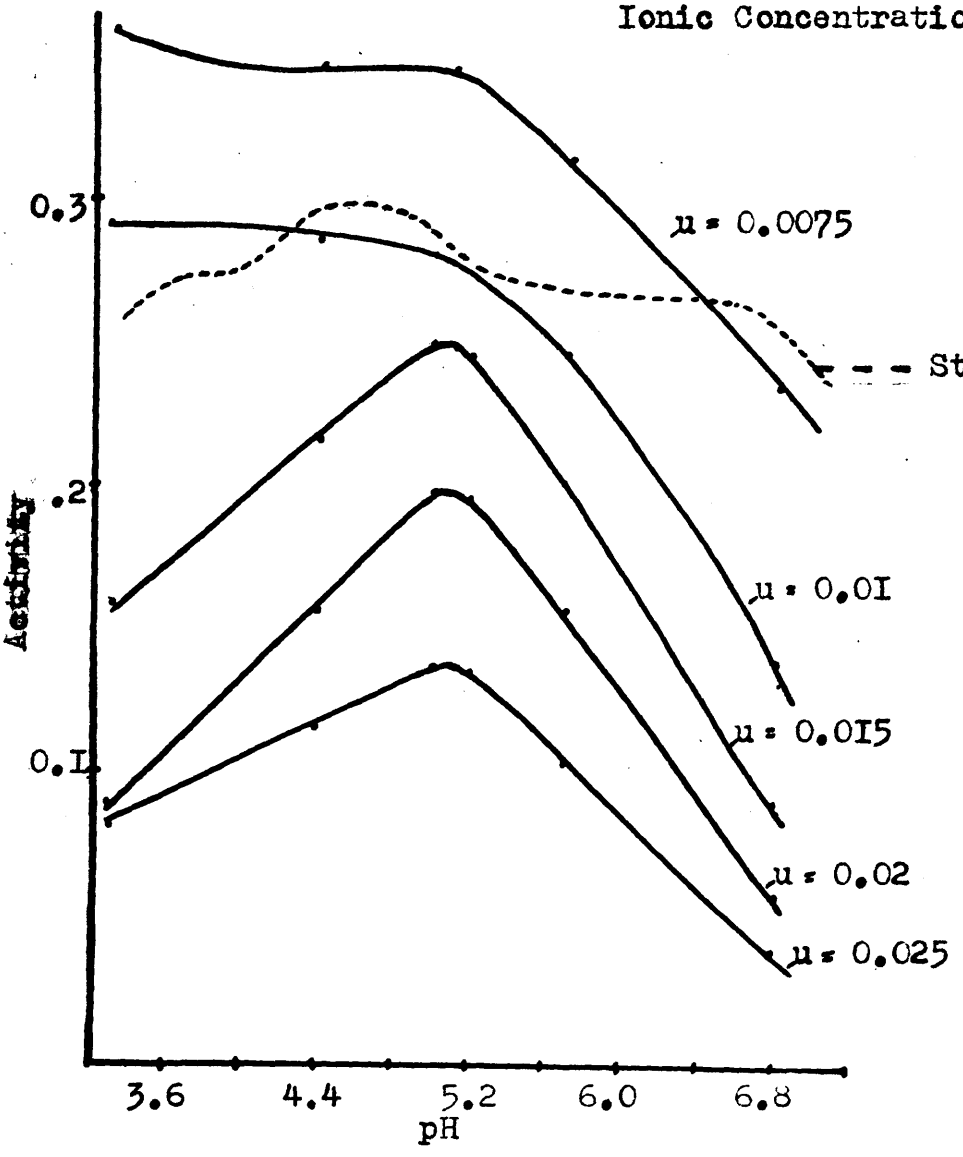
Figure 20

Fractionation of Ox Liver β - Glucuronidase

Acetone Concentration - 25%

pH 6.0

Ionic Concentration Variable



pH 3.4 - 5.8 Acetate Buffers

pH 5.8 - 6.8 Veronal-acetate Buffers

importance of highly standard conditions is thus once more emphasised. One system of fractionation evolved however, appeared to be relatively independent of the variations encountered in the Stage C preparation, and gave consistent evidence of the partial separation of the glucuronidase entities. The only variable employed in this scheme was that of solvent concentration, an ionic concentration of 0.01 and a pH of 5.5 being maintained throughout.

To 50 ml. of a Stage C liver preparation of ionic strength and pH defined was added sufficient 80% acetone (μ = 0.01) to give a final concentration of 35%. The precipitated enzyme was successively eluted with 2 x 10 ml. volumes of 30%, 25%, 20%, 15% amd 10% acetone (μ = 0.01) and the separate eluates dialysed at 0°C for 24 hours. Ionic concentrations are expressed in terms of added sodium acetate, and a temperature of -5°C. was maintained throughout fractionation in the manner previously described. While the method is fundamentally simple in relation to the single variable employed, reproducibility of results could only be obtained at the ionic strength and pH specified, any variation in either factor markedly reducing the degree of separation of enzymic activity expressed in Figure 21.

Further composite fractionation studies based on the principles outlined in the purification of ox liver β-glucuronidase (metallo-protein interactions combined with organic solvent precipitation) were probably the most effective in demonstrating the degree of separation which could be attained under a variety

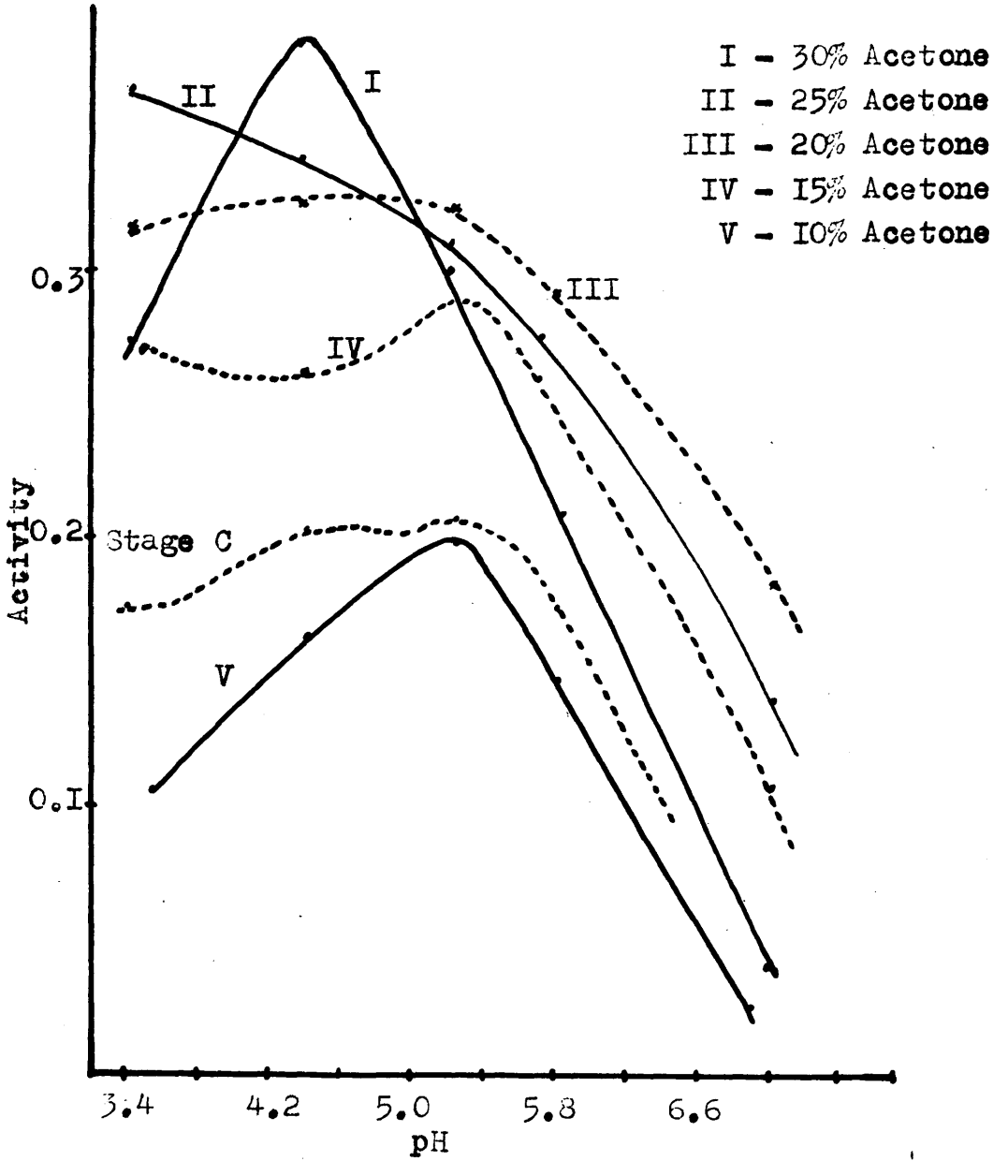
Figure 2I

Acetone Fractionation of Ox Liver β - Glucuronidase

μ = 0.0I (Na Acetate)

pH 5.5

Acetone Concentration Variable



pH 3.4 - 5.8 Acetate Buffers

pH 5.8 - 6.8 Veronal-acetate Buffers

of circumstances. No attempt will be made to outline the considerations involved in the choice of reacting conditions. The latter were, more often than not, arbitrarily chosen from a wide range of possibilities, and although expressed in isolated terms, each experiment serves to illustrate the extent of fractionation whixh can be achieved under diverse conditions.

Method I.

Method I is outlined in full in Figure 22, and the corresponding results obtained by assay of the various fractions are graphically represented in Figure 23. A marked separation into two fractions having pH optima at 4.5 and 5.2 (acetate buffer) is evident. A factor which may have contributed in some measure to the above separation was the relative deficiency of enzyme activity at pH 6.5 in the original Stage C solution.

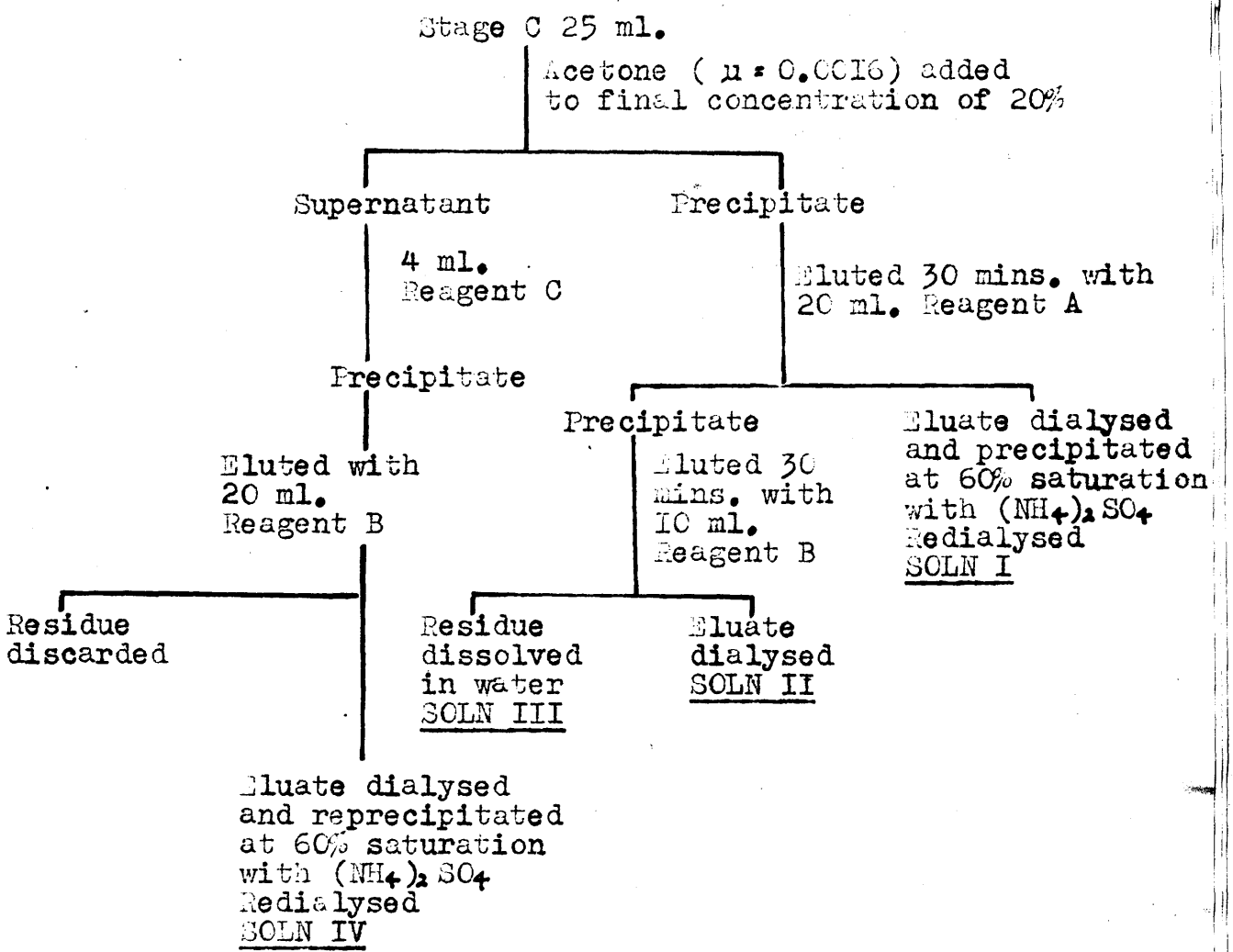
Method II.

A barium Eluate obtained in the manner described for the purification of ox liver β-glucuronidase was fractionated with $(NH_4)_2SO_4$ using the Celite adsorption technique and the individual fractions so acquired (1.6 M., 1.4 M. and 1.2 M.) separately dialysed against running tap water for 24 hours. The resultant precipitates were centrifuged off at 0°C. and eluted overnight with 2 ml. volumes of 0.2 N. acetate buffer pH 4.0. The interesting variations in activity curve of the individual eluates are shown in Figure 24. Each eluate was sufficiently active per se to allow of considerable dilution for assay (1/200)

Figure 22

Fractionation of Ox Liver β - Glucuronidase

Method I



Reagent A : 20 ml. acetone
0.26 g. barium acetate
2.0 ml. 0.8 M. sodium acetate
0.73 ml. M. acetic acid
to 100 ml. with water
pH 5.5

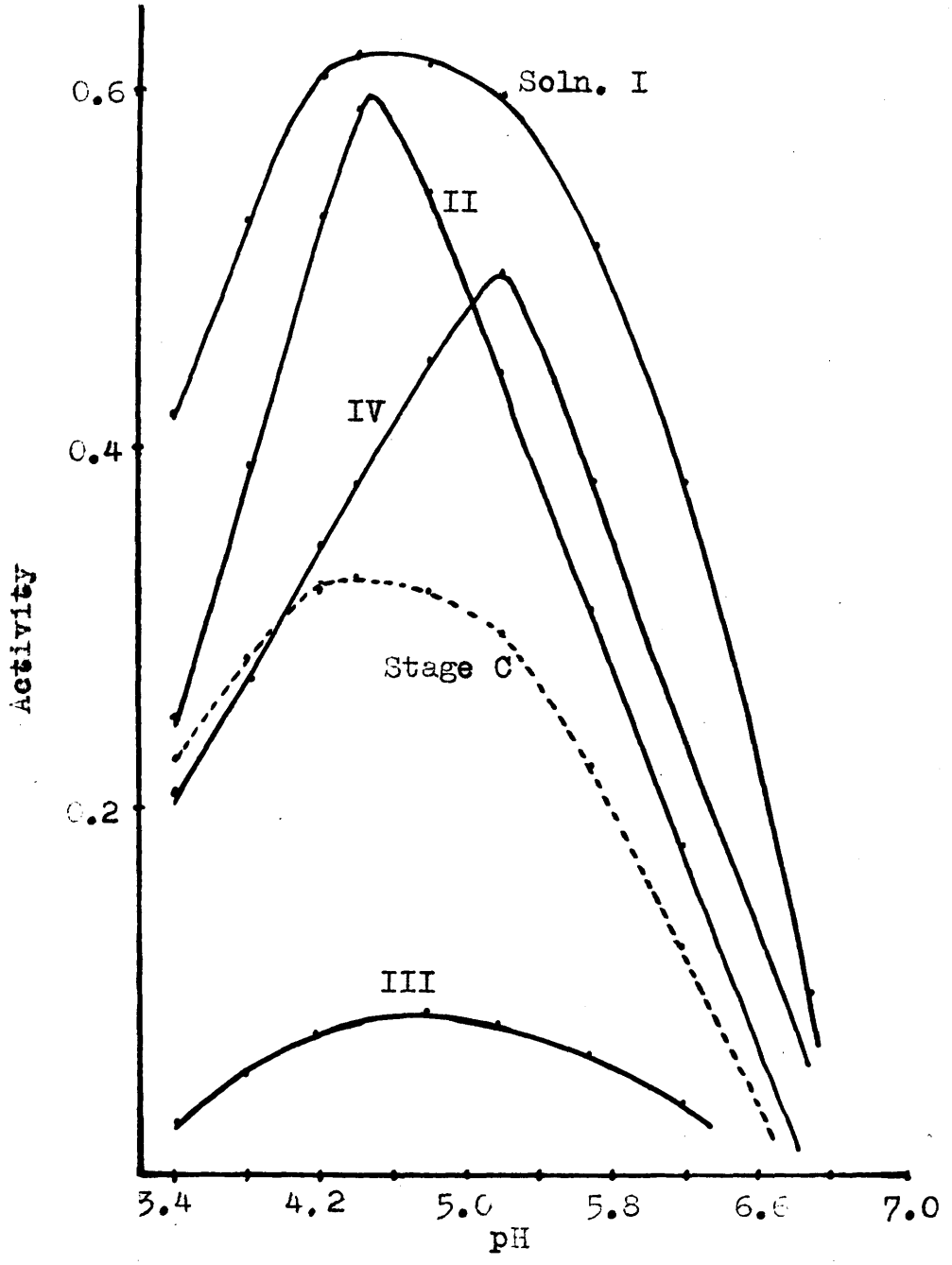
Reagent B : 16 ml. acetone
0.26 g. barium acetate
2.0 ml. 0.8 M. sodium acetate
0.73 ml. M. acetic acid
to 100 ml. with water
pH 5.5

Reagent C : 20 ml. acetone
5.48 g. zinc acetate dihydrate
to 100 ml. with water

Figure 23

Fractionation of Ox Liver β - Glucuronidase

Method I



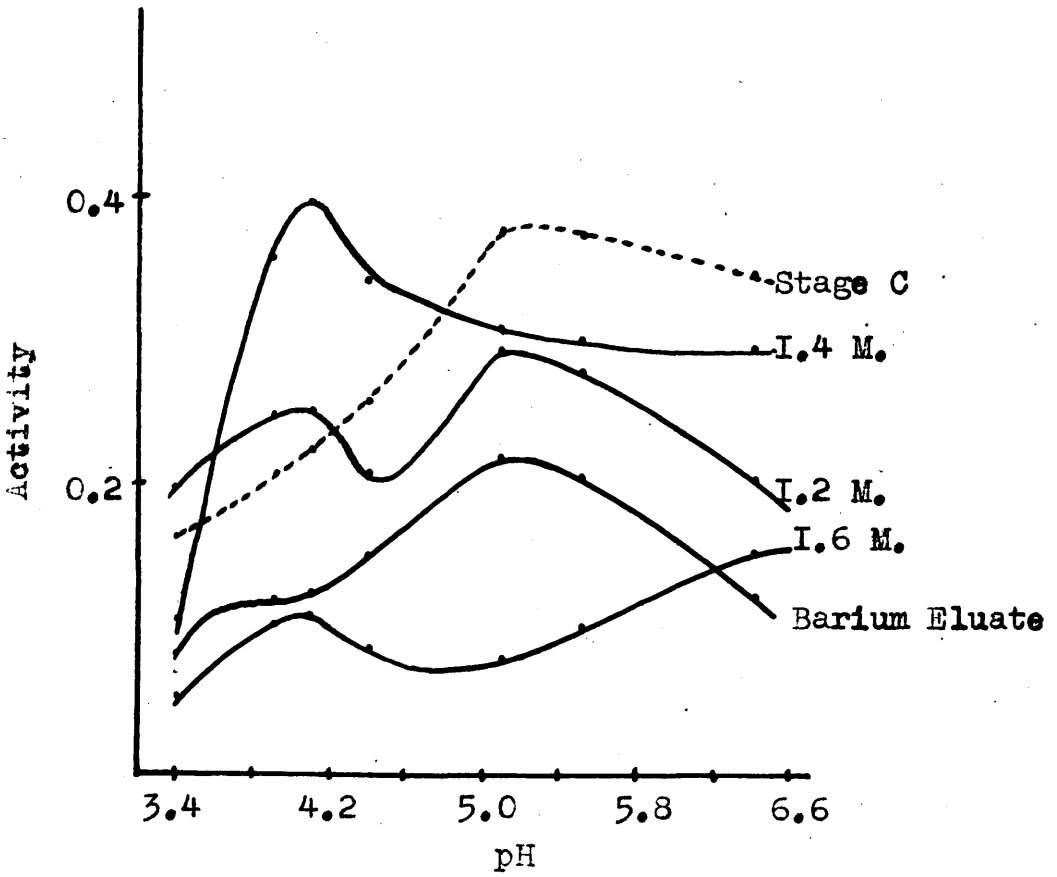
pH 3.4 - 5.8 Acetate Buffers

pH 5.8 - 7.0 Veronal-acetate Buffers

Figure 24

Fractionation of Ox Liver β - Glucuronidase

Method II



pH 3.4 - 5.8 Acetate Buffers

pH 5.8 - 6.6 Veronal-acetate Buffers

thus minimising possible alterations in the shape of the curve due to the presence of high concentrations of salt.

The possibility was considered that a controlled dialysis of one specific $(NH_4)_2SO_4$ fraction would provide equally interesting results. Again using the barium solution as starting material, glucuronidase was precipitated from a 1.4 M. Celite eluate of the latter at a concentration of 3 M. $(NH_4)_2SO_4$, centrifuged off at $0^oC.$, dissolved in the minimum volume of water and fractionally dialysed against several changes of distilled water at $0^oC.$ The precipitates successively obtained with a 12 hour, 48 hour and 96 hour dialysis were eluted in the manner described above and the proportionality of enzyme activity in each eluate determined at varying pH's. The residual supernatant also exhibited a substantial degree of activity and is included in Figure 25 for comparative purposes. Marked alterations in activity curve were again apparent. While further investigation of the use of fractional dialysis in enzyme separation might well prove informative, the tendency of glucuronidase to denature with prolonged dialysis in this instance appreciably limited the scope of investigations, and further experiments along these lines were rendered ineffectual by the subsequent rapid loss of enzyme activity.

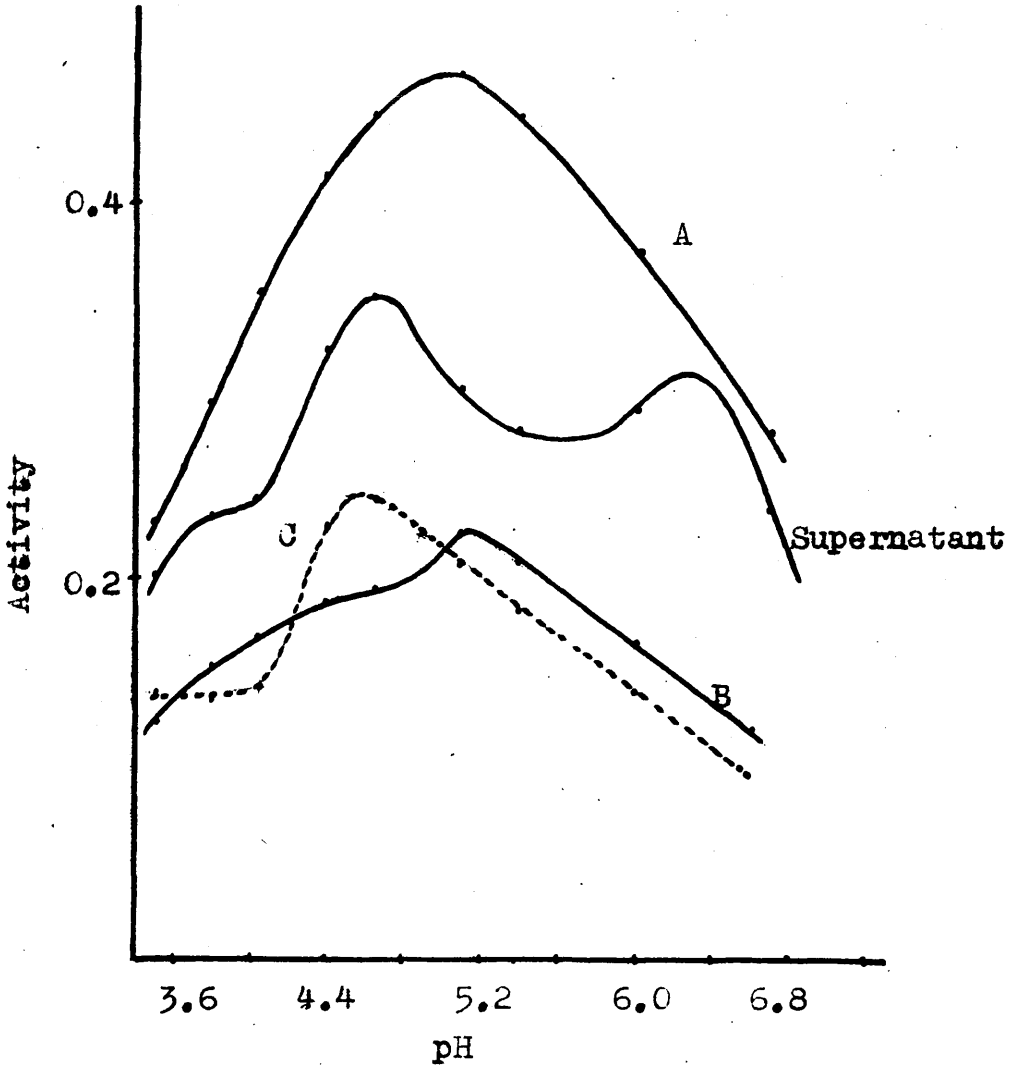
Application of Electrophoresis to Glucuronidase Fractionation.

Electrophoretic studies on β-glucuronidase using either the Tiselius method or that of paper electrophoresis leave one

Figure 25

Fractionation of Ox Liver β - Glucuronidase

Controlled Dialysis of I.4 M Fraction



pH 3.4 - 5.8 Acetate Buffers

pH 5.8 - 6.8 Veronal-acetate Buffers

A 12 hour dialysis
B 48 hour dialysis
C 96 hour dialysis

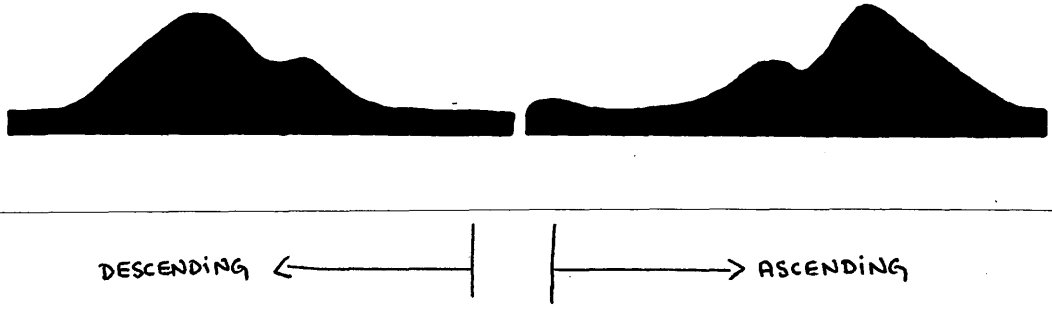
in some doubt as to whether the enzyme migrates as a single or complex protein. Fractionation studies by means of the above techniques, while supplying no solution to the problem, nevertheless provided some interesting information on the lability of the enzyme under applied current.

Figure 26 shows the electrophoretic pattern of a 1.6 M spleen preparation obtained by the Celite elution technique from the Stage C. solution of Mills (1948). In terms of purity (20,000 units activity per mg. protein N) the preparation was not comparable to the ultimate value obtained for the liver enzyme, but the overall concentration of protein present established a protein - buffer boundary suitable for electrophoretic analysis.

Electrophoretic fractionation indicated that enzyme activity was associated almost entirely with the fast fraction. Further examination of the activity curve of this sectioned material indicated a pH optimum of 5.2 (acetate buffer) for the preparation which was distinctly at variance with that of the enzyme previous to electrophoresis (Figure 27.). The alteration could not be attributed solely to prolonged contact with phosphate buffer pH 8.0, as the control curve presented was that of the enzyme dialysed against the above buffer for a period of 4 days to establish ionic equilibrium for electrophoretic analysis. While this alteration in activity curve was an invariable feature of free electrophoresis of either spleen or liver preparations, the effect was even more pronounced when enzyme migration was

Figure 26.

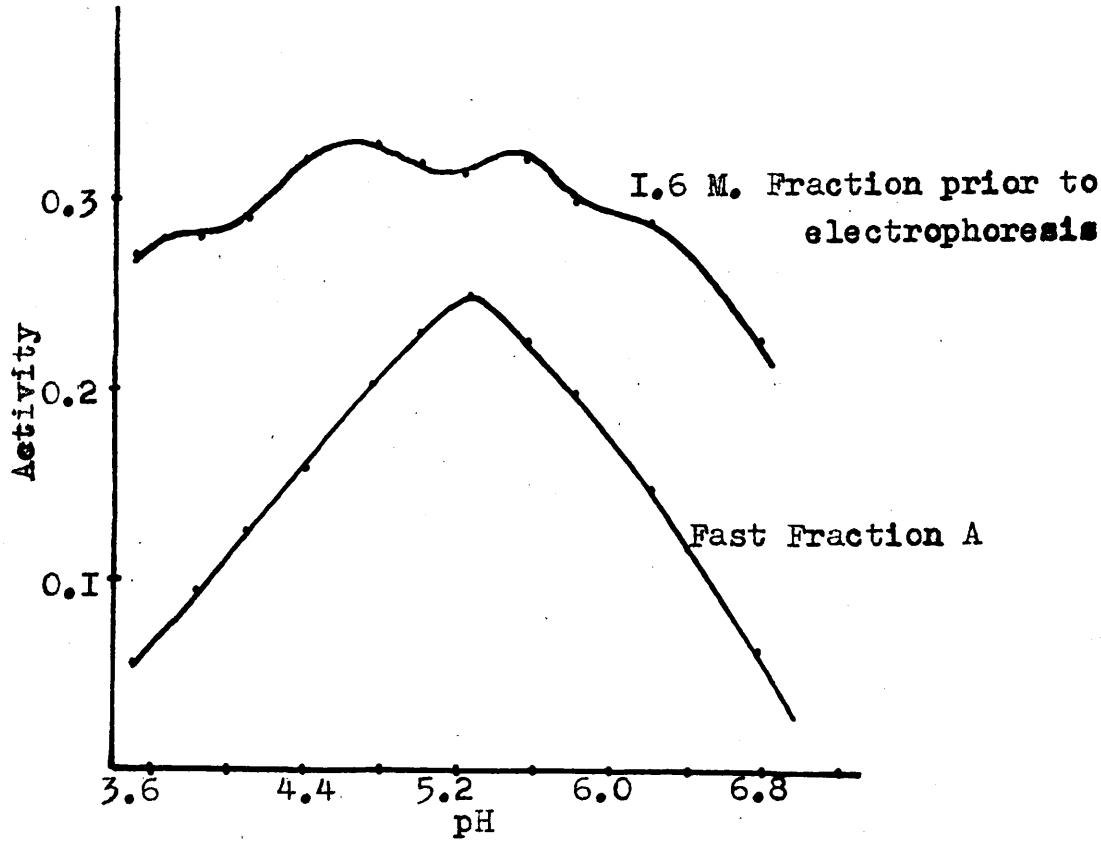
Electrophoretic Analysis (Tiselius Method) of Ox Spleen /β- Glucuronidase (I.6 M.Ammonium Sulphate Fraction ; 20,000 units per mg. Protein N)



Phosphate buffer pH 8.0 ; μ = 0.2

Figure 27

Electrophoretic Fractionation of Ox Spleen β - Glucuronidase (Tiselius U Tube)



pH 3.4 - 5.8 Acetate Buffers

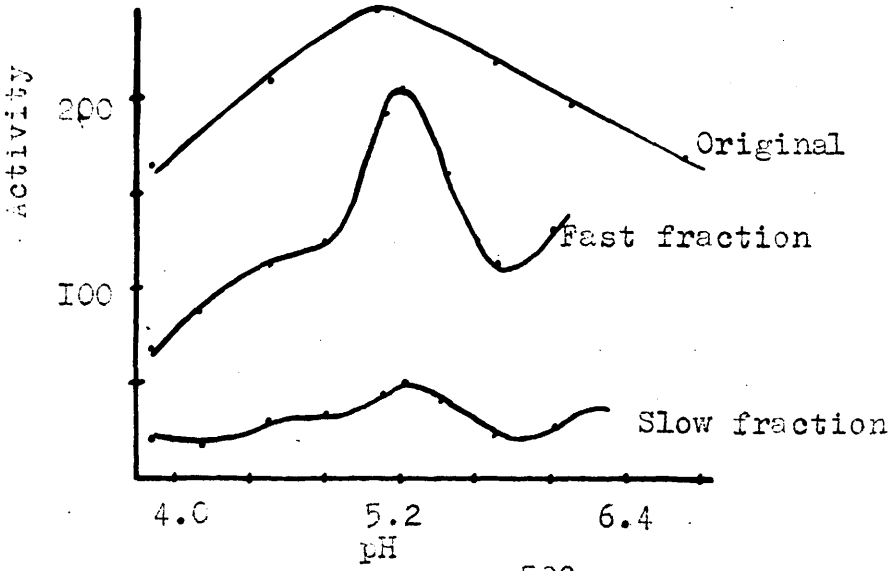
pH 5.8 - 6.8 Veronal - acetate Buffers

effected on filter paper strips, the latter experiments providing some interesting ground for speculation on the nature of the enzyme system.

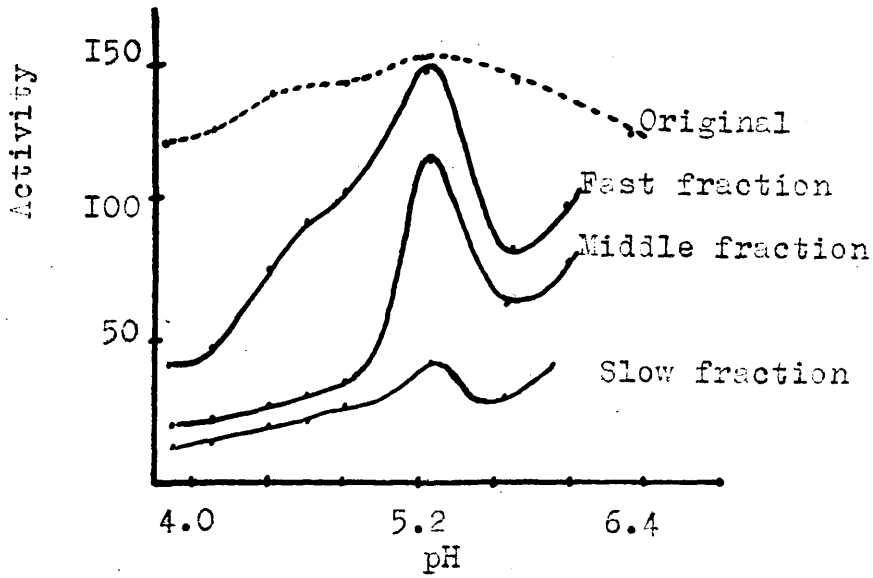
Figure 28 (a,b and c) illustrates the specificity of this effect obtained from electrophoresis on paper of three different enzyme preparations. Glucuronidase activity was localised chromogenically on a control strip, and the extent of migration thus determined, the enzyme was eluted from another strip run simultaneously by soaking the requisite section of the latter in 2 - 3 ml. water for 20 - 24 hours. In most instances the area of activity localised was not compact and suggestive of the non-homogeneity of the enzyme protein. Each section was accordingly divided into two parts prior to elution, the so-called FAST fraction (Figure 28) representing the area primarily adjacent to the anode, and the SLOW fraction that area immediately behind the former. Such individual eluates showed no difference in pH optima on assay although the relative proportions of enzyme activity at varying pH's were not comparable. Any tendency towards partial separation may well have been obscured by the transformation of activity from several well defined optima to the predominating optimum at pH 5.2. Reference to Figure 28 (c) also indicates a very sharp optimum of activity at pH 6.3. Citrate buffers were employed for assay of electrophoretic eluates over the pH range 5.8 - 7.0; it has since been shown by the present author that such buffers induce marked activation of glucuronidase activity above pH 5.4 accounting

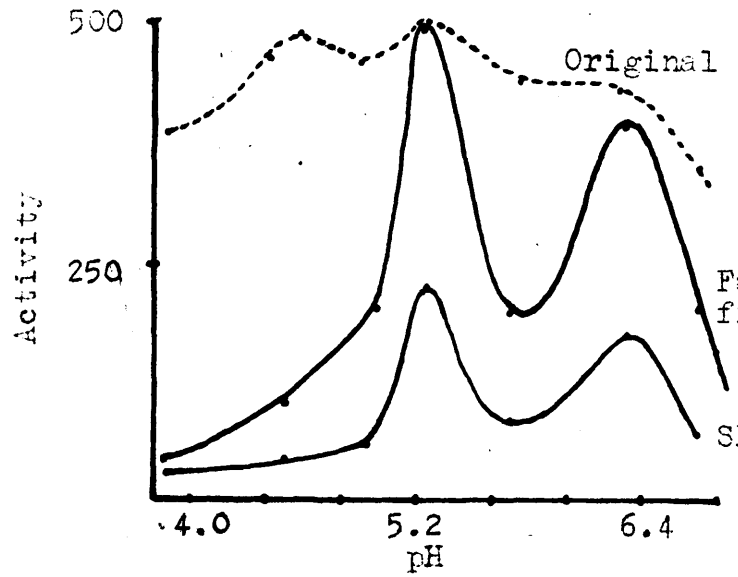
Figure 28. Paper Electrophoresis.

(a) I.4 M Liver Fraction(PO_4 Buffer pH 8.0; μ = 0.04 : 3 V./cm. ; 20 hours)



(b) I.4 M. Liver Fraction (PO_4 Buffer ; pH 8.8 ; μ = 0.03 ; 5 V/cm.; 20 hrs.





(c) I.4 M Spleen Fraction PO_4 buffer ; pH 8.0 ; μ = 0.04 ; 5 v./cm. I7 hours.

for the duality of optima in this instance. Where veronal-acetate buffers were employed (pH 5.8 - 7.0) no such activation was apparent, all assays featuring the single optimum at pH 5.2.

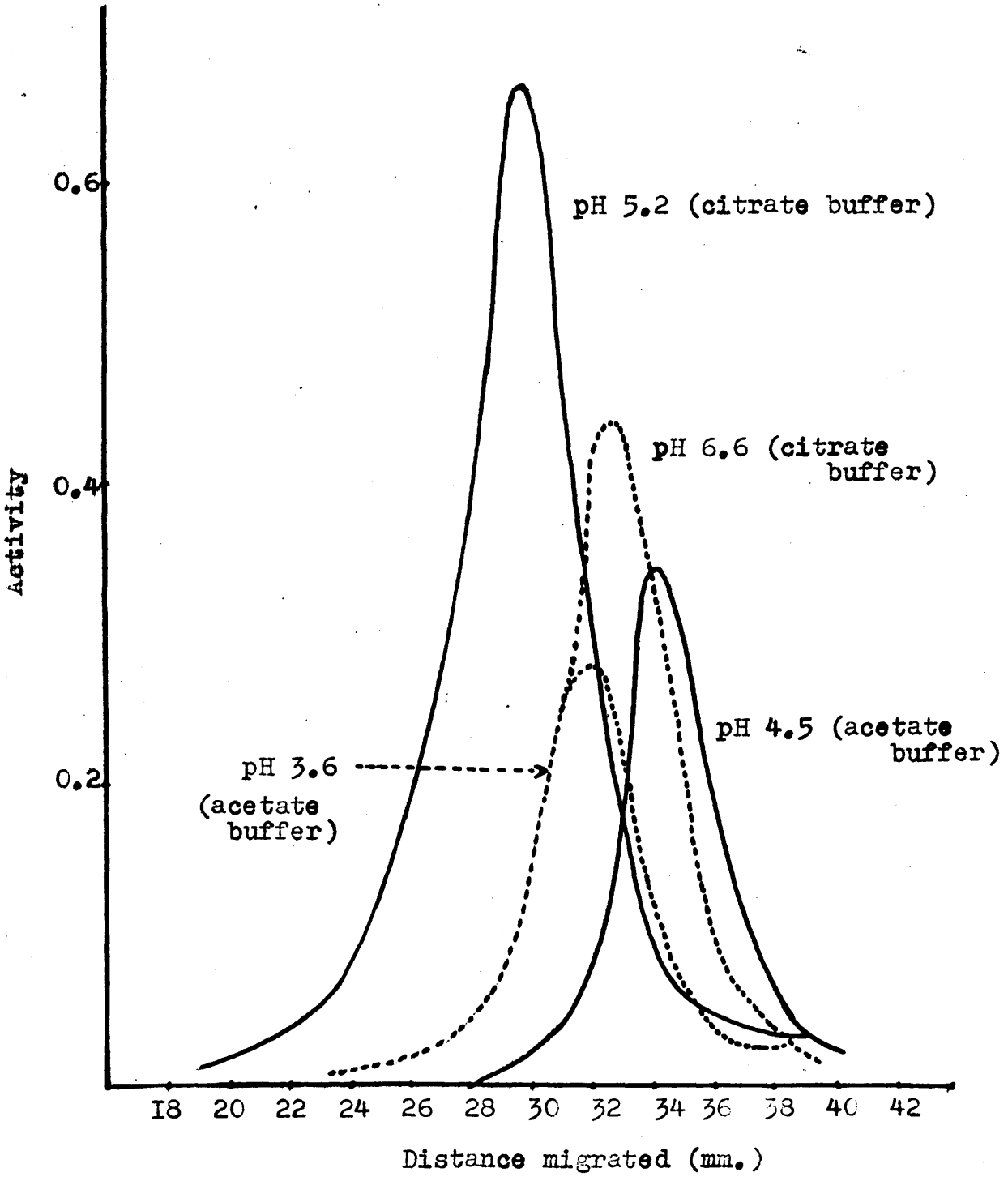
An attempt was made to analyse an electrophoretic run more critically in terms of the following experiment:

A 1.4 M. spleen preparation obtained by the Celite elution technique from the Stage C preparation of Mills (1948) was thoroughly dialysed, freeze-dried, and taken up in the minimum volume of water. 40 μ.l. were applied to paper and migration effected at a potential of 2.6 v./cm. for 37 hours. At the end of the run the paper was sectioned into 5 mm. strips and each strip assayed for glucuronidase activity.

Four such runs were done in parallel and each run assayed at a different pH value (pH 3.6 and 4.5 acetate buffer; pH 5.2 and 6.6 citrate buffer.), the resultant plots of enzyme activity against distance migrated giving a "Schlieren" analysis of the distribution of glucuronidase as shown in Figure 29. While homogeneity of the enzyme was apparently indicated in this instance, the problems of adsorption on paper and protein-protein interactions necessitate a certain amount of caution in the interpretation of such results.

Figure 29

Migration of Ox Spleen β - Glucuronidase on Filter Paper



Phosphate Buffer : pH 8.0 ; u 0.04. 37 hrs. 2.6 v/cm.

4 Separate runs each assayed at the pH indicated

D. Kinetic Studies on β-Glucuronidase.

Kinetic studies on β-glucuronidase were not confined solely to the liver enzyme but also included an investigation of the reaction characteristics of spleen glucuronidase. The validity of the original kinetic studies by Masamune (1934), Oshima (1936), Fishman (1940), Talalay et al (1946), Kerr et al (1948) and Karuniaratnam and Levvy (1949) are open to criticism if the multiple nature of the enzyme is accepted. Such studies were made on preparations which probably contained mixtures of the enzymes in unknown proportions. Moreover the studies of Kerr et al (1948) and Karunairatnam and Levvy (1949) were conducted in citrate buffers; the latter in the molarities employed for assay (final concentration 0.1 M) is an effective inhibitor of β-glucuronidase within a certain pH range, a fact which was early recognised by Oshima (1936).

The kinetic data on both ox liver and ox spleen glucuronidases here presented were ascertained on preparations obtained in the course of fractionation showing an overwhelming degree of activity at either pH 3.4, 4.5 or 5.2 (acetate buffers). Little information can be presented on the characteristics of the proposed enzyme of optimum pH 6.5 as extreme difficulty was encountered in dissociating the latter from enzymic activity at pH 5.2, to give a sufficiently pure preparation for reaction studies. Most of the findings relative to the spleen enzyme now to be described have been published. (Mills and Paul 1949; Mills, Paul and Smith 1949; Mills, Paul and Smith 1952 a and b).

Phenolphthalein glucuronide was used throughout as substrate for the following kinetic experiments.

Enzyme Concentration.

Activity has been shown to be linear with enzyme concentration for bornylglucuronide (Fishman 1940), phenolphthalein glucuronide (Talalay et al 1946) and phenylglucuronide (Kerr et al 1948.). Using phenolphthalein glucuronide as substrate this has been confirmed for glucuronidase from both ox liver and spleen with the reservation that where very concentrated or impure preparations were used a certain deviation from linearity was apparent.

Effect of Substrate Concentration on β-Glucuronidase Activity.

The effect of variation of substrate concentration on reaction velocity was studied for the three ox spleen β-glucuronidases and for the liver enzymes of pH optima 4.5 and 5.2. Using the terminology employed in the publications of Mills et al (1952 a and b), the individual enzymes will subsequently be referred to as I, II and III, corresponding to the pH optima 4.5, 5.2 and 3.4 (0.2 N acetate buffers).

Substrate inhibition was apparent for all three spleen glucuronidases as shown in Figure 30, the degree of inhibition differing in each specific instance. A consideration of the liver enzymes however, (Figure 31) shows no inhibition of enzyme II with excess of substrate.

An evaluation of enzyme-substrate dissociation constants

Figure 30.

Relation Between Phenolphthalein Glucuronide Concentration and β- Glucuronidase Activity of Ox Spleen Fractions I, II and III.

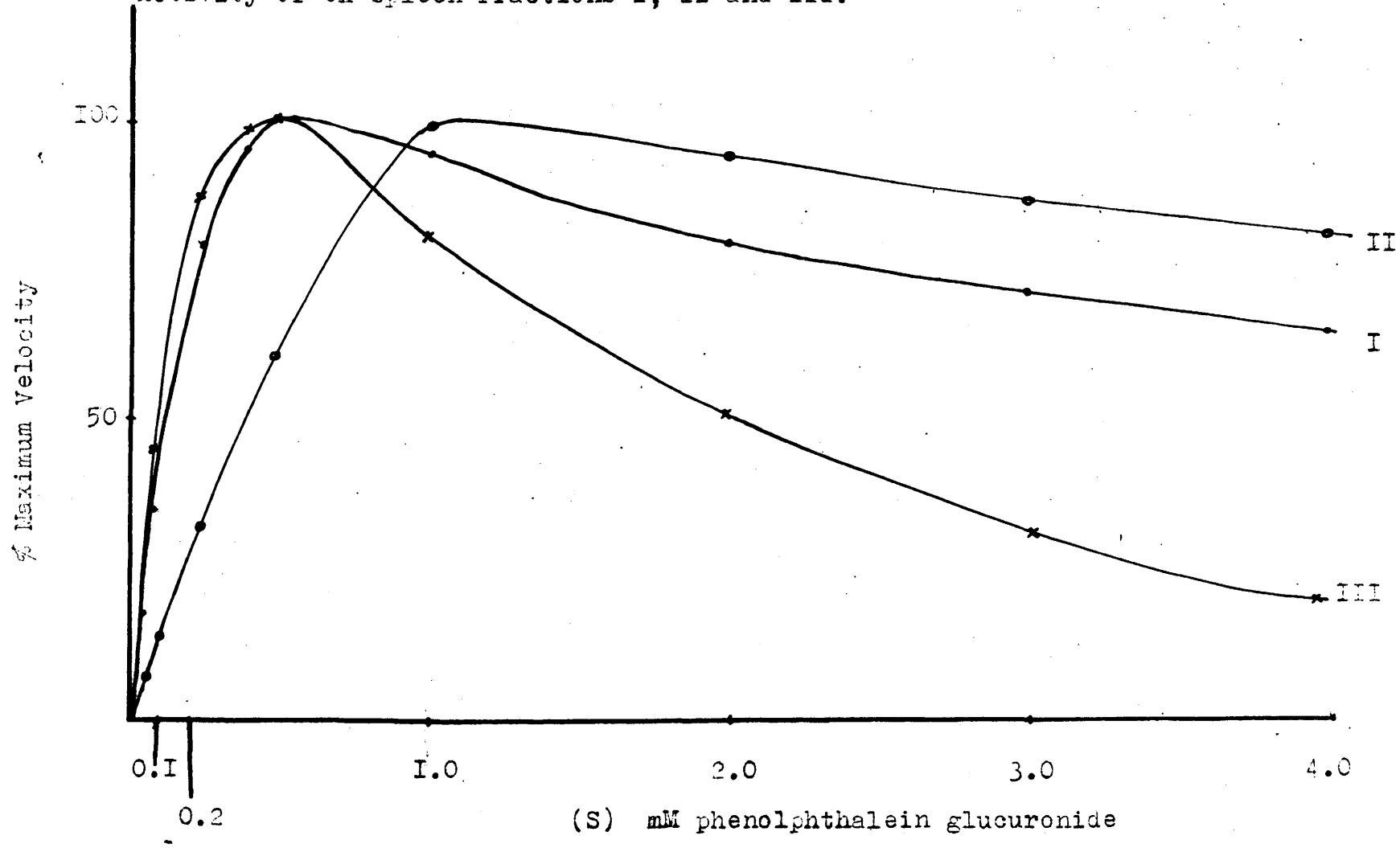
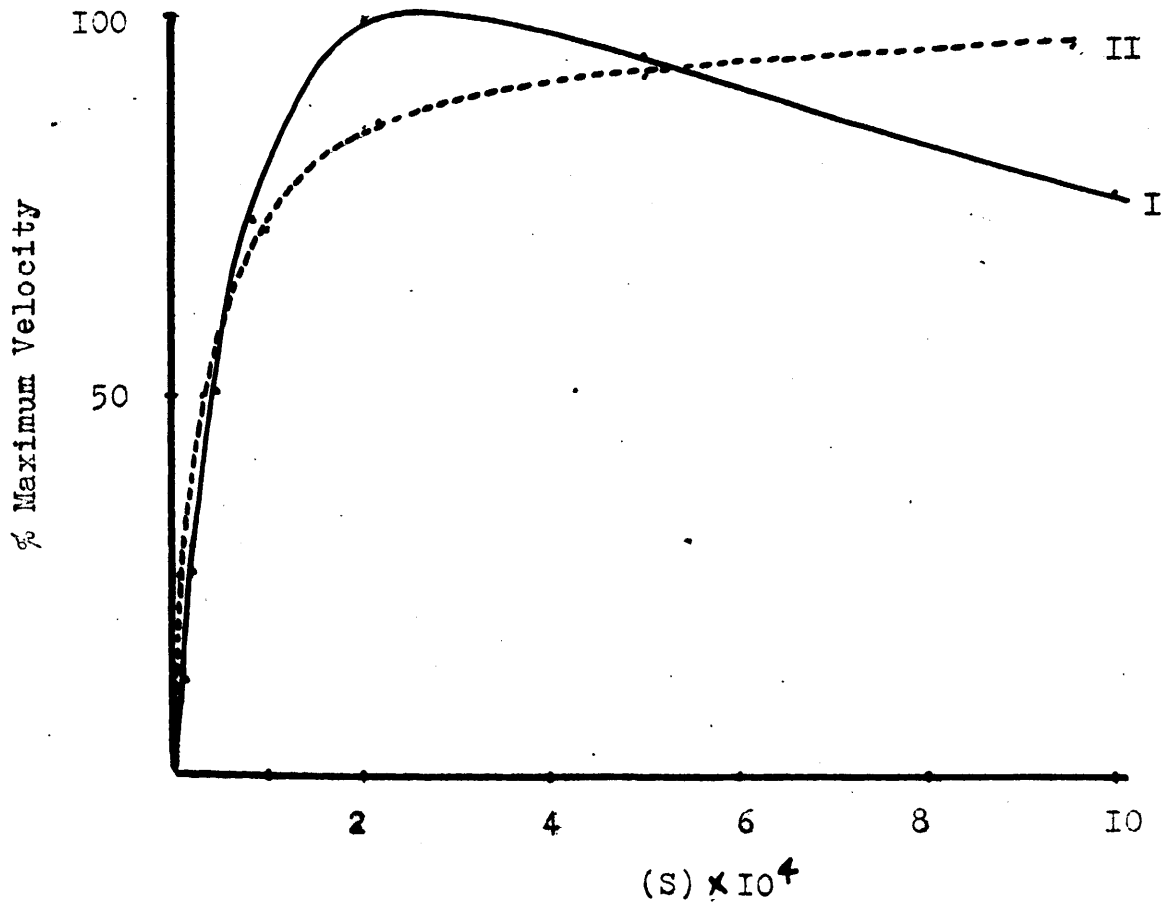


Figure 3I.

Relation Between Phenolphthalein Glucuronide Concentration and β - Glucuronidase Activity of Ox Liver Fractions I and II



by the method of Michaelis and Menten (1913) is not reliable where substrate inhibition is apparent, since the maximum velocity observed is not the true maximum velocity and values of K_m calculated from the substrate concentration for 50% maximum velocity will be too low. The present data has been analysed by the method of Lineweaver and Burk (1934) where the reactions involved in substrate inhibition are:

$$E + S \rightleftharpoons ES \quad \text{and} \quad ES + (n - I)S \rightleftharpoons ESn,$$

where E = enzyme, S = substrate, ES is the active and ESn the inactive enzyme substrate complex. The equation for general substrate inhibition derived by Lineweaver and Burk (1934) is:

$$(S)/V = I/Vmax \left((S) + Ks + (S)^n/K_2 \right) \qquad (I).$$

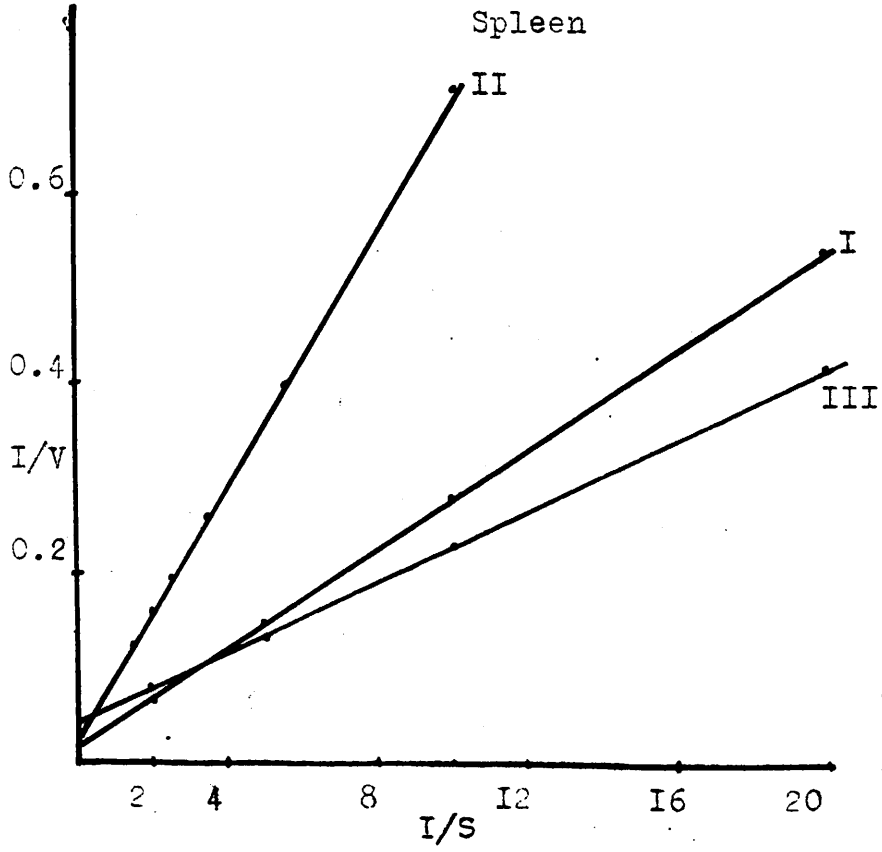
where S = substrate concentration, V = observed velocity, Ks = dissociation constant of the ES complex, K_2 = dissociation constant of ESn complex, Vmax = maximum velocity of the enzyme reaction assuming no inhibition by excess substrate, n = number of molecules of substrate combined with enzyme-substrate complex. At low substrate concentrations the value of $(S)^n/K_2$ will become negligibly small and in this case a plot of I/V against I/S will give a straight line with slope of Ks/Vmax and ordinate intercept of I/Vmax, from which it is possible to calculate the value of Ks, the true enzyme-substrate dissociation constant.

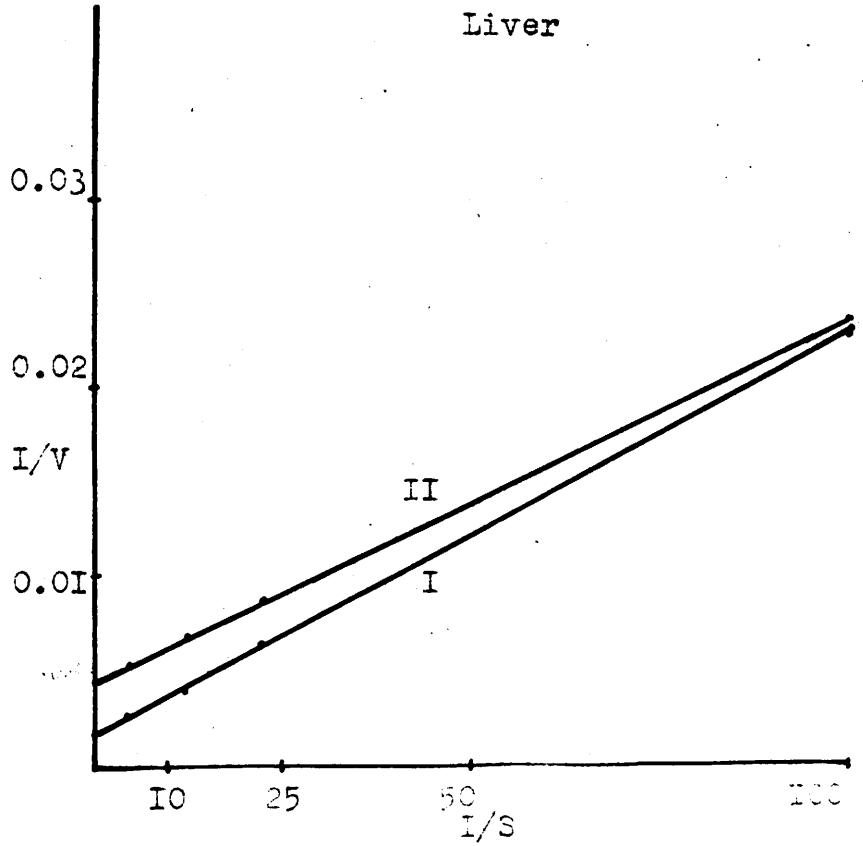
Analysis of the data by this means as shown in Figure 32 gave values of Ks for the fractionated glucuronidases as follows:

Figure 32.

Plot of Reciprocal of Reaction Velocity (I/V) Against Reciprocal of Substrate Conc.(I/S) for Ox Spleen Glucuronidases I, II and III, and Ox Liver Glucuronidases I and II

Substrate Phenolphthalein Glucuronide





Values of Enzyme-Substrate Dissociation Constants (K_s)

	β - Glucuronidase		
Source	I	II	III
Ox Spleen	0.8×10^{-3}	2.3×10^{-3}	1.0×10^{-3}
Ox Liver	1.2×10^{-4}	4.1×10^{-5}	

Values determined at 37°C using phenolphthalein glucuronide as substrate.

The relative substrate affinities of the individual enzymes differed not only inter se, but were also dependent on the source of enzyme material, providing confirmation of the original observation of Kerr et al (1948) that characterisation of the enzyme was dependent on the origin of the former.

Rearrangement of equation (I) into the form:

$\log((S)/V - Ks/Vmax - (S)/Vmax) = n \log (S) - \log K_2 \; Vmax$ (2).

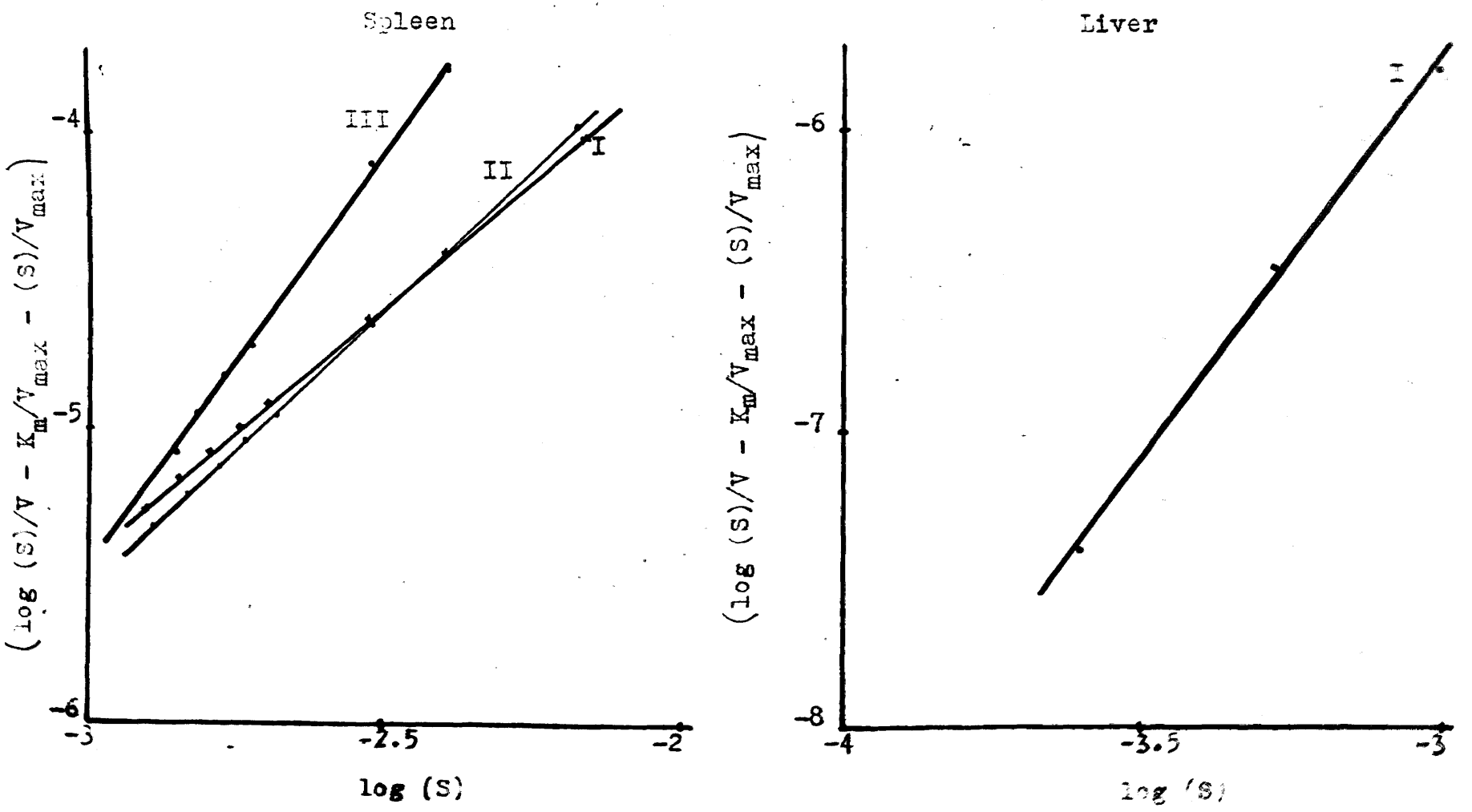
indicates that a plot of the left hand term against log (S) yields a straight line of slope = n and intercept at (S) = I of - log K_2 Vmax, which gives a value for K_2.

The values derived from Figure 32 were thus used to plot $\log((S)/V - Ks/Vmax - (S)/Vmax)$ against log (S), the graphs of which are given in Figure 33. From this Figure, the value of n, which is the slope drawn to the nearest whole number (Lineweaver and Burk 1934) can be determined.

The calculated values for n (number of molecules of substrate combined with enzyme-substrate complex) and K_2 (dissociation constant of inactive enzyme substrate complex)

Figure 33.

Plot of Log (S) Against $\left(\text{Log } (S)/V - K_m/V_{max} - (S)/V_{max}\right)$ for Spleen β - Glucuronidases I, II and III, and Liver Glucuronidase I . Substrate = Phenolphthalein Glucuronide.



and K_2 (dissociation constant of inactive enzyme substrate complex) are expressed in the following table:

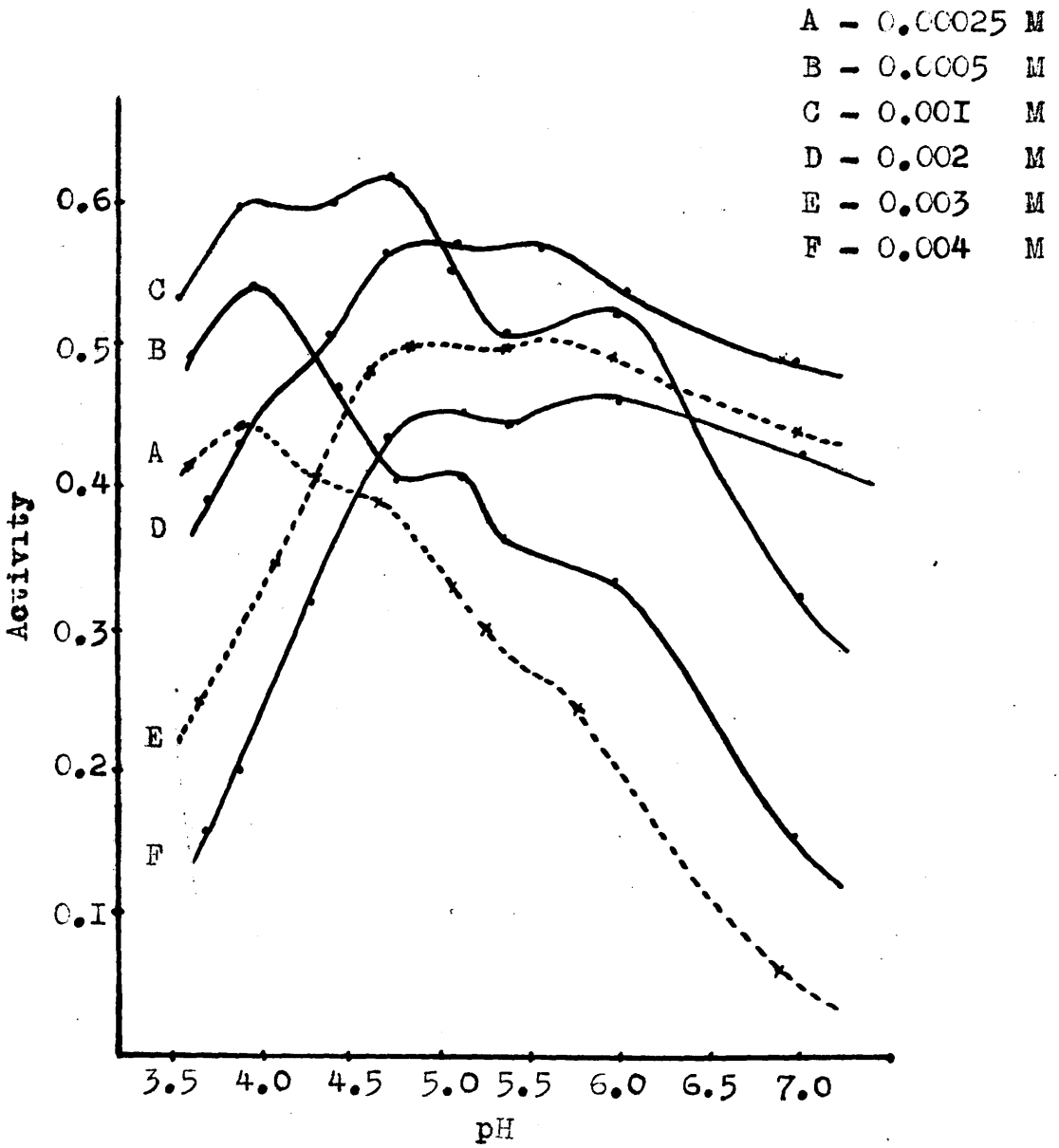
Enzyme	Opt. pH	n	K_2
Ox spleen β-glucuronidase I	4.5	2	2.9×10^{-3}
Ox spleen β-glucuronidase II	5.2	2	7.4×10^{-3}
Ox spleen β-glucuronidase III	3.4	3	3.1×10^{-6}
Ox liver β-glucuronidase I	4.5	3	4.1×10^{-3}

Due to the differemt values of these constants, it is possible in unfractionated glucuronidase preparations to obtain entirely different pH activity curves simply by varying the substrate concentration used in assay. pH activity curves were determined on a Stage C preparation (Mills 1948) from ox spleen, using varying concentrations of phenolphthalein glucuronide as substrate over the range 0.00025 M to 0.004 M.

The alterations in activity curve obtained in this manner are shown in Figure 34. Striking variations were apparent dependent on the choice of substrate concentration. Thus with low concentrations of the latter, enzyme III was particularly prominent, while at higher values the pH of optimum activity was nearer 6.0. The optimum pH of enzyme activity is thus obviously dependent on the choice of substrate concentration, a feature which is particularly apparent in the case of phenolphthalein glucuronide where inhibition by excess substrate is pronounced. The variations so obtained from unfractionated material compare favourably with the calculated kinetic constants

Figure 34.

pH Activity Curves of Ox Spleen β - Glucuronidase (Stage C) at Various Concentrations of Phenolphthalein Glucuronide



pH 3.4 - 5.8 Acetate Buffers

pH 5.8 - 7.0 PO_4/Citrate Buffers

for the isolated enzymes per se, providing some measure of evidence for the complex nature of the enzyme system.

Analysis of a highly purified liver preparation (170,000 units activity per mg. protein N) by similar means gave analagous results (Figure 35). It is difficult, under such circumstances, to envisage the pure enzyme in terms of a single system of optimum pH 4.5 as quoted by Sarkar and Sumner (1950). The activity curve of the purified liver material obtained by the latter authors showed no well defined optima but was characterised by a flat topped plateau with maximum activity between pH 4.2 and 5.6 (phenolphthalein glucuronide as substrate, final concentration 0,00042 M). A similar curve could be obtained by the present author by allowing a preparation as depicted in Figure 2, to stand for several weeks at 0°C. The gradual transition from individual peaks of activity to a plateau shaped curve has been attributed to instability of the purified material synonymous with enzyme denaturation. Analysis of the latter type of curve by assay at varying substrate concentrations however, again illustrated the overall effect apparent in Figures 34 and 35.

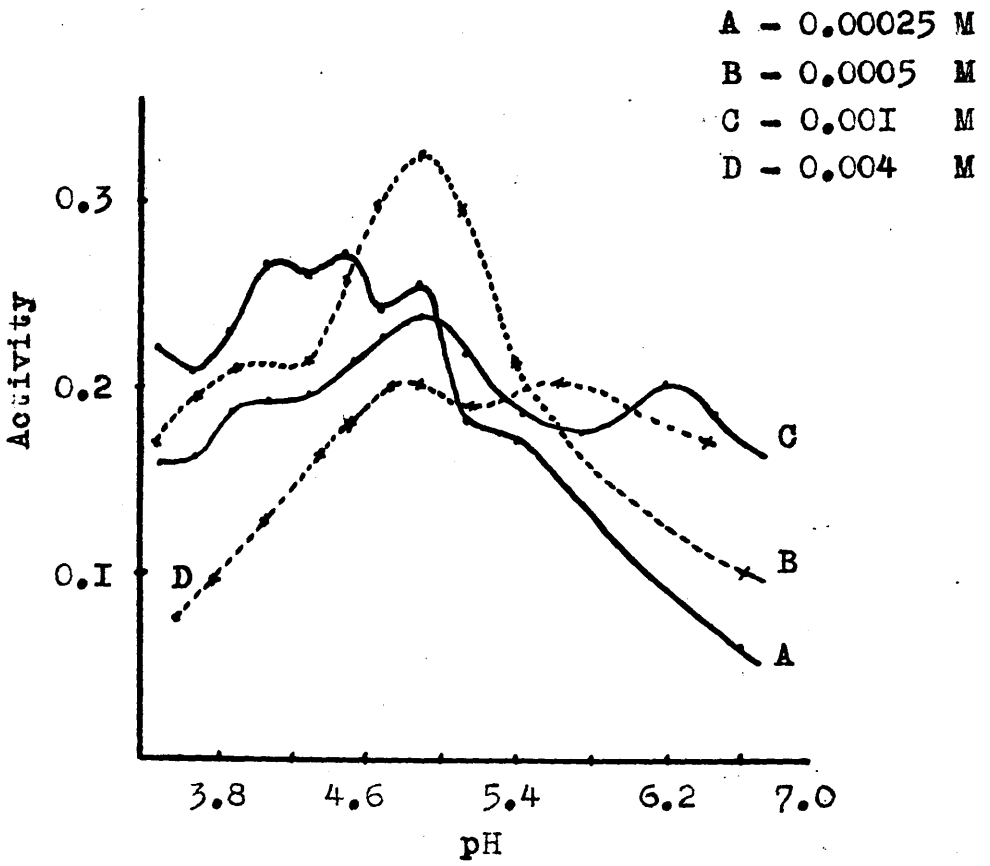
Inhibition by Carboxylic Acids.

Reference to Table 3 illustrates the inhibitory action of a number of aliphatic organic acids on the three ox spleen β-glucuronidases. The powerful inhibitory effect of mucic and saccharic acids on enzyme activity confirms the findings of Karunairatnam and Levvy (1949) that these two compounds are the

Figure 35.

pH Activity Curves of Purified Ox Liver β - Glucuronidase at Various Concentrations of Phenolphthalein Glucuronide

(I70,000 units per mg. P.N.)



pH 3.4 - 5.8 Acetate Buffers

pH 5.8 - 7.0 Veronal-acetate Buffers

two most powerful inhibitors of β-glucuronidase so far known.

Of the inhibitory substances listed, saccharate was examined in greater detail to determine more specifically the type of inhibition exerted.

As indicated by Mills (Mills et al 1952 b) a possible difficulty arises in determining the type of inhibition exerted by such compounds, where there is not only inhibition by an inhibitor, but also substrate inhibition to a greater or less degree depending on the substrate employed. Analysis of results by the method of Lineweaver and Burk (1934) is thus necessarily complicated by the latter factor. An equation has been derived (Mills et al 1952 b) featuring a combination of the equations portraying simple substrate inhibition and simple competitive inhibition as defined by Lineweaver and Burk (1934).

Assuming the three equilibria:

$$E + S \rightleftharpoons ES \quad \text{(active)} \qquad \text{(i)}$$

$$ES + (n-I)S \rightleftharpoons ESn \quad \text{(inactive)} \qquad \text{(ii)}$$

$$E + I \rightleftharpoons EI \quad \text{(inactive)} \qquad \text{(iii)}$$

where E = enzyme, S = substrate, I = inhibitor, ES = active enzyme - substrate complex, ESn = inactive enzyme - substrate complex, EI = inactive enzyme - inhibitor complex, and Ks, K_2, and K_I are the requisite dissociation constants for reactions (i), (ii) and (iii) respectively, it has been found (Mills et al 1952 b) that the velocity equation solves to:

$$V = Vmax\ (S) / \left[(S) + Ks + (S)^n/K_2 + Ks(I)/K_I \right]$$

At low substrate concentrations the term $(S)^n/K_2$ in the above

equation becomes negligible and the equation becomes:

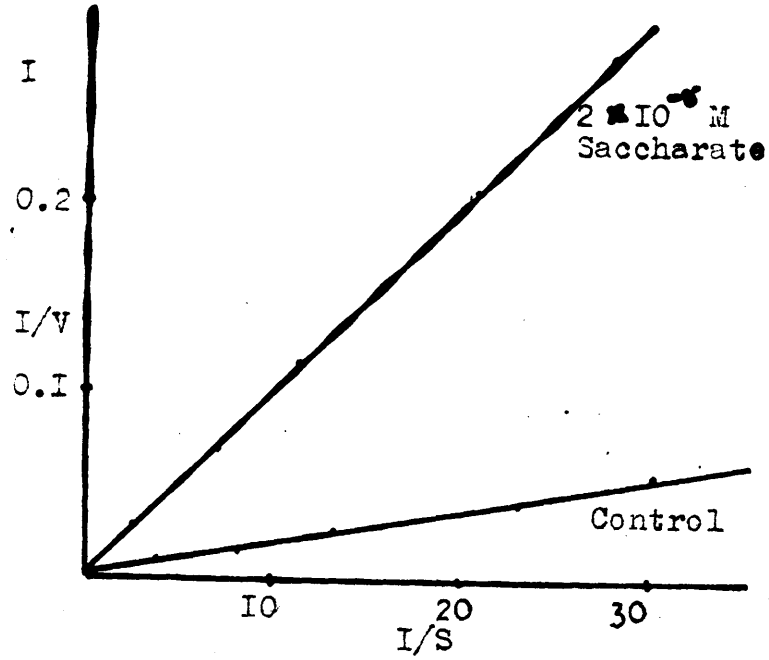
$$V = Vmax\ (S)/\ \left[\ (S) + Ks + Ks(I)/K_I\right]$$

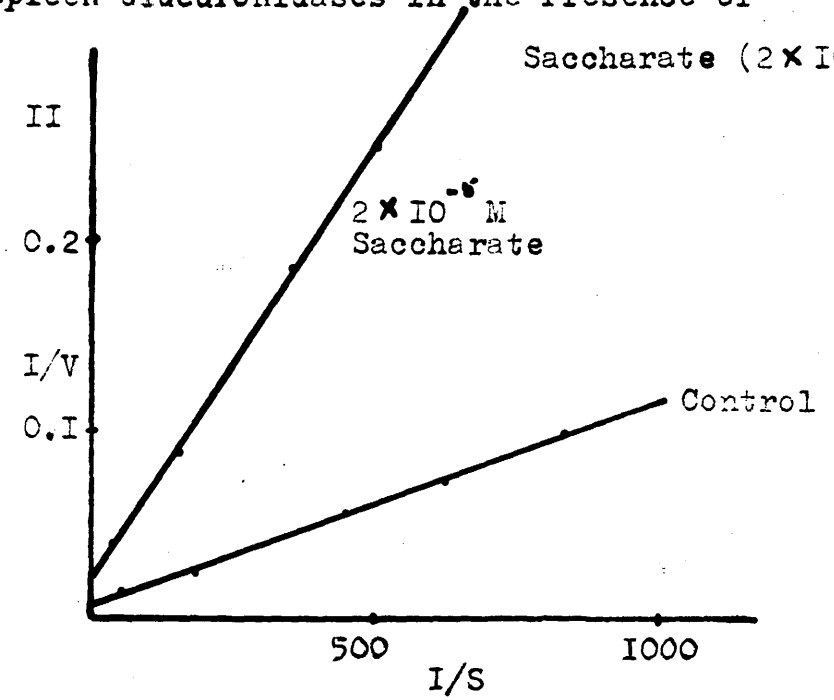
which is the equation for simple competitive inhibition as demonstrated by Lineweaver and Burk (1934). If low substrate concentrations are thus employed, the methods of analysis of Lineweaver and Burk (1934) are applicable to the present study. Hence by plotting the reciprocal of reaction velocity against the reciprocal of substrate concentration in the presence and absence of inhibitor, straight lines will be obtained indicating whether or not the equation for competitive inhibition is obeyed. If Vmax. remains unaltered in presence of the inhibitor and only the slope of the line is increased, the inhibition is competitive. If however Vmax decreases in addition to the slope of the line being altered, the inhibition is non-competitive in type.

Experiments were accordingly carried out with each of the three ox spleen β-glucuronidases and liver enzymes I and II, in which the velocity of reaction was determined at various substrate concentrations in the presence and absence of saccharate. The substrate concentrations chosen were such that a plot of (S)/V against (S) gave a straight line i.e. where the term $(S)^n/K_2$ was negligible.

In Figures 36 and 37 are presented the graphical analysis of such results by the procedure of Lineweaver and Burk (1934), where I/V is plotted against I/(S). The type of inhibition exerted in each instance may be summarised in the following table:

Figure 36. Plot of Reciprocal of Reaction Velocity (I/V) Against Reciprocal of Substrate Concentration (I/S) for the Three Ox Spleen Glucuronidases in the Presence of Saccharate (2×10^{-5} M)





I - Enzyme of Opt. pH 4.5 (Substrate - phenolphthalein glucuronide)

II - Enzyme of Opt. pH 5.2 (Substrate - phenyl - glucuronide)

III - Enzyme of Opt. pH 3.4 (Substrate - phenolphthalein glucuronide)

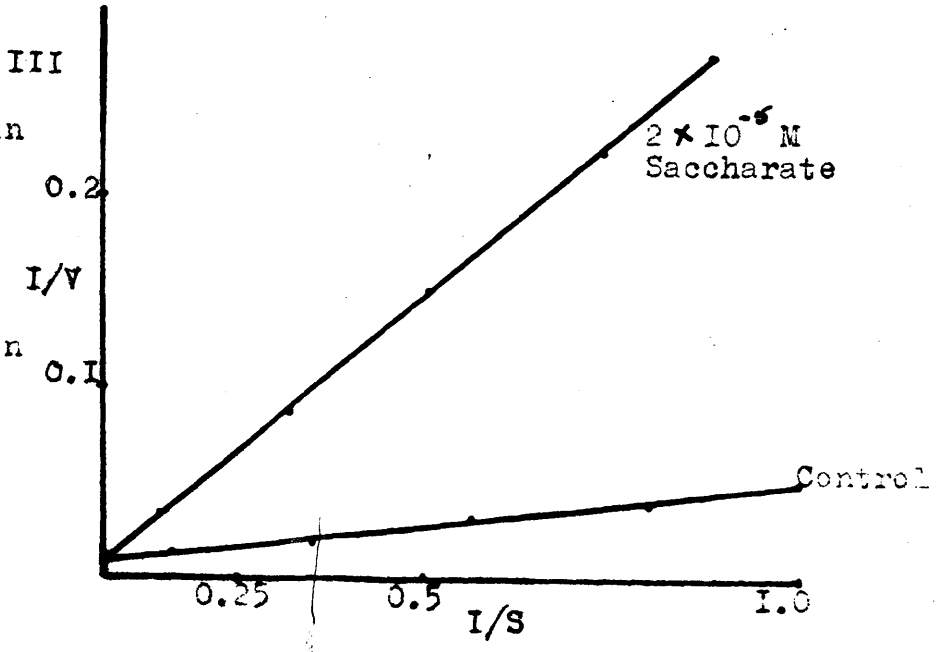
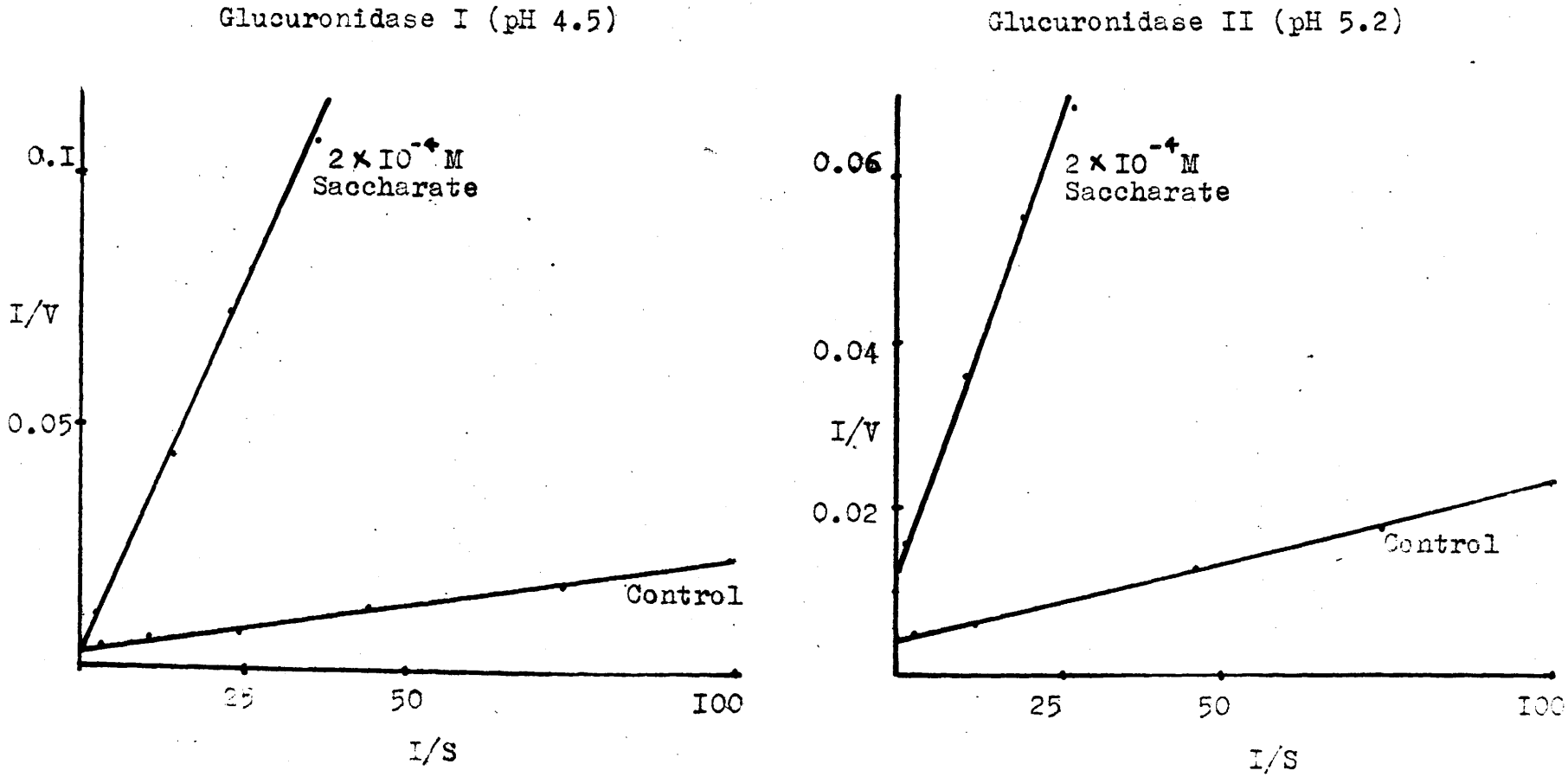


Figure 37



Plot of Reciprocal of Reaction Velocity (I/V) Against Reciprocal of Substrate Concentration (I/S) for Liver Glucuronidases I and II in the Presence of Saccharate ($2 \times IO^{-4}$ M)

(Substrate = Phenolphthalein Glucuronide)

Type of Inhibition Exerted by Saccharate on β-Glucuronidase

	β-Glucuronidase		
Source	pH 3.4	pH 4.5	pH 5.2
Ox spleen	Competitive	Competitive	NON-competitive
Ox liver		Competitive	NON-competitive

The calculated values of the enzyme-inhibitor dissociation constants (K_I) for the cases of competitive inhibition where the increase in slope of the I/V against I/(S) plot in the presence of inhibitor equals $Ks(I)/K_I$ are as follows:

Values of Enzyme-Inhibitor Dissociation Constants (K_I) for Saccharate.

	β-Glucuronidase	
Source	pH 3.4	pH 4.5
Ox spleen	5.7×10^{-5}	4.8×10^{-3}
Ox liver		8.6×10^{-3}

The non-competitive nature of saccharate inhibition at pH 5.2 serves to emphasise the independance of enzymic activity at this pH. That the three spleen enzymes also vary considerably in their sensitivity to saccharate is demonstrated in the following table:

Concentration of D-Saccharate	Spleen β-Glucuronidase % Inhibition		
M.	pH 3.4	pH 4.5	pH 5.2
10^{-3}	100	100	100
2×10^{-4}	100	100	70
10^{-4}	100	60	20
5×10^{-5}	90	20	0
10^{-5}	25	0	0

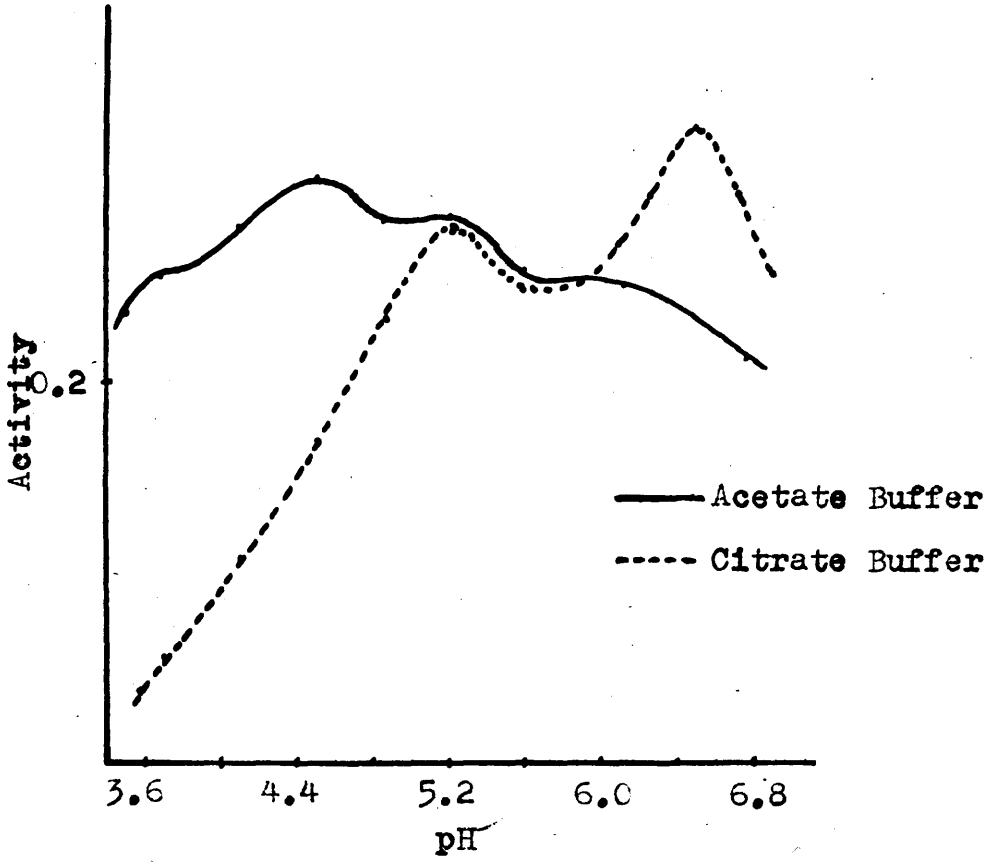
Reference has already been made to the inhibition of β-glucuronidase by citrate. Figure 38 illustrates this effect more specifically by a comparison of the activity curves of a Stage C (Mills 1948) liver preparation in 0.2 M. acetate and 0.2 M. citrate buffers. Marked inhibition of enzyme activity up to pH 5.2 is evident, concurrent with activation in the higher pH range. The pattern of inhibition was strikingly similar in all preparations studied, and in no instance did the presence of citrate affect enzyme activity at pH 5.2. Inhibition of the pH 3.4 and 4.5 enzymes determined on fractionated spleen preparations was shown to be competitive in both instances.

Activation Energies.

The effect of temperature on the rate of enzyme reaction in the region where heat inactivation is negligible has been found in many cases to fit the equation originally proposed by Arrhenius (1889) (c.f. Sizer 1943) namely:

Figure 38.

pH Activity Curve of a Stage C Liver Glucuronidase Preparation in 0.2 N Acetate Buffers and 0.2 N Citrate Buffers



$$\frac{d \ln k}{dt} = \frac{A}{RT^2}$$

Integration of this equation yields:

$$\ln \frac{k_2}{k_1} = \frac{A}{R} \left(\frac{I}{T_1} - \frac{I}{T_2} \right)$$

where k = reaction rate at Absolute Temperature T°
R = gas constant
A = energy of activation

Values of energy of activation (A) may be calculated from the slope of the graph in which log k is plotted against I/T. The slope multiplied by the constant 4.58 gives the value of A.

The reaction velocities were determined for each of the three ox spleen and ox liver enzymes at five temperatures between 0° and 40° C, using a substrate concentration of phenolphthalein glucuronide (0.001 M) sufficient to ensure maximum activity of the enzyme. The results of the plot of log k against I/T are recorded in Figure 39, and the values of A obtained by multiplying the slopes of these graphs by 4.58 are as follows:

Activation Energies of β-Glucuronidases

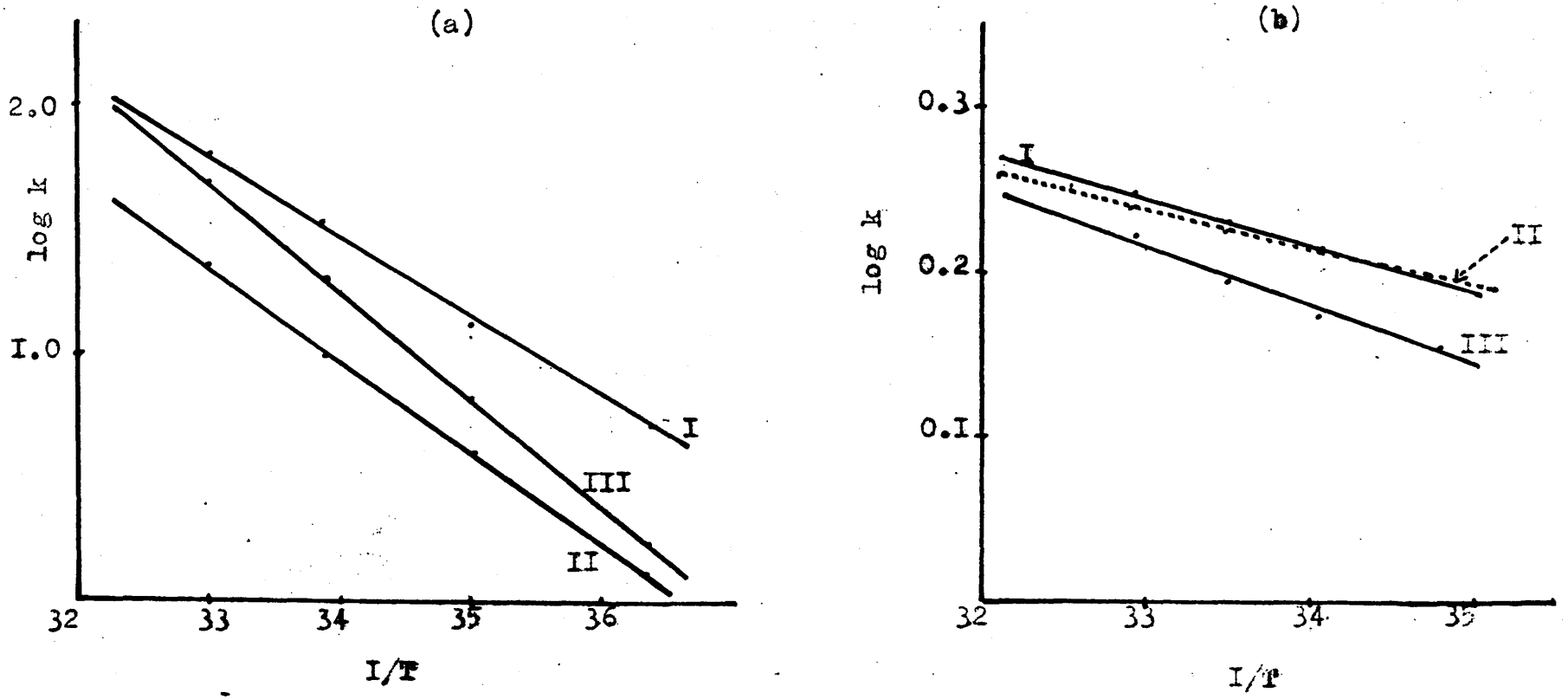
	Calories per mole.		
Source	pH 3.4	pH 4.5	pH 5.2
Ox spleen	18,900	14,400	16,200
Ox liver	16,000	13,700	12,000

The values of A for enzymes I, II and III (pH optima

Figure 39

Plot of Log Reaction Rate Against Reciprocal of Absolute Temperature (I/T) for the Three Ox Spleen (a) and Ox Liver β - Glucuronidases (b).

Substrate = Phenolphthalein Glucuronide



I Enzyme of Optimum pH 4.5

II Enzyme of Optimum pH 5.2

III Enzyme of Optimum pH 3.4

4.5, 5.2 and 3.4 respectively) are significantly different inter se and also appear to be dependent on the tissue of derivation.

That the purity of the enzyme plays no apparent rôle in determining the activation energy (c.f. Sizer 1943) was established by obtaining identical values of A for the enzymes at differing stages of purity. Unfractionated extracts likewise showed identical activation energies at the three pH optima of the enzymes (pH 3.4, 4.5 and 5.2 acetate buffers).

It might be anticipated that reagents which modify the catalytic surface of the enzyme would alter the activation energy as well. The marked activating effect of DNA (final concentration 0.06%) on β-glucuronidase activity at pH 4.5 and 5.2 (acetate buffer) was considered of interest in this respect, and activation energies were accordingly determined on the purified liver glucuronidase preparation of Figure 9 at the above pH values in the presence of DNA. No alteration in activation energies at either pH was apparent however. Assays conducted in the presence of albumin (0.05%), gum acacia (0.1%) and starch (0.2%) likewise showed no variation in the values of A.

Similar activation energies for the hydrolysis of phenyl-glucuronide have been obtained for the ox spleen β-glucuronidases (Mills, Paul and Smith, 1952a) indicating that the values of A are characteristic of the enzyme and independent of the substrate employed.

SECTION IV.

PHYSIOLOGICAL ROLE OF β-GLUCURONIDASE.

The function of β-glucuronidase *in vivo* has occasioned considerable speculation since the original postulate by Oshima (1934) that the enzyme was responsible for glucuronide conjugation. Most of the work in this connection has already been reviewed, and while such a function is of fundamental importance, it does not appear to be tenable in the light of the experimental evidence proffered. Mention has already been made of the work of Fishman and his colleagues (Fishman 1940; Fishman and Fishman 1944; Fishman 1947a), who observed increased glucuronidase activity in a number of organs in response to administered menthol and oestrogens. The inference was made that the enzyme was thus responsible for glucuronide synthesis *in vivo*; while the experimental results are *sans reproche* however they are capable of being interpreted in diverse ways. In this respect, Kerr Levvy and Campbell (1947), Kerr and Levvy (1947), and Levvy, Kerr and Campbell (1948) verified the findings of Fishman (1940) that repeated feeding of menthol to mice induced increased β-glucuronidase activity in both liver and kidney, but preferred the explanation that such increases were secondary to the toxic action of menthol on such organs and bore no relation to its glucuronidogenic properties. In general the increase in enzyme activity was found to be associated with active cell proliferation produced by injury rather than with injury itself, and a close parallelism was drawn between glucuronidase activity

in an organ and the amount of tissue growth in progress in that organ. Further confirmation of the usefulness of β glucuronidase as an index of biochemical growth was provided by the observation that high enzyme values were apparent in the livers of adult mice regenerating after hepatectomy, and in the livers, spleen and kidneys of infant mice. Various factors causing proliferative changes in liver and kidney were subsequently studied by Kerr, Campbell and Levvy (1948) and the increase in enzyme activity so induced again appeared to be associated with mitotic activity.

Increased β -glucuronidase activity has been reported in numerous instances in tissues excised from malignant neoplasms of various organs. (Fishman and Anlyan 1947 a and b; Fishman, Anlyan and Gordon 1947; Odell and Burt 1949; Odell, Burt and Bethea 1949; Odell and Burt 1950; Fishman, Kasdon and Homburger 1950 a, b and c; Kasdon, Fishman and Homburger 1950; Fishman and Bigelow 1950; Fishman 1951; Cohen and Huseby 1951 a and b). While the requisite individual facets of such literature will be discussed elsewhere, Levvy, Kerr and Campbell (1948) consider that the validity of their conception of β -glucuronidase as an index of biochemical growth is considerably enhanced by such reports.

In the present work, the activity of rat liver β -glucuronidase was studied under a variety of experimentally induced circumstances in an endeavour to substantiate the more important claims made concerning the physiological significance of the enzyme in vivo. The first of such studies was the effect of

partial hepatectomy on liver β-glucuronidase activity.

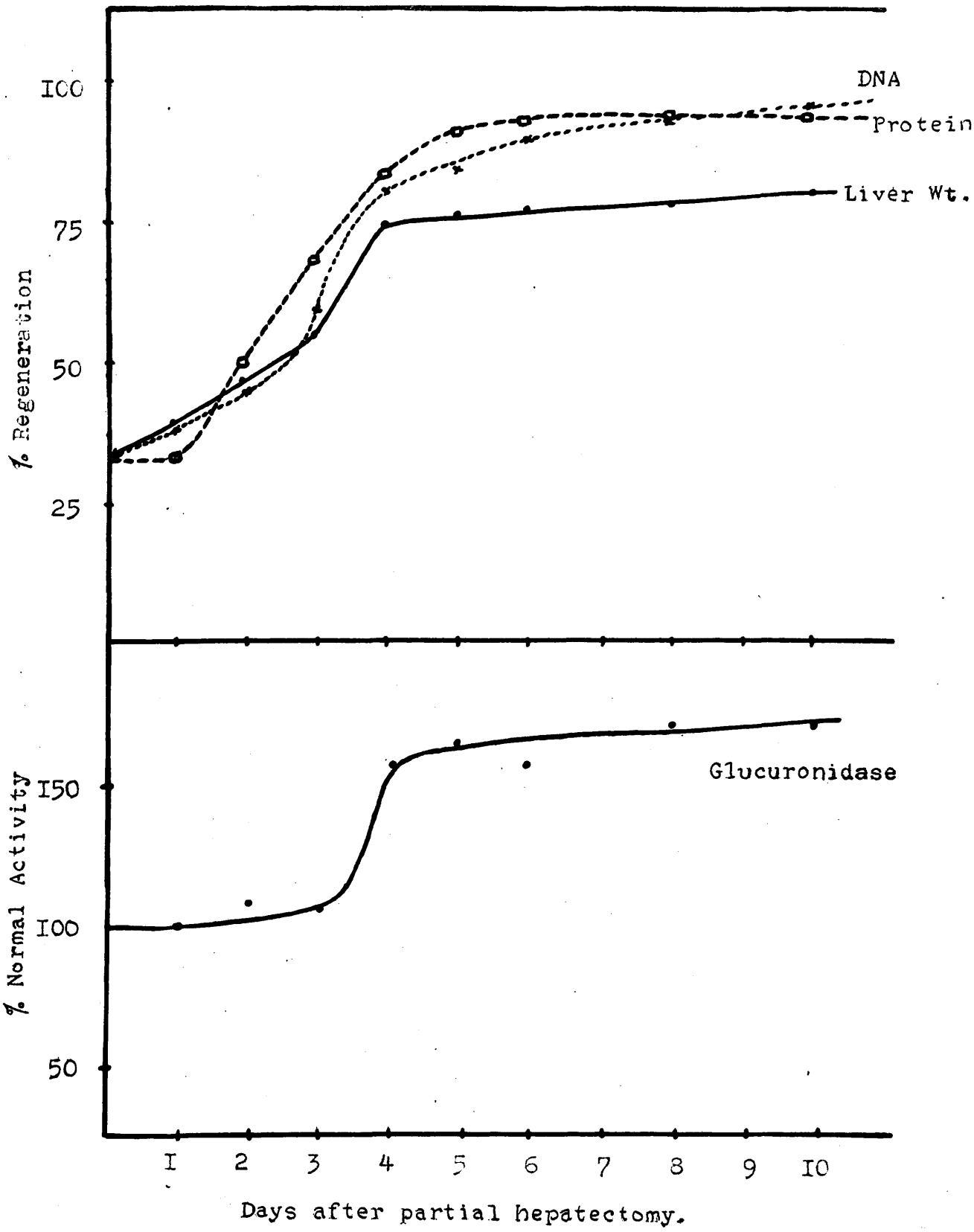
Variations in β-Glucuronidase Activity Subsequent to Partial Hepatectomy.

In order to verify the report of Levvy, Kerr and Campbell (1948) that increased glucuronidase activity following partial hepatectomy was a reflection of the degree of cellular proliferation in the regenerating liver, a similar study was conducted on a series of rats in the following manner:

70 male rats (Hunter strain) were subjected to partial hepatectomy by the procedure of Higgins and Anderson (1931) whereby two-thirds of the liver was removed, and these animals were studied over a period of 10 days from the date of operation. A minimum of 6 animals was examined at any one time and the results averaged. Glucuronidase activity was determined at pH 4.5 (acetate buffer) on whole liver homogenates using phenolphthalein glucuronide as substrate, and simultaneous analyses were made for DNA, RNA and protein N.

The results so obtained are tabulated in Figure 40 where liver glucuronidase activity is shown as a percentage of normal along with the degrees of regeneration of liver weight, DNA and protein. A consideration of the latter criteria indicates that the period of most rapid growth was apparent 2-4 days after hepatectomy, at which time liver weight was 75%, protein N 83% and DNA 81% of normal, while glucuronidase activity had commenced to increase only at this time. After 4 days when enzyme activity was at least 60% - 70% above normal, the rate of growth was

Figure 40.


100
75
50
25
% Regeneration
DNA
Protein
Liver Wt.
150
100
50
% Normal Activity
Glucuronidase
1
2
3
4
5
6
7
8
9
10
Days after partial hepatectomy.

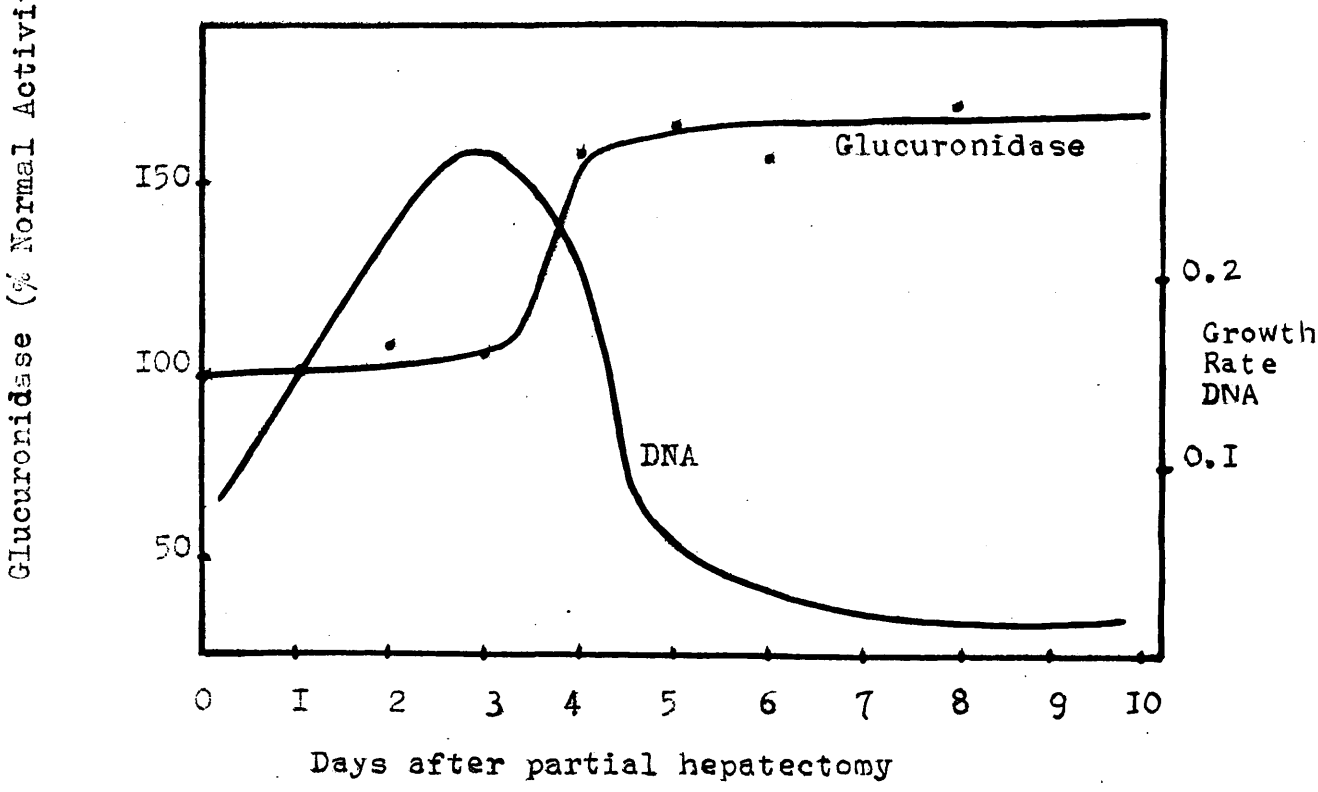
considerably retarded reaching 100% regeneration at 14 days.

Davidson and Leslie (1950 a and b) have demonstrated the value of the constancy of the DNA content of the cell nucleus for a single species as an index of reference when studying growth phenomena. This enables the DNA content of the tissue to be used as a valuable criterion of the number of cells in that tissue, and reference to Figure 41 shows the results obtained when glucuronidase activity is related to cellular proliferation as measured by the DNA growth rate. No correlation can be visualised between the two factors, while Table 4 further expresses glucuronidase activity numerically as a function of DNA (i.e. per cell) and as a function of protein N. In the former instance, the glucuronidase/DNA ratio was constant to 4 days rising only after this period, while the glucuronidase/protein N ratio dropped initially with a subsequent rise at 4 days.

Such findings, while confirming the observation of Levvy et al (1948) that increased glucuronidase activity is apparent in liver tissue regenerating after partial hepatectomy, do not permit the conclusion that this increase is in any way related to cellular proliferation, and another explanation must be sought for the rise in activity occurring only when rapid cell proliferation is over.

In view of the shock syndrome associated with all operative procedures it was considered advisable to control the hepatectomy experiments with a series of animals which had been subjected to a similar operative technique leaving the liver intact. Such laparotomised animals showed the post-operative variations in

Figure 4I.



$$\text{DNA Growth Rate} = \frac{I}{W} \cdot \frac{dW}{dt}$$ where W = DNA concentration

Table 4.

Effect of Partial Hepatectomy on Glucuronidase Activity, DNA and Protein N Content of Rat Liver.

Days After Partial Hepatectomy	Glucuronidase Activity / DNA	Glucuronidase Activity / Protein N	Protein N / DNA
Before operation	66	62	93
1	66	68	96
2	70	63	83
3	63	51	122
4	101	87	115
5	98	85	115
6	90	80	115
8	109	106	102
10	104	101	102

All concentrations expressed per gm. of fresh liver.

liver β-glucuronidase activity summarised in Table 5. A minimum of 5 experimental animals were studied at any one period, and a statistically significant fall in liver glucuronidase was apparent 1-2 days after operation, with a return to normal activity at 4 days. Both protein concentration and the protein/DNA ratio were raised at this period, normal values again being apparent at four days after operation, while no significant alteration in the number of cells per liver of 100 g. rat was obvious throughout the control experiment.

It was necessary in the course of investigations, to use a different strain of rat for the control experiments than had been employed for hepatectomy purposes, and the interesting point was established that a strain difference exists in the liver glucuronidase of this species. The Tuck strain of the laparotomy experiments was some 60% - 70% higher in liver glucuronidase activity (expressed as units per g. tissue) in comparison with the Hunter strain originally employed. Similar differences in mice have already been reported by Morrow, Greenspan and Carroll (1949; 1950), who observed consistently low glucuronidase activity in the liver, kidney and spleen of all C_3H sublines relative to 8 other strains investigated, and the possibility was envisaged (Morrow et al 1950), that such differences could be genetically determined. A further sex difference in the kidney glucuronidase of inbred mice was also reported by Morrow, Carroll and Greenspan (1951); in all strains studied the kidney glucuronidase activity of adult male mice was 1.3 to 2.6 times greater than that of the adult female, although such differences were not apparent to

Table 5.

Glucuronidase Activity of Rat Liver in Relation to Other Constituents After Sham Operation

Days After Operation	Glucuronidase Activity (% Normal)	Glucuronidase Activity / DNA	Glucuronidase Activity / Protein N	Protein N / DNA
Before operation	100	130	112	115
1	80	111	91	118
2	83	117	85	142
3	89	121	93	131
4	100	111	108	100

Ratio concentrations expressed per gm. of fresh liver.

Levvy et al (Levvy, Kerr and Campbell 1948), who specifically reported no sex variation in the kidney glucuronidase of mice drawn from several different colonies.

Further comparative studies in this field obviously demand singularity in choice of species and sex, a fact which was fully recognised in further biological investigations.

The Effect of Subcutaneous Injection of Carbon Tetrachloride on Liver Glucuronidase Activity.

In a series of experiments on mice, Levvy, Kerr and Campbell (1948) studied the variations in liver β-glucuronidase activity associated with the injection of a variety of agents causing proliferative changes in that organ. Liver damage was provoked by such agents as yellow phosphorus, chloroform and carbon tetrachloride, and a close parallelism was observed between mitotic division and glucuronidase activity in the damaged organ. Repair processes were invariably associated with high glucuronidase values, the rise in enzyme activity preceding the first appearance of cell division. Whether β-glucuronidase was actually concerned with cell proliferation, or whether the observed increase in activity was a reflection of increased metabolic activity was not apparent, but the inference was made that glucuronidase levels per se were a valuable index of biochemical growth in an organ.

To test the validity of this hypothesis, a similar series of experiments were carried out on adult male rats (Hunter strain) using CCl_4 to provoke liver damage. 0.5 g. CCl_4 in arachis oil

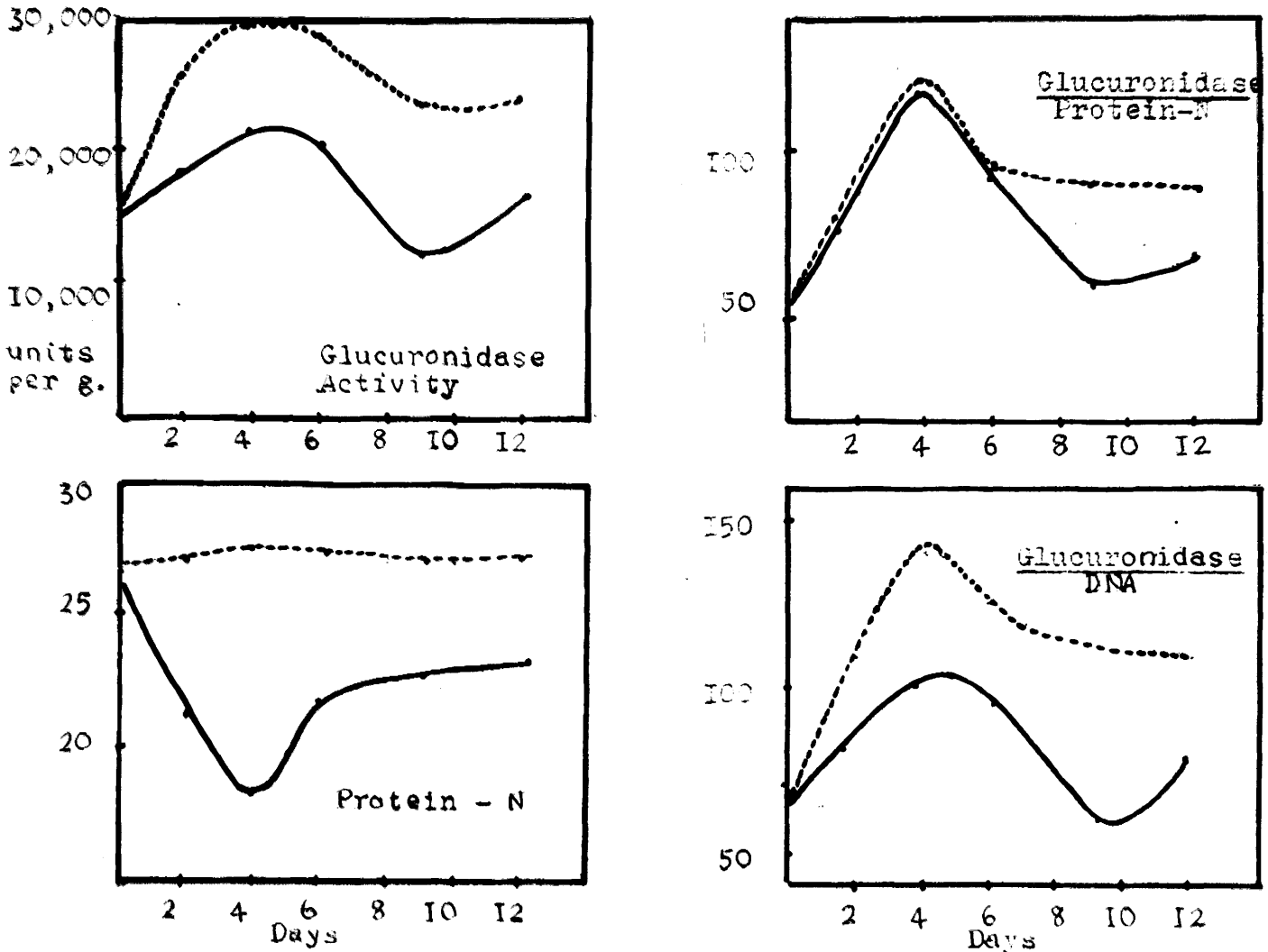
(1 ml. per 100 g. body weight) were administered subcutaneously to a group of such animals (25 in number), and the glucuronidase activity, protein N and DNA of the liver estimated at two day intervals over a period of 12 days on groups of 4 animals. A control series of animals (25 in number) of the same sex and strain simultaneously received subcutaneous injections of arachis oil alone. The results so obtained are expressed collectively in Figure 42.

The most striking feature of such experiments was the greater rise in glucuronidase activity apparent in the control series of animals receiving injections of arachis oil alone, such controls showing no histological evidence of liver damage throughout the 12 day period. Although such findings are again at variance with the results of Levvy et al (1948) who report no increase in liver enzyme activity 2 days after injection of arachis oil or olive oil to mice, the vehicle for administration of the toxic agent in this instance appeared to play a very significant part in influencing enzyme values. Reference to Figure 42 also indicates a fall in the protein concentration in the livers of animals receiving CCl_4, with no attendant variation in the case of the control animals, while both groups showed a similar rise in the glucuronidase/protein ratio up to a period of 6 days.

If one considers the collective significance of the above facts, the difference in the glucuronidase activities after administration of CCl_4 in arachis oil and arachis oil alone may

Figure 42.

The Effect of Subcutaneous Injection of Carbon Tetrachloride on Liver Constituents of Adult Male Rat (Hunter Strain).



——— CCl_4 in Arachis Oil

------ Arachis Oil.

imply an actual loss in glucuronidase from the liver, since the protein concentration did not alter with administration of arachis oil and decreased with CCl_4 injection, while the glucuronidase/protein ratios remained similar in both instances.

The histological pattern of liver damage following administration of CCl_4 in arachis oil is shown in Plate B. Maximum liver damage was apparent at two days with repair processes commencing 6 days after injection of the toxic agent. When such repair processes were histologically most evident (6 - 9 days), the general pattern of liver constituents was steadily approaching a more normal value. From such results it is difficult to accept the postulate that increases in liver glucuronidase following the administration of CCl_4 is a reflection of the degree of cellular proliferation in that organ, particularly when variations in enzyme activity as induced by arachis oil were associated with no histological abnormality in liver structure.

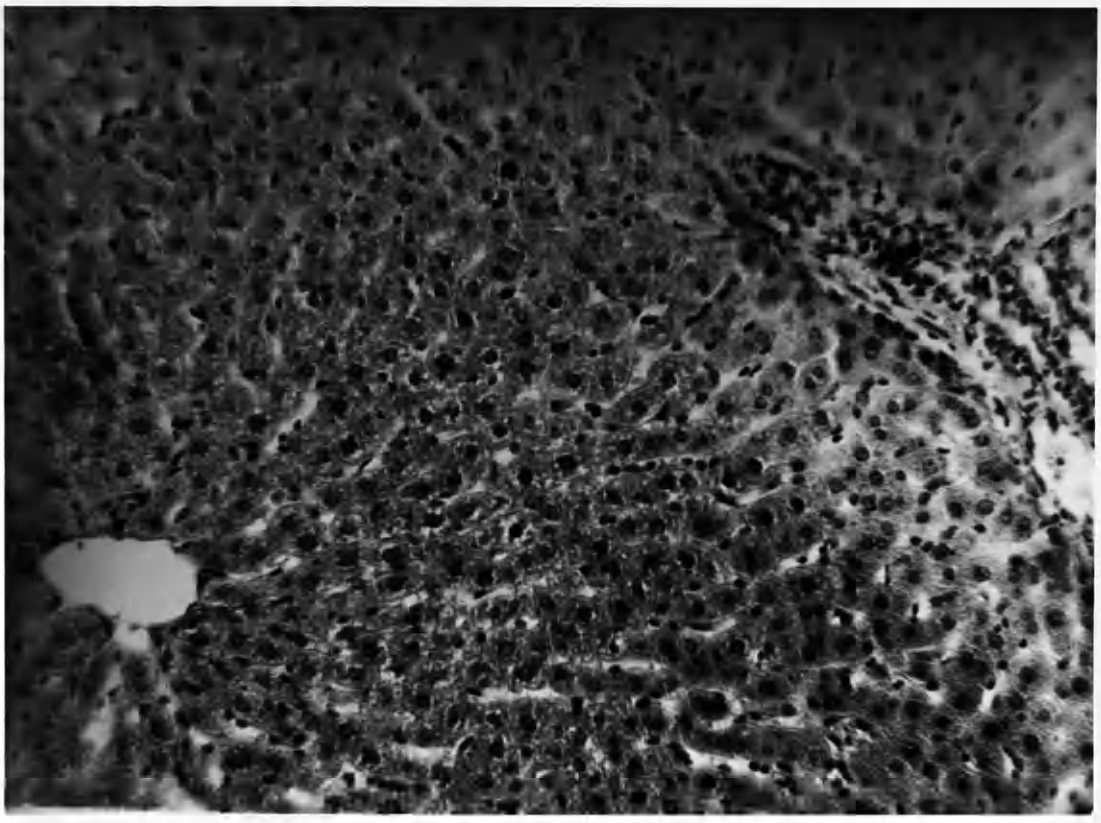
Fatty Infiltration of the Liver and Glucuronidase Activity.

The subcutaneous injection of CCl_4 in arachis oil was invariably associated with a certain degree of fatty infiltration of the liver, and the effect of such infiltration *per se* on glucuronidase activity was thus considered of some interest.

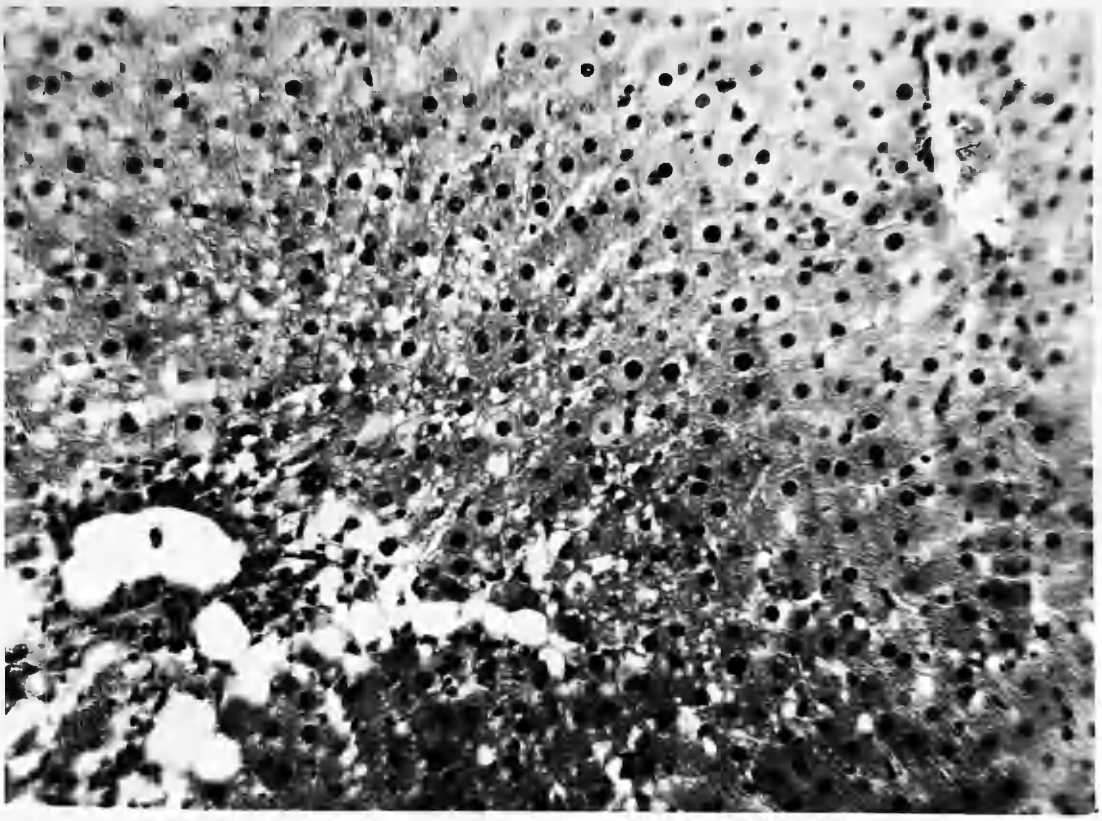
A total of 24 male rats (Hunter strain) were maintained on the low protein, low choline diet of Channon, Mills and Platt (1943) and groups of 6 animals killed at 5, 10, 16 and 20 day intervals. The excised livers were analysed for

Plate B.

Histological Pattern of Normal Rat Liver and Liver Changes Induced by the Subcutaneous Injection of Carbon Tetrachloride.



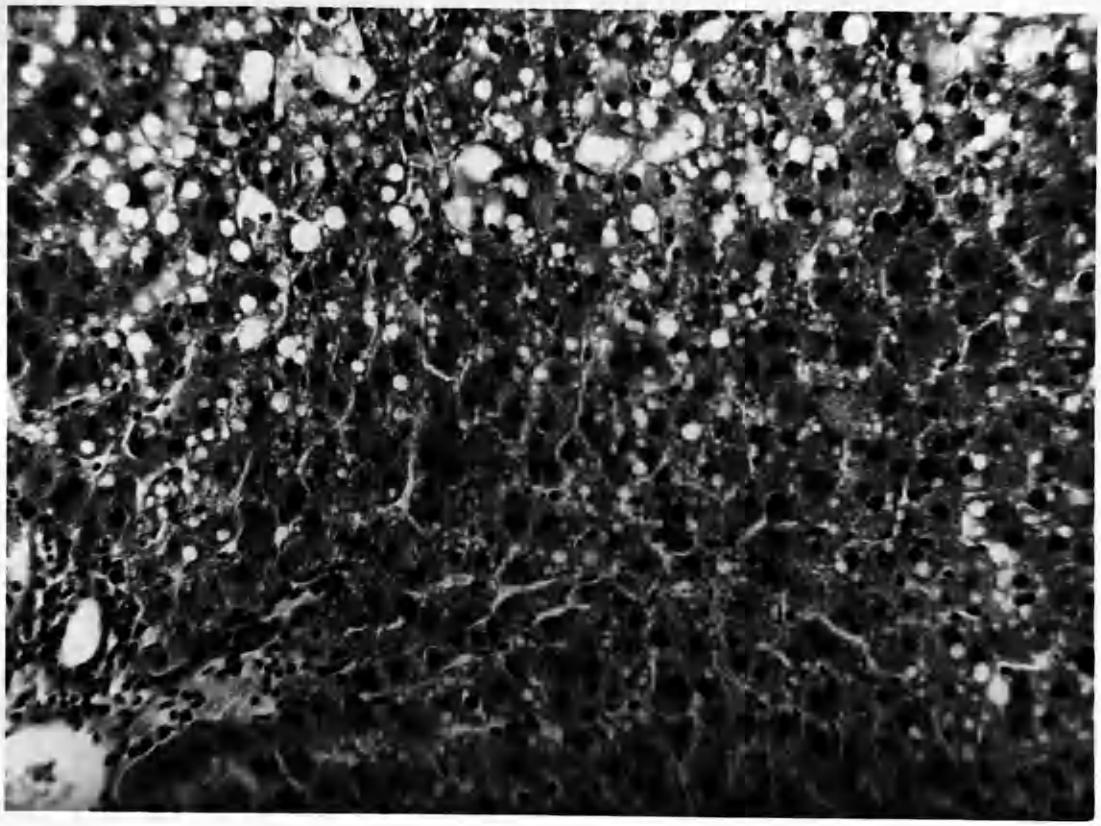
Normal liver



2 Days after CCl_4 injection

Plate B (Contd.)

4 Days after CCl_4 injection



6 Days after CCl_4 injection

glucuronidase activity, protein N, DNA and percentage fat content and the results so obtained are shown in Table 6.

A slight increase in glucuronidase activity was apparent over this period which did not appear to be statistically significant, nor was this increase related to the percentage fat in the liver. While the number of cells per liver of 100 g. animal was found to remain fairly constant, the observed variations in other constituents expressed as glucuronidase/DNA, glucuronidase/protein N and protein N/DNA ratios are not without interest, there being a definite increase in the glucuronidase/protein ratio and a fall in the protein N/DNA ratio.

Effect of Adrenlectomy on Glucuronidase Levels in the Rat.

In view of the fall in liver glucuronidase activity observed after laparotomy, and the elevated enzyme levels induced by subcutaneous injection of arachis oil with no attendant damage to the liver, the possibility of variations in enzyme activity associated with a general shock syndrome was visualised. The influence of adrenalectomy on the glucuronidase content of the liver, kidney and spleen was accordingly investigated. 20 male rats (Tuck strain) were adrenlectomised under light ether anaesthesia and maintained in the post-operative condition on a diet of wholemeal bread, rat cubes and a plentiful supply of water containing 1% NaCl. The glucuronidase activity of liver, kidney and spleen was determined daily on groups of 4 animalsm over a period of 4 days, with simultaneous analyses on liver tissue for DNA, RNA and protein N.

The rather surprising fact came to light that while the

Table 6.

Glucuronidase Activity of Rat Liver in Relation to Other Constituents During Feeding on Fatty Liver Diet.

Days on Diet	Glucuronidase Activity (% Normal)	% Fat* in Liver	Protein N mg./g.	Glucuronidase / DNA	Glucuronidase / Protein N	Protein N / DNA
0	100	3.0	26.5	63	56	113
5	106	6.5	23.0	81	69	117
10	125	13.2	16.4	99	114	86
16	124	17.0	17.9	100	104	95
20	136	20.0	17.2	86	120	73

* Fat = Fatty acids plus unsaponifiable material.

Ratio concentrations expressed per gm. of fresh liver.

laparotomised animals used as control again exhibited a statistically significant fall in glucuronidase activity similar to the results previously expressed, no alteration was apparent in the liver, kidney or spleen enzyme activities of the adrenalectomised rats during the course of the experiment. 2 isolated animals which survived the operation for a period of 21 days likewise conformed to the normal enzyme pattern at the end of this time. RNA and protein N values remained constant throughout, while DNA values showed an increment of 40% over the 4 day post-operative period.

The Glucuronidase Activity of Chemically Induced Rat Hepatoma.

Reference has already been made to the recent extensive literature submitting evidence that elevated β-glucuronidase levels are characteristic of cancer cells. The summation of data implies that β-glucuronidase is intimately involved in processes of malignant growth with a close correlation between enzyme activity, state of cellularity and mitotic activity.

A major difficulty in the interpretation of work on tumours is to obtain adequate controls of a comparable cell type. Whereas an organ may be a complex of tissues, only one of these is usually involved in neoplasia, and in many cases the cell type of tumour is very different from that of the tissue of origin. Few tissues however, lend themselves so admirably to comparative studies as do the liver and hepatomas, and many agents have been employed successfully in the experimental induction of the latter in several species of animals.

As indicated by Greenstein (1947) such experimentally induced neoplastic growths are nearly indistinguishable from those which arise spontaneously at the same sight, and their biological behaviour is also nearly identical. Experiments were accordingly carried out on chemically induced rat hepatoma to determine whether any increase in glucuronidase activity was a specific accompaniment of neoplasia.

Stock Albino rats were maintained on the diet of Griffin, Nye, Noda and Luck (1948), containing 0.06% dimethylaminoazobenzene (DAB) for 5 months, and a control group was fed on a similar diet without DAB for the same time. An additional control group of animals was maintained on normal laboratory diet (Lever's rat cubes). β-Glucuronidase activity was determined on a 1/100 homogenate of the tissue in water and simultaneous analyses were made for DNA, RNA and protein N.

At the end of 5 months multiple hepatoma were apparent and portions of such tumours showing no necrosis were examined. Occasionally hepatoma induction was characterised by a series of small diffuse tumours, and in such instances, (which are separately indicated in the relevant tables), the whole liver was analysed. The results so obtained are presented in Table 7, while Plate C shows a typical histological pattern of a normal and DAB induced neoplastic liver.

It will be noted from Table 7 that the β-glucuronidase activity of the hepatomas was considerably lower than that of the control livers, while those livers with diffuse tumours (Group 3) were intermediate in activity. Use was again made

Table 7.

Glucuronidase Activity, Nucleic Acid and Protein Content of Rat Hepatoma induced by DAB. Duration of Feeding 21 weeks.

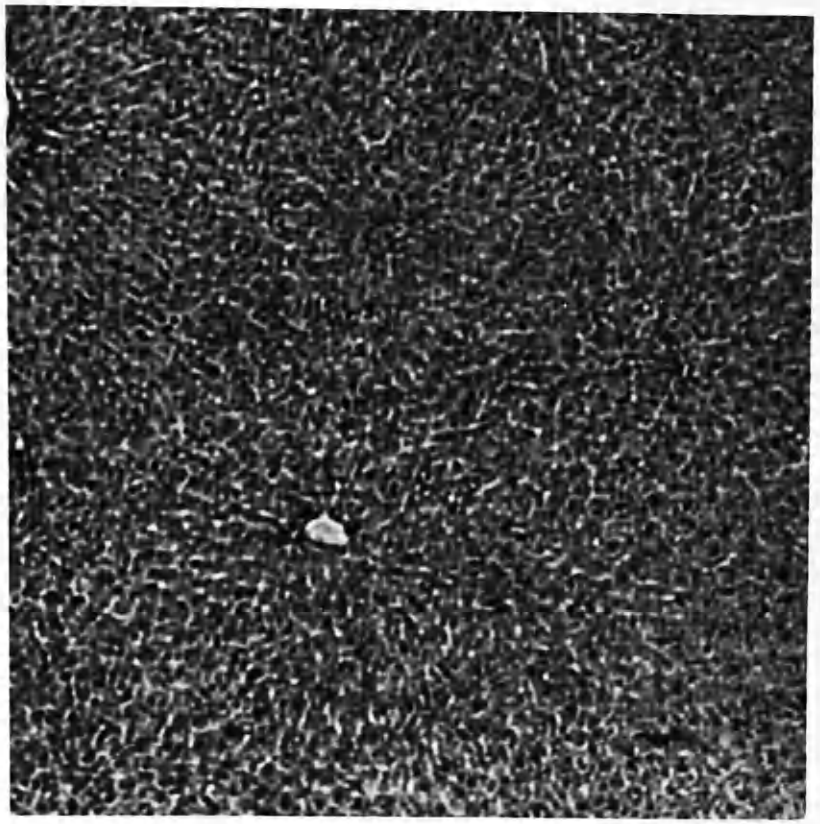
	Number of Animals	Glucuronidase Activity u./g.±S.E.	DNA µ.g.P/g. ± S.E.	RNA µ.g.P/g. ± S.E.	Protein N mg./g. ± S.E.
1. Controls: Stock Diet	10	29,000±999	227±9	871±25	26.1±0.6
2. Controls: DAB Free Diet	4	30,410±3210	255±8	833±22	23.8±0.9
3. Livers with Diffuse Tumours	6	22,100±1935	371±16	711±26	20.4±0.4
4. Hepatoma	10	15,420±1070	378±18	733±34	20.4±0.5

All concentrations expressed on a fresh liver basis.

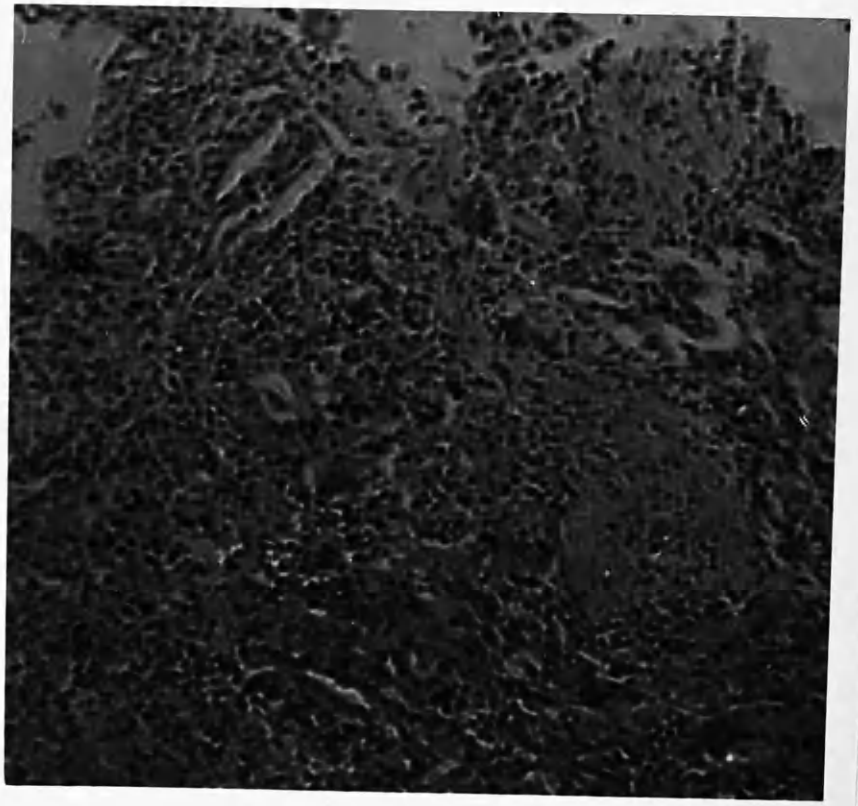
S.E. = Standard error of the mean.

Plate C.

Histological Pattern of Normal Rat Liver and Hepatoma Induced by Feeding p - Dimethyl aminoazobenzene.



Normal liver



Neoplastic liver

of the proposal of Davidson and Leslie (1950 a and b) that the DNA content of a tissue is a valuable criterion of the cell number, and glucuronidase/DNA ratios were evaluated, representing in effect the β-glucuronidase activity per cell. Alterations in the protein content of the tumour cell relative to the normal liver cell were further observed, and enzyme activity was also related to the protein N content of the tissue. These ratios are presented in Table 8.

It will be clearly seen that the β-glucuronidase activity/DNA ratio was greatly lowered, from which it may reasonably be inferred that the enzyme activity per cell is less in the tumour than in the normal liver. Less protein was also observed in the tumour cell than in the normal liver cell, concurrent with a low glucuronidase/protein ratio in the tumour cell. Reference to Table 8 also indicates that while those livers with diffuse tumours (Group 3) have lost almost as much protein per cell as the hepatoma, the β-glucuronidase loss has not been so great in proportion, suggesting that in the change from liver cells to hepatoma cells, not all proteins are lost at the same rate.

In view of the reduced β-glucuronidase activity of chemically induced rat hepatoma it would be unwise to propose a too facile correlation between elevated β-glucuronidase levels and neoplastic processes. It is more probable that the raised glucuronidase activities observed in some tumours are related to changes in cell type and are not characteristic of the cancer tissue itself, while considerable caution should also be exercised in associating elevated glucuronidase levels with mitotic activity.

Table 8.

Glucuronidase Activities of Rat Hepatoma and Normal Rat Liver Relative to DNA and Protein N Contents

Group	Glucuronidase Activity / DNA	Glucuronidase Activity / Protein N	Protein N / DNA	RNA / DNA
1	130	112	115	3.9
2	120	129	93	3.3
3	60	108	63	1.9
4	41	87	54	1.9

All Concentrations expressed on a fresh liver basis.

SECTION V.

BACTERIAL GLUCURONIDASE.

The possibility that bacteria may possess glucuronidase activity was suggested by the work of Marrian (1934) and Patterson (1937) who showed that the oestrogens of pregnancy urine were liberated by putrefaction, and further observations to this effect were made by Venning (1938) and Bucher and Geschickter (1940). The latter authors, in a study of the sodium pregnandiol glucuronidate content of human urine during pregnancy and the menstrual cycle, observed a hydrolysis of the glucuronide to free pregnandiol when such urine was allowed to stand at room temperature for several hours. The hydrolytic agent was shown to be bacterial in origin as liberation of pregnandiol did not occur in boiled, sterile urine, while transference of the factor from a specimen in which it was active to a sterile sample of urine containing sodium pregnandiol glucuronidate again induced hydrolysis. Barker, Brooksbank and Haslewood (1948) conducted a more specific investigation on the type of organism responsible for the destruction of this glucuronide in unpreserved human urine, and showed that, while a number of common bacteria were without effect, hydrolysis occurred when certain strains of Staphylococcus albus were grown in sterile urine to which the glucuronide had been added. Their experiments also suggested that more than a simple hydrolysis was involved in the action of the organism as glucuronic acid itself could not always be detected concomitant with the aglycone.

Buehler, Katzman and Doisy (1949 a and b; 1951) were the first to suggest that hydrolysis was effected by the enzyme β-glucuronidase. When certain strains of Escherichia coli were grown in a beef-extract medium, assay of the culture fluid in the presence of phenolphthalein glucuronide indicated the presence of a glucuronide decomposing enzyme as determined by the liberation of free phenolphthalein. While the enzyme was initally detected in the absence of an adaptive substrate, addition of menthylglucuronide to the growth medium stimulated enzyme production considerably, particularly when combined with constant agitation of the culture flask; optimal enzyme production also necessitated bacterial growth at pH 7.3 for a period of 8 days at 25°C.

The enzyme produced under such conditions from a random strain of Esch. coli isolated from a case of cystitis was characterised by an optimum pH of 6.2 (phosphate buffer) for the hydrolysis of phenolphthalein glucuronide, using as assay material the thoroughly dialysed culture fluid.

Classification of the enzyme as a β-glucuronidase on the basis of aglycone liberation is subject to a certain amount of criticism and it must also be shown that the sole reaction product liberated from phenolphthalein glucuronide apart from phenolphthalein, is glucuronic acid. This requisite of glucuronidase classification was emphasised by Karunairatnam and Levvy (1951); the above authors, in a study of the glucuronide decomposing enzyme obtained from rumen micro-organisms, suggested

that hydrolysis could be effected by the combined action of a decarboxylase and xylosidase or some related complex process.

Irrespective of this complicating factor, a characterisation of the enzyme or enzyme systems responsible for glucuronide decomposition was made by Karunairatnam and Levvy (1951). The source of the enzyme in this instance was not a pure culture, but a mixed suspension of microorganisms obtained by differential centrifugation from the rumen liquor of sheep. Such suspensions were subjected to cellular disintegration, and the enzyme so liberated fractionated from the cell-free supernatant with $(NH_4)_2SO_4$ within the limits 30% - 70% saturation. The precipitated material was dissolved in water and dialysed overnight against running water, providing the active enzyme extract for assay.

While the use of mixed suspensions of micro-organisms is also open to the criticism that final enzymic activity may be influenced by indeterminate factors contributed by more than one type of organism, Karunairatnam and Levvy (1951) have published kinetic data for the hydrolysis of phenolphthalein glucuronide by the above extract which appeared to be derived from a single reaction - either a simple hydrolysis of the glucuronide or the rate determining reaction in a more complex process. On the assumption that the rate of liberation of phenolphthalein from the glucuronide was governed by this single reaction, a value of 3.05×10^{-5} M. was obtained for the dissociation constant of the active enzyme-substrate complex. Excess

substrate caused pronounced inhibition of the enzyme, the inactive complex containing two substrate molecules per active enzyme centre with a second dissociation constant of 0.0126 M.

The optimum pH for the decomposition of phenolphthalein glucuronide by the enzyme was 6.1 (phosphate-citrate buffer) corresponding closely to that reported by Buehler et al (1949 a and b; 1951) for Esch. coli extracts. The rumen enzyme was inhibited only slightly by saccharate (8% inhibition at 10^{-2} M.) at the above pH optimum.

A feature of the biological studies reported in Section IV of the present work was the contention of Odell and his colleagues (Odell, Burt and Bethea 1949; Odell and Burt 1950; Odell, Priddle and Burt 1950) that vaginal secretions abnormally high in β-glucuronidase activity could be used as a diagnostic aid in the detection of female genital malignancy. Increased enzyme activity was frequently apparent however in the secretions of patients free from cervical cancer, and appeared in such instances to be related to vaginal infection with Trichomonas vaginalis. The elaboration of β-glucuronidase by vaginal micro-organisms was thus further considered by Lorincz, Novelli, McGoogan and Odell (1951), and Green, Burt, Hesseltine and Odell (1951), who observed a significant liberation of β-glucuronidase activity not only from the T. vaginalis of infected fluid, but also from all the organisms isolated from a culture plate of the vaginal secretion. Biologically the most significant feature of this work was the fact that detection of enzyme

activity was dependent on the presence of menthylglucuronide (0.7%) in the growth medium. When grown in media in the absence of the glucuronide no enzyme was detectable over a period of 1 to 35 days. It was thus suggested by Green et al (1951) that a glucuronide of tissue origin secreted into the vagina might stimulate enzyme production. While a rapid diminution in the ability of the vaginal organisms to produce the enzyme in the absence of glucuronide is thus inferred, considerable doubt was shed on this hypothesis by Kasdon, McGowan, Fishman and Homburger (1951). The latter authors incubated a fresh, sterile sample of vaginal fluid for two weeks in the presence of a multiplying strain of Esch. coli, with companion growth of the same organisms in a culture medium containing menthylglucuronide. While glucuronidase production was apparent in the latter instance, vaginal fluid per se induced no enzyme stimulation. Under such circumstances it is difficult to evaluate the relative importance of micro-organisms in contributing directly to the glucuronidase activity of vaginal secretions.

Apart from the biological implications inherent in such work, Green et al (1951) made the further observation that the liberation of β-glucuronidase was closely associated with the initial growth phase of bacteria. Bacterial counts on stock cultures of Esch. coli grown in the presence of menthylglucuronide indicated a close correlation between the initial increase in the number of bacteria and the overall production of enzyme. While the strain of Esch. coli used also produced the enzyme in the

absence of menthylglucuronide, no attempt was made to evaluate the relation between bacterial growth and glucuronidase activity in the absence of the adaptive agent. In view of the further unconsidered toxic action on bacterial growth of enzymically liberated menthol, and the inadequately controlled conditions of assessment, the correlation of β-glucuronidase activity with growth processes in bacteria is subject to considerable criticism.

The present study on the glucuronide-decomposing enzyme of Esch. coli was undertaken in an attempt to determine whether or not the enzyme was in effect a glucuronidase, and if so, to determine a few of its characteristics relative to the glucuronidases of animal origin.

EXPERIMENTAL

Materials and Methods.

The author is indebted to Dr. H.J. Buehler, St. Louis School of Medicine, U.S.A. for provision of the strain of Esch. coli used in his investigations (Buehler, Katzman and Doisy 1949 a and b; 1951). A further random strain of Esch. coli was isolated from faecal material and characterised in the following manner: fermentation with glucose, maltose and lactose; no fermentation with sucrose; methyl red, nitrate, indole and motility tests positive; Vosges-Proskauer test and growth on citrate negative. Isolation and characterisation of the latter strain was effected by Dr. Iwo Lominski, Department of Bacteriology, The University of Glasgow, to whom the author is also indebted. Such strains (henceforth arbitrarily referred to as B and L strains respectively) were maintained on agar slopes prior to inducement of enzyme activity by transference to more specific media.

Media Employed.

Enzyme production was stimulated in both the B and L strains of Esch. coli by growth in either 1% peptone water or a 1% casein digest medium (Pronutrin; Herts Pharmaceuticals Ltd.), both media containing 0.5% menthylglucuronide (prepared biosynthetically by the method of Quick (1924)). The controlling conditions necessary for optimal enzyme production in such media will be discussed elsewhere.

Preparation of the Enzyme.

Enzyme extracts suitable for assay were obtained in the following manner:

The requisite culture medium was centrifuged at 0° C. to sediment bacterial cells and débris and the supernatant menthol removed by filtration. To the filtrate was added sufficient solid $(NH_4)_2SO_4$ to ensure 60% saturation, with the addition of Celite (519 A; 2% (w/v)) to adsorb the precipitated material. Following filtration the filter cake was eluted three times with minimal volumes of distilled water, the combined eluates so obtained constituting the active enzyme extract. Dialysis against distilled water has been shown by the present author to result in a radical loss of enzyme activity sufficient to render kinetic studies impracticable. All assays were accordingly conducted on undialysed material; although the presence of $(NH_4)_2SO_4$ in the latter instance is undesirable, the concentration of salt present was minimised to a certain extent by the ability to dilute such extracts at least 1/20 prior to assay. Furthermore the validity of reaction kinetics on undialysed material is equally open to question in view of the, as yet, unknown nature of the phenomenon.

Enzyme Assay.

Enzyme digests consisting of 0.5 ml. buffer, 0.2 ml. 0.00025 M. phenolphthalein glucuronide (Na salt) and 0.3 ml. enzyme solution were incubated for a specific time period and the reaction terminated by the addition of 2 ml. glycine-NaOH

buffer of pH 10.4. 2 ml. aliquots of the solution were added to a further 6 ml. glycine-NaOH buffer and the resultant colour of the free phenolphthalein determined in the Spekker photo-electric absorptiometer using an Ilford 605 yellow-green filter.

The method defined was essentially that outlined in Section II of the present work, and the unit of activity under such conditions is similarly defined as:

1 phenolphthalein unit = 1 µg. phenolphthalein liberated per hour.

The following buffers were employed unless otherwise indicated:

pH 3.4 - 6.0 0.2 M Acetate buffers.

pH 6.0 - 8.0 Phosphate-citrate buffers (Mixtures of 0.2 M Na_2HPO_4 and 0.1 M citric acid).

RESULTS.

Conditions of Growth and Enzyme Production.

Considerable variation in ability to produce the glucuronide-decomposing enzyme was apparent depending on the strain of Esch. coli employed, and the complex medium and conditions requisite for maximal production by the B strain were found to be superflous when applied to the L strain. The conditions outlined by Buehler *et al* (1951) in the former instance are as follows:

Enzyme production was stimulated in a medium consisting of:

1.0% Difco Bacto-peptone

0.3% Difco Bacto-beef extract

0.5% NaCl

3.0% Sodium glycerophosphate

0.7% Menthylglucuronide

pH adjusted to 7.3

By constant agitation of the medium (75 - 100 r.p.m.) combined with aeration, optimal enzyme activity (1500 - 2000 phenolphthalein units) was apparent within 8 days as determined by assay of the straight culture fluid.

An enzyme preparation of similar potency (activity assessed from the cell free culture fluid) could be obtained from the L strain in the following manner. Following growth for 24 hours in 1% peptone, the latter strain was sub-cultured into a similar medium containing 0.1% menthylglucuronide. Enzyme activity was detectable in this medium within 24 hours, and

transference of the organism after this period to 1% peptone containing 0.5% menthylglucuronide resulted in maximal enzyme production within 3 days. Further stimulation was limited by the susceptibility of the organism to the free menthol liberated into the medium, the toxicity of the latter proving fatal as determined by the inability to sub-culture in 1% peptone after this period. Analagous conditions employing a 1% casein digest instead of peptone water were equally effective in elaborating the enzyme.

It was frequently found that the organism, once adapted to enzyme production, would de-adapt when transferred to an agar slope for any length of time. In such instances the ability to develop the enzyme could only be reinduced by a number of successive daily subcultures in 0.1% menthylglucuronide. The organism was accordingly maintained in the adaptive state by growth in a medium containing 0.1% menthylglucuronide, the concentration of free menthol liberated in such instances being insufficient to affect the viability of the strain.

Growth was sustained throughout at a temperature of 37°C. and a pH of 7.0. Any deviation in the latter towards acidity or alkalinity considerably reduced the yield of enzyme. At a pH of 6.0 and 8.0 enzyme production was respectively 20% and 40% less, but whether this was due to instability of the enzyme, inhibition of bacterial growth, or a combination of the two factors was not determined.

A study of the B strain indicated that while enzyme activity could be induced under circumstances similar to the

above, at least 8 - 10 days growth in a medium containing 0.5% menthylglucuronide was required to obtain a reasonably active preparation. While Buehler et al (1949 b; 1951) have reported enzyme production by this strain of Esch. coli in the absence of menthylglucuronide, this apparently did not apply to growth in the more simple media employed by the present author as no enzyme activity could be detected in either strain in the absence of adaptive substrate.

In an endeavour to obtain a more pure enzyme preparation for kinetic studies, growth conditions were simplified by using the following synthetic medium:

$Na_2HPO_4 . 12 H_2O$	16.5 g.
KH_2PO_4	1.5 g.
NH_4Cl	2.0 g.
$CaCl_2$	0.01 g.
$FeSO_4 . 7 H_2O$	0.0005 g.
$MgSO_4 . 7 H_2O$	0.2 g.
Glucose	10.0 g.
Distilled water	1000 ml.

Adjusted to pH 7.0 with NaOH.

While growth was well sustained in such a medium no enzyme activity could be induced from either strain over a period of 35 days with continued sub-culturing in the presence of added 0.1% or 0.5% menthylglucuronide. Elimination of glucose from the medium and provision of menthylglucuronide as the sole source of carbon also failed to support bacterial growth even in organisms well adapted to enzyme production by previous growth

in a casein digest, substrate-containing medium.

Karunairatnam and Levvy (1951) have shown that the glucuronide-decomposing enzyme of rumen micro-organisms is intracellular in nature. In the present study, the presence of activity in the culture fluid is suggestive of extracellular liberation of the enzyme, although the possibility cannot be excluded that such activity is due to cellular lysis by menthol. When a viable, enzyme-producing culture of the L strain was subjected to ultra-sonic disintegration however, no increase in enzyme activity was apparent in the medium following cellular destruction, from which it may be inferred that enzyme production in this instance was extracellular in nature.

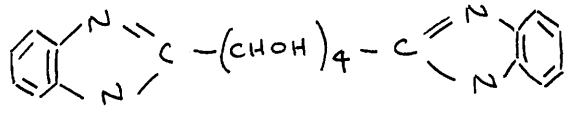
An endeavour was made to induce enzyme activity from 8 different strains of atypical coli (aerobacter) using the stimulative technique employed for faecal coli. No activity could be detected in any instance however with growth periods of 1 to 10 days in substrate containing medium.

Identification of the Enzyme.

To determine whether or not substrate decomposition was due to hydrolysis of the glucosidic link by a β-glucuronidase, identification of the glucuronic acid liberated under such circumstances was considered necessary. Whether glucuronic acid is the sole product of enzymic decomposition is obviously difficult to establish, as it is conceivable that other enzymes causing destruction of the hexuronide may well be present; if glucuronic acid can be detected *per se* however, it is reasonable

to assume that it has arisen by initial hydrolysis of the glucuronide irrespective of subsequent decomposition reactions.

To identify the possible hexuronic acid residue arising from enzymic decomposition of menthylglucuronide, recourse was made to the method of Lohmar, Dimler, Moore and Link (1942) whereby oxidation of the former to saccharic acid was followed by condensation with o-phenylenediamine to yield the dibenziminazole derivative of D-glucosaccharic acid.



The technique employed was as follows:

A potent enzyme extract was induced from the L strain of Esch. coli by growth of the latter in 250 ml. 1% casein digest medium in the manner previously described. Cellular material was removed from the culture fluid by centrifugation, menthol removed by filtration at 0°C. and the enzyme subsequently precipitated from the supernatant at 60% saturation with $(NH_4)_2SO_4$. Adsorption of the precipitated material on Celite 519 A (2% w/v) was followed by successive elution of the filter cake with 5 x 10 ml. volumes of water, the combined eluates so obtained constituting the enzyme extract. Use of a casein digest as culture medium in preference to peptone water considerably reduced the total protein present in the above extract, thus facilitating the later stages of condensation with o-phenylenediamine.

To the 50 ml. enzyme solution so obtained was added a

further 140 ml. water and 1 g. menthylglucuronide. pH adjustment to 6.3 was effected with a few drops of 2 N. NaOH plus 10 ml. veronal acetate buffer of similar pH, and the mixture was incubated at 37°C. for 3 days. The substrate concentration employed (0.5%) was found to be the most suitable for reaction purposes, any concentration in excess of this causing considerable inhibition of enzymic activity.

The 3-day incubation was followed by filtration of the solution at 0°C. to remove menthol, with subsequent adjustment of the filtrate pH to 2.0 with 10 N H_2SO_4 and extraction with peroxide-free ether for 6 hours in a continuous extractor to remove further traces of menthol and unreacted substrate. The presence of glucuronic acid in the resultant solution was demonstrated by the colour reaction given with naphthoresorcinol, using the method of Hanson, Mills and Williams (1944) as modified by Paul (1951). Control experiments showed that no glucuronic acid was liberated in the absence of enzyme under the conditions of the experiment, and that menthylglucuronide was completely removed from aqueous solution by the extraction process.

Isolation of the dibenziminazole derivative was accomplished in the following manner:

The residual solution from hydrolysis was adjusted to pH 8.0 with 2 N.NaOH and condensed in vacuo to the smallest possible volume, which was then refluxed with 25 ml.N HBr containing 1 ml. bromine for 2 hours. Following removal of bromine by aeration, excess HBr was removed by distillation in vacuo at 40°C. to a

volume of 2 ml. Elimination of the last traces of excess HBr was facilitated by the addition of 25 ml. ethanol, distillation in vacuo to small volume and repetition of this latter stage.

Condensation was brought about by the addition of 0.85 g. o-phenylenediamine, 0.6 ml. syrupy H_3PO_4 and 2 ml. diethylene glycol, the mixture being maintained at a temperature of $135^{\circ}\pm5^{\circ}$ on an oil bath for 2 hours. The syrupy product so obtained was dissolved in 10 ml. water, charcoaled and filtered; following alkalisation of the warm filtrate with concentrated NH_4OH, the solution was allowed to stand at $0^{\circ}C$. overnight. The crystals obtained in this manner were washed with water, acetone and ether, suspended in hot water, acidified with dilute HCl, decolourised with charcoal and again crystallised by alkalisation with excess NH_4OH.

A comparison of the melting point of the above crystals with that of an authentic dibenziminazole derivative of D-glucosaccharic acid is as follows:

	Substance Isolated	Authentic Specimen	Mixture
Dibenziminazole	m.p.236°C	m.p.238°-240°C	m.p.238°C

From such results it would appear that the hydrolytic action of the enzyme is associated, at least in part, with rupture of the glucosidic link of the glucuronide. No attempt was made to interpret the experiment on a quantitative basis in terms of the glucuronic acid liberated by enzymic hydrolysis, as a number of other condensation derivatives were also obtained under the conditions of the experiment. Such derivatives were readily

separable from the dibenziminazole conjugate of saccharic acid by their insolubility in acid solution, and while their precise nature has not yet been determined it is conceivable that a number of interfering substances of bacterial origin may well be present in the original enzyme extract. No derivative corresponding to the condensation of pentose with o-phenylenediamine could be identified.

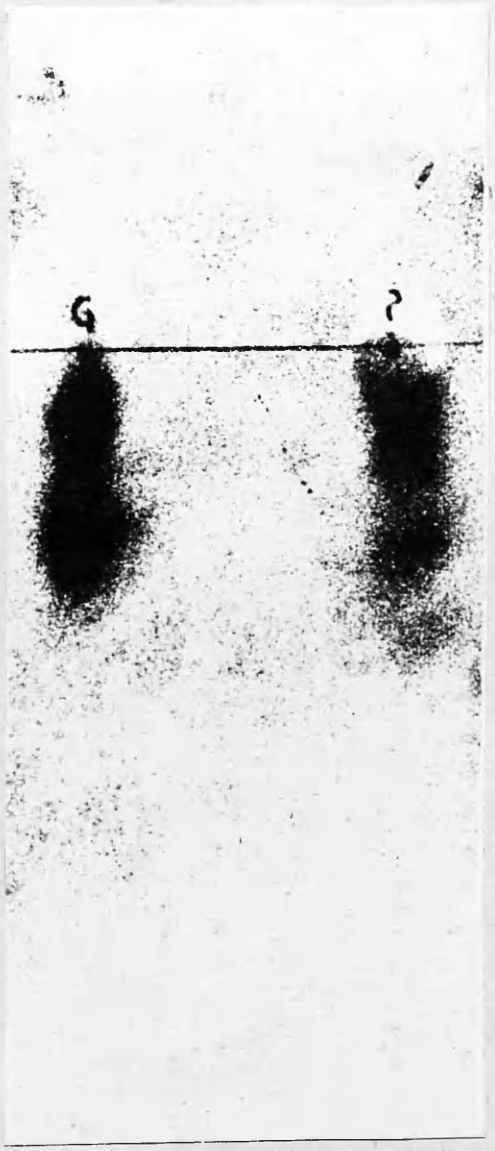
An experiment analagous to that described was carried through to the stage previous to oxidation to saccharic acid, condensed in like manner to a volume of 10 ml., freeze dried, and the residue extracted overnight with 1 ml. ethanol acidified with 6 drops glacial acetic acid. The addition of the latter facilitated the conversion of glucuronic acid to the lactone and a one-dimensional chromatogram of the extract was run in the butanol-NH_3 solvent of Partridge and Westall (1948) using pure glucurone as control. Location of the spots was effected by spraying with ammoniacal $AgNO_3$ and heating to 120°C for several minutes. While the result shown in Figure 43 is not in itself a conclusive demonstration of the presence of glucuronic acid, it provides a useful supplement to the evidence already obtained on the nature of enzymic hydrolysis.

Properties of the Enzyme.

In view of the facility with which enzyme activity was induced in the L strain of Esch. coli relative to the B strain, further studies on the reaction characteristics of the enzyme were confined to the former.

Figure 43.

One Dimensional Chromatogram of the Product of Hydrolysis of Menthylglucuronide by the Glucuronide Decomposing Enzyme of Esch.Coli.



Solvent = Butanol - NH_3

G = GLUCURONE

? = TEST

Effect of Dialysis.

The first notable feature apparent from a study of this enzyme was the radical loss in activity associated with dialysis against tap or distilled water. Buehler et al (1949 b) made no reference to this phenomenon in preliminary reports on the enzyme induced from the B strain, while Karunairatnam and Levvy (1951) specifically asserted that no loss in enzyme activity of rumen preparations occurred with a 20 hour dialysis against running tap water. In a later publication however, Buehler et al (1950) reported a 50% reduction in enzyme activity concurrent with dialysis for several hours against distilled water.

In the present instance, dialysis for periods ranging from 4 to 12 hours was invariably associated with a 90% loss in activity in both the straight culture fluid and fractionated material. Dialysis against small volumes of water and addition of the total dialysate to the enzyme was without effect in restoring activity, while control of temperature likewise appeared to play no part in this phenomenon.

A considerable lowering of pH during the dialysis of salts against water has been reported by Gilbert and Swallow (1950), who have suggested that such changes in pH arising from the ion selectivity of the cellophane membrane may be great enough to endanger proteins or other substances which are sensitive to acid. An enzyme preparation was accordingly dialysed against small volumes of buffer solution of varying pH in an endeavour to counteract this possible source of inactivation; the data

obtained from this experiment is summarised in Table 9.

While such results suggest that the irreversible loss in enzyme activity occurring during dialysis against water may be related in part to a temporary fall in pH, the actual nature of the dialysis phenomenon is still a matter of conjecture. The full extent of pH control by buffer solutions during dialysis was not thoroughly investigated, while it is conceivable that loss in enzyme activity is dependent on factors other than variation in hydrogen ion concentration. The addition of metallic ions (zinc acetate, $MnCl_2$ and $MgSO_4$; final concentration 10^{-1}M.) and boiled culture fluid to dialysed enzyme preparations also failed to restore activity.

pH Activity Curve.

Figure 44 shows the effect of varying pH on the rate of decomposition of phenolphthalein glucuronide by an enzyme preparation. A well defined optimum at pH 6.3 (phosphate-citrate buffer) was apparent in all assays on both dialysed and undialysed material, while variation in substrate concentration caused no alteration in the optimum pH of the enzyme. Activity at pH 6.3 was shown to be the same in phosphate-citrate, phosphate, citrate and veronal acetate buffers.

Suramin Inhibition.

Application of the method of Wills and Wormall (1950) to a determination of the isoelectric point of an enzyme preparation gave the results shown in Figure 45. The pH of 50% inhibition

Table 9.

Effect of Dialysis on the β-Glucuronidase of Esch. Coli.

pH of assay	Original Activity	Preparation Dialysed Against:					
		Tap H_2O	Distilled H_2O	0.2 N Acetate Buffer pH 3.8	0.2 N Acetate Buffer pH 4.4	0.2 N Acetate Buffer pH 6.0	Veronal Acetate Buffer pH 8.0
4.0	28	0	0	0	0	25	0
5.2	305	38	40	0	0	135	70
6.3	370	50	48	0	0	160	80
6.6	354	40	42	0	0	100	52

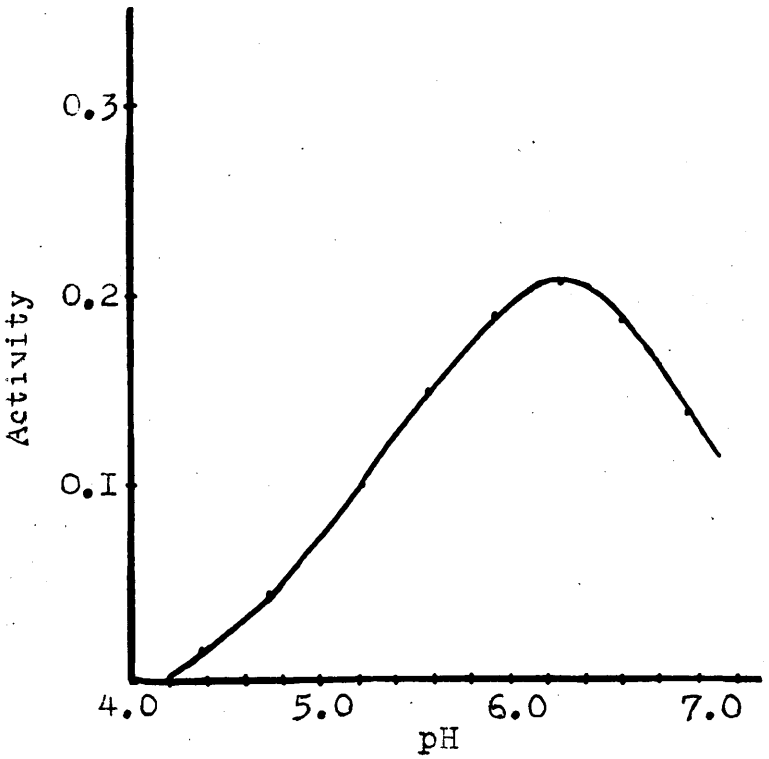
Results expressed as direct Spekker Readings.

Volume of dialysis fluid = 10 ml.

Figure 44.

pH Activity Curve of Esch.Coli β - Glucuronidase.

(L Strain).

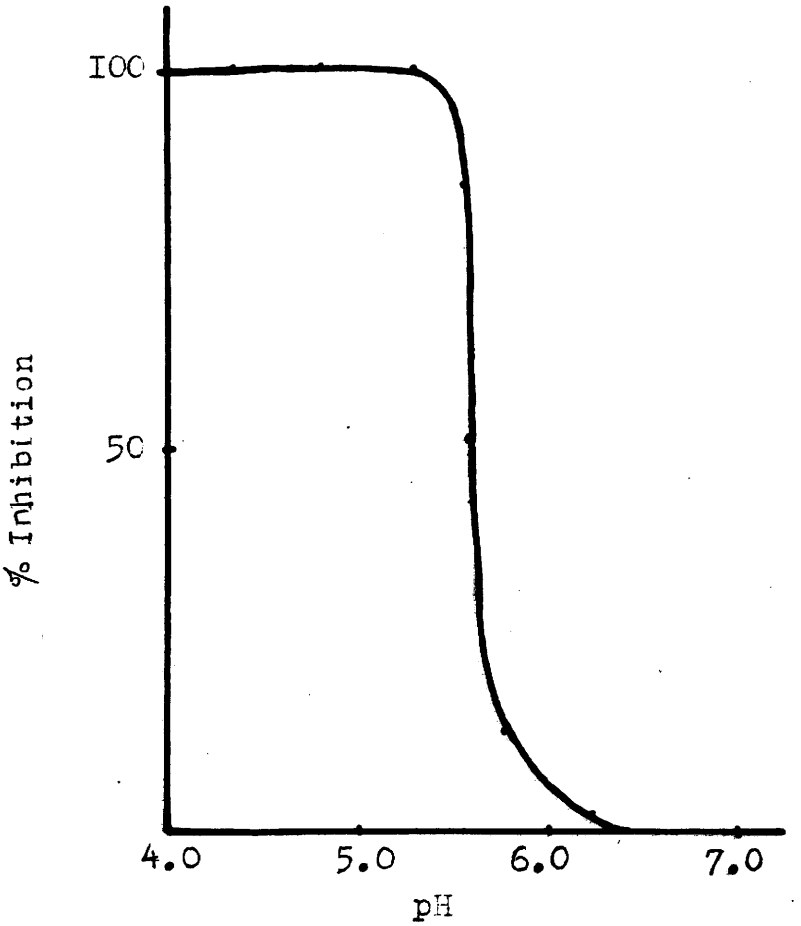


pH 4.0 - 5.8 Acetate Buffers

pH 5.8 - 7.0 Veronal - acetate Buffers

Figure 45.

Inhibition-pH Curve of Esch.Coli/β - Glucuronidase in the Presence of Suramin (10^{-3} M.)



PO_4 Citrate Buffers

by suramin was apparent at 5.6 at which point the enzyme may be considered isoelectric in terms of the interpretation of the above authors. The fact that inhibition occurs so sharply over a narrow and well-defined pH range also suggests that a single enzymic reaction rather than a combined series of mechanisms is responsible for initial decomposition of the substrate.

Variation in Activity with Substrate Concentration.

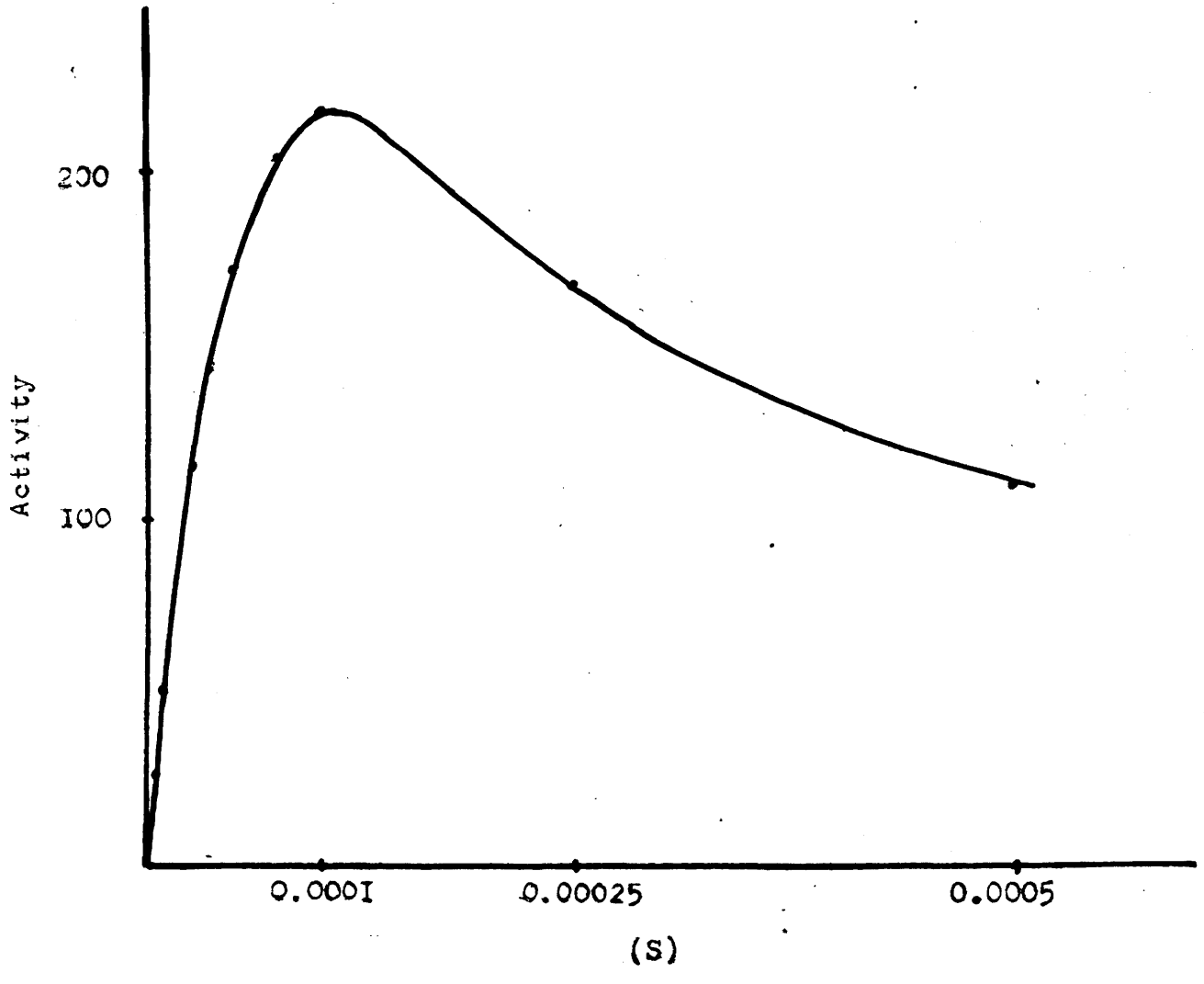
The effect of varying the substrate concentration on the decompositiom of phenolphthalein glucuronide by an enzyme preparation in phosphate-citrate buffer pH 6.3 is shown in Figure 46. Pronounced inhibition by excess substrate was apparent. Analysis of results by the method of Lineweaver and Burk (1934) gave a value of 4.2×10^{-5}M for K_S the dissociation constant of the active enzyme-substrate complex. (Figure 47).

Using the analytical methods outlined in Section III D of the present work for derivation of n (the number of substrate molecules per active enzyme center in the inactive complex) and K_2 (the dissociation constant of the inactive complex), values of 2 and 1.05×10^{-5} were respectively obtained for such constants.

Such results are sufficiently comparable to those reported by Karunairatnam and Levvy (1951) for rumen preparations to imply that a similar enzyme is responsible for substrate hydrolysis in both instances. The specific loss in activity on dialysis was not reproduced by rumen extracts however, and a further anomaly was evident when the effect of added saccharate on enzyme activity was considered. Karunairatnam and Levvy (1951)

Figure 46.

Relation Between Phenolphthalein Glucuronide Concentration and β- Glucuronidase Activity of Esch.Coli (L Strain)



report only slight inhibition of the rumen enzyme by saccharate, and the results obtained by the above authors are compared with present findings in the following table:

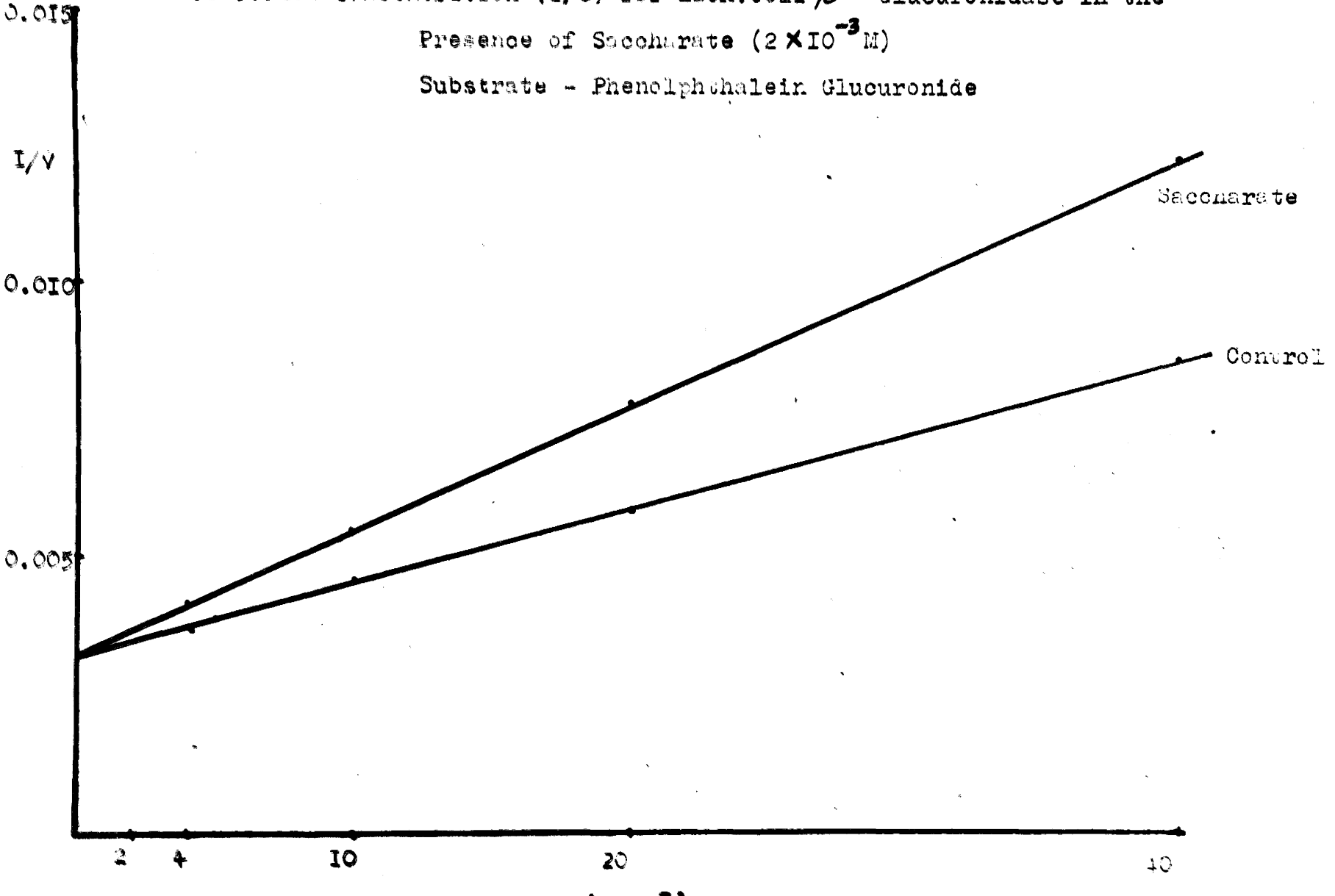
	% Inhibition	
Concentration of Saccharate	Rumen Preparation (Karunairatnam & Levvy 1951)	Esch. Coli preparation (L Strain)
10^{-1} M.	44	100
10^{-2} M.	8	90
10^{-3} M.		60

Using the method of analysis previously employed, inhibition by saccharate for the enzyme induced from Esch. coli was shown to be competitive in nature (Figure 47), with a calculated value of 0.91 for K_1, the enzyme - inhibitor dissociation constant.

Figure 47.

Plot of Reciprocal of Reaction Velocity (I/V) against Reciprocal of Substrate Concentration (I/S) for Esch.Coli β - Glucuronidase in the Presence of Saccharate (2×10^{-3} M)

Substrate - Phenolphthalein Glucuronide



SECTION VI.

DISCUSSION.

Physical Properties of β-Glucuronidase

When considering the physical nature of β-glucuronidase, it is initially a matter of some importance to determine the precise nature of the enzyme system under investigation. While some measure of dissention is apparent in this field, the complex number of factors affecting glucuronidase activity must inevitably lead to disagreement over the most fundamental of points. Such factors as choice of buffer and substrate concentration for assay can materially alter the pH activity curve and kinetic results, and many of the anomalies evident in the literature can be traced to the reaction conditions employed by the various authors. Until such time as the complexity of factors affecting enzyme activity is fully realised, little measure of agreement can be anticipated.

The first point at issue is whether or not we are dealing with a complex enzyme system. While the initial work of Mills (1948) has been substantiated in part by independent investigators (Kerr, Campbell and Levvy 1949; Morrow, Greenspan and Carroll 1950), Bernfeld and Fishman (1950a) and Sarkar and Sumner (1950) have failed to adduce any evidence in support of the multiplicity of the glucuronidases, preferring to interpret enzyme activity in terms of a single entity of optimum pH 4.5. The data accumulated in the course of the present study however,

does not permit acceptance of such a postulate, and it is proposed to discuss the evidence for a complex enzyme system along with other more general considerations, by a sub-division of the major features at issue in the following manner:

A. Characteristics of the purified enzyme

B. Kinetic studies on fractionated β-glucuronidases

C. The effect of activators and inhibitors on β-glucuronidase activity, with a general summary (D) on the proposed nature of the glucuronidase system.

A. Characteristics of the Purified Enzyme.

Sarkar and Sumner (1950) have asserted that the type of pH activity curve shown by their highly purified liver preparation is, in itself, ample indication of the single nature of the enzyme. The plateau-shaped curve showed maximum activity between pH 4.2 and 5.6, but when subjected to a more critical examination it is apparent that a curve with independent peaks of activity at pH 4.5 and 5.2 could equally well fit the published data, a fact which has already received the comment of Paul (1951). Moreover the substrate concentration chosen for assay was sufficiently low to suppress enzymic activity in the lower pH range with a simultaneous tendency to accentuate activity around pH 4.5. Nevertheless, taking such facts into consideration, the well-defined peaks of activity obtained by the present author for the purified enzyme were infinitely more pronounced than those expressed in the activity curve of Sarkar and Sumner (1950). Are we therefore to concede that such peaks are artefacts, and that the curve of

Sarkar and Sumner is a true reflection of enzyme activity?.

Probably the most salient feature in this respect was the observation by the present author that all highly purified enzyme preparations when allowed to stand for any length of time (2-3 weeks) gradually assumed the plateau-shaped curve illustrated by Sarkar and Sumner (1950). The pH activity curve of any enzyme is the resultant of a number of factors including enzyme stability, and it might reasonably be assumed that the latter factpr plays a predominant part in determining the activity curve of the highly purified glucuronidase preparation. Such preparations were invariably associated with very low protein contents, and over a period of time the enzyme may have been rendered more susceptible to pH changes in the absence of the protective effect exerted by extraneous protein, with a resultant plateau-shaped curve dependent on the stability of the enzyme within a certain pH range. This postulate receives support from the observation that enzyme preparations of a lesser degree of purity than that ultimately obtained, and characterised by a lower activity/protein ratio were infinitely more stable with respect to pH activity curve, maintaining the well-defined optima even on standing for periods of several weeks. Whether enzyme stability relative to protein concentration entirely explains the transition phenomenon is obviously difficult to evaluate, as other factors such as coincident variation in activators and inhibitors may well be operative.

If the original pH activity curve may be accepted as valid

however, it is difficult to visualise enzyme action in simple terms, and the original Michaelis concept of enzyme activity relative to pH infers that here we are dealing with a multiple system. It may well be argued that glucuronidase activity at any one pH is dependent on a variety of extraneous factors, but the most reassuring feature is the consistent demonstration of activity at the four pH optima of the enzyme irrespective of the purity of the material. Straight tissue homogenates, partially purified material and the finally purified product are all characterised by the same pH optima, enzyme activity varying in degree at such optima but always being well-defined. It is difficult therefore to regard the ultimate pH activity curve as an artefact under such circumstances.

Another feature of the purified preparation was the accentuation of enzyme activity at the defined optima with varying substrate concentration. This was also apparent in preparations showing the plateau-shaped curve, although to a lesser degree as might be anticipated if enzyme stability is competing with such factors as substrate affinity in determining the ultimate shape of the activity curve. This variation in activity curve with substrate concentration was also reflected in straight tissue homogenates serving to demonstrate the original complexity of the system before purification. While the apparent K_s may change with pH if the latter influences the velocity of combination of enzyme with substrate, it has been shown in fractionation studies that cleanly separated glucuronidase preparations showing optima at say pH 5.2 showed no difference in K_s value

when assayed at pH 4.5. Hence changes in pH *per se* have little direct influence on substrate affinity, and the alterations in activity curve of the highly purified preparations with varying substrate concentration are entirely dependent on the different affinities of the individual enzymes for substrate, suggesting the independent nature of enzymic activity at the four pH optima.

B. Significance of Kinetic Studies on Fractionated β-Glucuronidases.

The most convincing demonstration of the complexity of the glucuronidase system is the separation of different entities showing overwhelming activity at one or other of the pH optima of the enzyme. Fractions have been obtained by widely diverse methods showing particularly well-defined optima at pH 4.5 and 5.2 (acetate buffer), and although separation of the 3.4 enzyme has not been attended with the same measure of success, preparations markedly rich in activity at this pH can readily be obtained. A highly significant feature of many such separations is that quantitative summation of the pH activity curves of the individual fractions can satisfactorily account for the pH activity curve of the starting material, thus confirming the fact that such separations are indeed valid reflections of enzyme fractionation and not possible artefacts, while kinetic studies on the fractionated material provide a sound argument for the individuality of the enzymes at at least three of the four pH optima of the system.

A feature of the activation energies of any enzyme system is that such values are essentially unaffected by changes in pH.

Sizer (1937) has found that the activation energy of yeast invertase was unaltered by changes in pH from 3.2 - 7.9, and numerous other examples can be cited in this respect (c.f. Sizer 1943). Significantly different values for A (activation energy) have been obtained however for both liver and spleen β glucuronidases at pH 3.4, 4.5 and 5.2 in unfractionated material, values which are faithfully reflected for the separated entities. Furthermore if fractionation is associated with any modification of the catalytic surface of the enzyme, such modifications should be apparent in changed values of A. This is not obvious in the separated glucuronidases, implying that the resultant pH activity curves are not due to structural alterations in the enzyme per se.

While the activation energies differ inter se, variations are also apparent in the individual enzymes from different sources. The liver enzymes I and II (pH optima 4.5 and 5.2) have significantly lower activation energies than the analagous spleen enzymes; the lower the value of A the more efficient is the catalyst, and the possible implication arises that the liver glucuronidases are functionally more efficient than their spleen counterparts. Although it should be remembered in this respect that the substrate employed for in vitro assay is not necessarily an in vivo reactant, it appears that the same activation energy characterises the action of an enzyme on different substrates (c.f. Sizer 1943), the glucuronidases themselves showing no difference in the values of A for either phenolphthalein glucuronide or phenylglucuronide. Furthermore the liver glucuronidases collectively have much lower K_s values than the analagous spleen enzymes, indicating a much

greater affinity of enzyme for substrate in the former instance. If the glucuronidases of the liver are thus catalytically more efficient than those of the spleen, possible functional differences between these organs in vivo may well be implied.

The different activation energies of the liver and spleen glucuronidases also implies some structural differentiation between the two enzyme systems. This implication is interesting in view of the fact that fractionation procedures applicable to the liver glucuronidases were relatively unsuccessful when applied to the spleen system and vice versa. Inasmuch as most fractionation techniques are also dependent on the proportionality and type of other protein constituents initially present however, such independent factors must also be taken into consideration.

Other points of evidence in favour of a complex enzyme system are the different K_S values for the separated enzymes and the different degrees of substrate inhibition exerted towards each enzyme. That changes in pH have little direct influence on the velocity of combination of enzyme with substrate has already been indicated, and the fact that the K_S values for the separated entities are unchanged over a very wide pH range is a very strong basis indeed for emphasising not only the individuality of the enzymes, but also the degree of separation that has been achieved. Such clear-cut results, while applying to the enzymes of pH optima 4.5 and 5.2 (acetate buffer) were difficult to reproduce with the enzyme of optimum pH 3.4, indicating the heterogeneous nature of this fraction, as is

indeed apparent from the pH activity curves of the fractionated material. The kinetic data for this enzyme may be substantially correct however, as identical results were obtained for a variety of different enzyme preparations showing maximal activity at pH 3.4. That little measure of agreement is evident in the literature over the fundamental reaction characteristics of β-glucuronidase no doubt has its origin in the unrecognised complexity of the system, and the use of unfractionated material for kinetic studies must inevitably lead to differences of opinion in this respect.

A consideration of the type of inhibition exerted by saccharate on the separated fractions again suggests the individual nature of such entities. In the case of the glucuronidases of pH optima 3.4 and 4.5 inhibition is competitive, while non-competitive inhibition is unquestionably apparent with the enzyme of optimum pH 5.2. A comparison of the isoelectric points of the fractionated enzymes by the suramin inhibition method of Wills and Wormall (1950) indicates that there is little difference in such values among the individual glucuronidases, and it might be assumed that, in the case of the 5.2 enzyme where inhibition by saccharate was determined at a pH value alkaline to that of the isoelectric point, the type of inhibition might differ from that at pH values on the acid side of this point. The actual type of inhibition may however be independent of factors affecting either the net charge on the molecule or the degree of dissociation of the enzyme or inhibitor, with a possible structural difference in

the active centre of the 5.2 enzyme as compared with those of lower pH optima.

C. The Effect of Activators and Inhibitors on β-Glucuronidase Activity.

It is apparent that activation and inhibition studies on β-glucuronidase cannot be classified with any convenience into two specific groups, as compounds causing inhibition at one pH were frequently associated with activation at another. A measure of simplification can be introduced however by considering such studies in terms of non-specific and specific effects - the former including such features as activation by proteins and polysaccharides, and the latter the more specific chemical effects of structurally related analogues on enzyme activity. While activation studies on β-glucuronidase contribute little to the general argument on the complexity of the enzyme system, some useful information is gained on the multiple factors affecting enzymic activity.

The nature of glucuronidase activation by extraneous protein is somewhat enigmatic. The unspecific nature of the mechanism is evident from the fact that activation appears to be independent of both the protein concentration and the nature of the protein molecule - crystalline albumin, electrophoretically separated α, β and γ globulins and whole serum giving remarkably analagous results. No modification of the catalytic surface of the enzyme can be implied as this would be reflected by changes in the energies of activation in the presence of the

activator, while electrophoretic studies also demonstrate that no protein-protein complex is formed between albumin and the glucuronidase protein. A distinct analogy can also be drawn between the effect of added protein and that of gum acacia or starch. The only similarity between two such structural extremes is the colloidal nature and high molecular weight of the compounds under study, from which it may possibly be inferred that the enzyme is protected in some manner by the presence of such additives. The protection afforded by albumin during dialysis however is not seen in the case of gum acacia or starch, which suggests that the protective effect in this case is not the same as the common activating effect of all these substances.

The most striking anomaly in the present work was the inability to reproduce the inhibition of β-glucuronidase activity by plasma or serum reported by Fishman et al (1948). The presence of an in vivo inhibitor is of considerable biological importance, and the lack of success in confirming the results of Fishman et al (1948) and the independent work of Paul (1951) is a disturbing complication. A discrepancy might be sought in the physical state of the enzyme under study or in the source of serum employed. Paul (1951) has shown that different enzyme preparations show different degrees of inhibition towards the same serum, but the present diverse experiments on straight tissue homogenates, partially purified enzyme and the highly purified product were ineffectual in demonstrating inhibition even when assayed in the presence of sera from different sources. Some indication that the inhibitor itself may be a

variable factor was provided by the fact that sera from identical human sources to those employed by Paul (1951) showed no inhibition of the enzyme at the later date of study of the present author. Fishman (1950) was also aware of several discrepancies in this field and has indicated that in about 10% of all sera investigated, activation was obtained instead of inhibition. Whether this effect was a feature of entirely different sera, or whether it was subject to variations in individual sera was not indicated however. The activating effect of added plasma or serum is readily explicable in terms of the general protein effect previously observed, and if periodic variations in inhibitor concentration in any one individual is indeed the critical factor, further work on the biological implications of this phenomenon might well prove remunerative.

Inhibition by Suramin and DNA.

Wills and Wormall (1950) have shown that many enzymes are inhibited by suramin in acid solution but not in neutral or alkaline solution, the inhibition - pH curve falling sharply at or about the isoelectric point of the enzyme. It was suggested by the above authors that suramin does not combine directly with the active centres of such enzymes, but that inactivation involves the combination with, or the association of, the sulphonic acid groups of suramin with basic groups lying near or perhaps on opposite sides of the active centre, with a resultant "bridge" formation protecting the latter.

While the isoelectric point of liver β-glucuronidase

METHOD IS SIMILAR TO THAT OBSERVED ELECTROPHORETICALLY, INHIBITION

(pH 4.6) obtained by the suramin inhibition by other polysulphonic acids does not appear to be completely independent of combination with the active centre of the enzyme. In the case of urease it was shown by Wills and Wormall (1950) that inhibition was dependent on the size of the suramin molecule, smaller molecular weight analogues of the latter showing progressively decreasing inhibitory effects, while 1 - naphthylamine - 4,6,8, trisulphonic acid was relatively ineffective as an inhibitor of this enzyme. Such observations were considered good evidence in support of the theory of masking of the active enzyme centre by bridging with the large suramin molecule. With glucuronidase however, compounds such as 2-naphthol 6, 8 disulphonic acid caused very marked inhibition of enzyme activity at low pH values; in this instance no conceivable masking of the active enzyme centre in terms of the postulate of Wills and Wormall (1950) could have occurred, and it must be inferred that polysulphonic acids of this type actually combine with the functionally active groupings of glucuronidase. If suramin were also involved in actual combination with the active centre of the enzyme a complicating factor is thus introduced into systems where basic groups are involved in the combination of enzyme with substrate; in such instances inhibition will not be solely dependent on the net charge on the molecule, and the pH of 50% inhibition may not necessarily be a true indication of the isoelectric point of the system. It is interesting in this connection to recall the work of Kendal (1949) who has suggested that mushroom tyrosinase is an enzyme or enzyme complex with independent centres of activity

associated specifically with monophenolase and catecholase function. Wills and Wormall (1952) observed two entirely different values for 50% inhibition of this enzyme by suramin depending on the substrate employed. Using catechol as substrate, 50% inhibition was apparent at pH 3.9, while the same enzyme acting on p-cresol was 50% inhibited by suramin at pH 4.4. It thus appears possible that suramin inhibition may be a measure of the isoelectric point of the functionally active grouping rather than of the whole molecule. While the isoelectric points of the fractionated glucuronidases as estimated by this method all appeared to be similar (around pH 4.6), some instances were observed in the case of unfractionated material where significantly different values were obtained, the inhibition - pH curve showing a number of breaks. This might suggest that the presence of other proteins influences the combination of suramin with the active centres of the enzyme.

An analogy has already been drawn in Section III B of the present work between the effect of suramin and that of DNA on β-glucuronidase activity. In the latter instance inhibition was also apparent over a very narrow pH range (0.3 pH units) with 50% inhibition occurring at pH 4.03. The fact that DNA shows no inhibition at pH 4.2 where the enzyme possesses a net positive charge, again suggests the specific nature of inhibition, with possible combination of the polyphosphoric acid groupings of DNA with the active centre of the enzyme. While depolymerisation of DNA as indicated by the appearance of free nucleotides and nucleosides was associated with a complete loss of inhibitory power,

the full extent of molecular degradation could not be determined, leaving little ground for speculation on the structural relationships involved in the inhibition.

If we assume that the independence of activity at the four pH optima of the enzyme is associated with different dissociation constants for each active enzymic centre, the specific inhibition by DNA at lower pH values may be related to combination with one individual active grouping. Such a postulate is necessarily highly speculative however, and precise data on the dissociation constants of the enzyme, substrate and DNA are required before such theorising can have any practical significance.

A more definite approach to the possible function of DNA as coenzyme of β-glucuronidase can be forthwith adopted, as little evidence is forthcoming from the present work to show that DNA is in any way an essential complement to glucuronidase activity. In the first instance, a determination of enzyme activity at varying dilutions provides no indication of a coenzyme dissociating from the parent molecule at high dilutions. With the highly purified liver preparations it was possible to dilute to a final protein N concentration of 0.05 µg. protein N per ml. (Figure 6), at which dilution the E/P ratio was still relatively constant. While a slight falling off in this ratio was observed in the case of spleen β-glucuronidase, this occurred at a concentration of 6 µg. protein N per ml., which is in no way comparable to the results expressed by Bernfeld and Fishman (1950b). Irrespective of such anomalies, it is difficult to visualise DNA functioning simultaneously both as an inhibitor and a coenzyme of

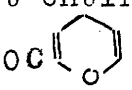
β-glucuronidase. While little doubt exists concerning the activating effect of DNA at pH 4.5 reported by Bernfeld and Fishman (1950b), the inhibitory effect of this compound at lower pH values was obviously not apparent to the above authors. Furthermore the effect of activation at pH 4.5 can be reproduced with equal facility by a variety of other agents such as albumin, gum acacia and starch, with remarkably analagous results to those obtained by DNA when the plot of E/P ratio against enzyme protein concentration is employed. The properties of a coenzyme are of necessity highly specific and unlikely to be shared by a number of such diverse compoumds. Whether activation by DNA is a non-specific effect similar to that exerted by other colloids is a debatable point, but the summation of results in no way permits the assertion that DNA functions as coenzyme of either liver or spleen β-glucuronidase.

Inhibition of β-Glucuronidase by Aromatic and Aliphatic Compounds.

In all inhibition studies on β-glucuronidase considerable care was exercised in neutralising the relevant acids with NaOH prior to assay, thus excluding the possibility that variations in enzymic activity at any one pH were due solely to alterations in pH of the buffer solution. A further check was made on the final pH of the solution in the presence of inhibitor and the results obtained may thus be considered valid reflections of the effect of specific compounds on enzymic activity.

From a consideration of the available dissociation constants of some of the compounds listed in Table 2, it would appear that

the degree of dissociation of any one acid has little relevance to the measure of inhibition at any one pH. 2:5 $(OH)_2$ benzoic acid (K_a=1.3 x 10^{-3}) and phthalic acid (primary K_a = 1.3 x 10^{-3}) show significantly different effects at the same molarities, while p-nitrophenol (K_a = 6.5 x 10^{-2}) and 2:6 $(OH)_2$ benzoic acid (K_a = 5.0 x 10^{-2}) are equally variable in this respect. Rather would it appear that inhibition is dependent on certain structural necessities within the molecule. This possibility is further enhanced by the fact that benzoic acid (K_a = 6.5 x 10^{-5}) is an activator of β-glucuronidase while the OH substituted derivatives of this acid show entirely different effects with little change in the individual K_a values. 3:5 $(OH)_2$ benzoic acid (K_a = 9.1 x 10^{-5}) and 3:4 $(OH)_2$ benzoic acid (K_a = 3.3 x 10^{-5}) cease to function as activators, and 3:4:5 $(OH)_3$ benzoic acid inhibits the enzyme at all pH values.

The introduction of an OH group in a position ortho to a COOH group in the benzene ring results in pronounced inhibition of glucuronidase activity at pH 3.4. An attempt to explain this in terms of increase in acid strength by chelation of the OH group with the adjacent COOH group is not entirely satisfactory. If such is the case, chelidonic acid (HOOC  COOH) should theoretically be a less efficient inhibitor, chelation of the two COOH groups with the intervening O in this instance resulting in depression of ionisation of the acidic groupings. Chelidonic acid has, however, considerable inhibitory action.

It is interesting in this respect to note that citric acid ($HOOC.CH_2.C(OH).COOH.CH_2.COOH$) inhibits glucuronidase at

pH 3.4 to the extent of 75% at 10^{-2}M., while tricarballylic acid (HOOC.CH_2.CH.COOH.CH_2.COOH) where the OH adjacent to the COOH group is lacking, inhibits only very feebly at this pH and molarity (Table 3), repitition of such assays invariably giving the same results. The effect may be considered anomalous in the sense that all other dicarboxylic acids, whether aliphatic or aromatic, inhibit to some extent at low pH values; indeed all compounds containing more than one acid grouping function as enzyme inhibitors at such pHs.

While the activating effect of such compounds at higher pH values is somewhat difficult to interpret, it may reasonably be inferred that the acid groups of such inhibitors enter into combination with functionally active basic groups on the enzyme molecule. The introduction of OH groups into the molecule considerably enhances the inhibitory effect of the latter with little alteration in K_a value, as already indicated in the case of benzoic and OH substituted benzoic acids. The importance of OH groups is also illustrated in the case of malonic and tartaric acids (primary K_a values 1.6×10^{-3} and 1.1×10^{-3} respectively), where the latter is the more powerful inhibitor. It might be assumed that once initial combination between enzymic basic groups and acidic groups of the inhibitor had been established, further hydrogen bonding could occur by virtue of additional positively charged basic groups of the protein accepting a pair of electrons from the oxygen of the OH group. In the case of the non-resonating structure 2,3,5,6, tetrahydroxycyclohexanecarboxylic acid, the capacity to donate

electrons is considerably reduced, which may account for the small inhibitory effect exerted by this compound.

The specific configuration of the OH groups in the inhibiting molecule is also of some importance. While mucate and saccharate are powerful inhibitors of β-glucuronidase, the latter is more efficient in this respect, and the nearer the configuration to that of glucuronic acid the greater is the inhibitory effect. Furthermore while inhibition by dicarboxylic acids per se is confined to the lower pH range, the introduction of OH constituents extends the inhibition to the four pH optima of the enzyme.

A further isolated observation can be made that basic compounds do not function as inhibitors of β-glucuronidase. A large number of such compounds was investigated none of which showed either activation or inhibition of the enzyme, implying that the functionally active groups of the latter are entirely basic in nature.

While some tentative theories may be formulated from such work, it is obvious that very much more information is required to enable one to draw definite conclusions concerning the mode of action of enzyme and inhibitors. The precise significance of features such as inhibition of activity by ortho OH substitution of acids, and activating effects concurrent with inhibition, is elusive, and will remain so until such time as a more exhaustive study can be undertaken. Comparative investigation of inhibition at varying molarities over the entire pH range together with information on whether such inhibition is reversible or irreversible, competitive or non-

competitive would provide a more sound basis for theorising, although the present work is not without interest in indicating the possible mode of action of the enzyme.

D. The Possible Nature of the β-Glucuronidases.

From the foregoing considerations it is difficult to regard the enzyme β-glucuronidase in terms of a single system of optimum pH 4.5. The complexity of the system is initially suggested by the consistent demonstration of well-defined peaks of activity at the four pH optima of the enzyme irrespective of the purity of the material, by the accentuation of enzyme activity at such optima with varying substrate concentration, and by the different activation energies apparent at such optima. Fractionation by diverse methods yields preparations of distinctly different pH optima which on summation account satisfactorily for the pH activity curve of the original material. Well-nigh complete separation of entities of pH optima 4.5 and 5.2 has been achieved, although considerable difficulty has been encountered in dissociating enzyme activity at pH 3.4 and 6.5 from contiguous activity around pH 4 - 5. Preparations overwhelmingly rich in activity at the former pH have been obtained however, but even partial separation of the latter enzyme is somewhat of a problem. Kinetic studies on the fractionated material provide very good evidence for the individual nature of enzymic activity at such pH optima, entirely different methods of fractionation giving very comparable results.

It remains to formulate an opinion on the nature of the

enzyme system involved, and two possibilities can be envisaged in this respect. Firstly that the enzyme system is a composite of closely related protein entities with very similar physical properties, or secondly that the enzyme is composed of a parent carrier molecule having independent prosthetic groupings, possibly protein in nature, constituting the functionally active centres.

Electrophoretically it is difficult to determine whether glucuronidase migrates as a single or complex protein, but reference has already been made to the work of Cohn et al (1946; 1950) who have shown that apparently homogeneous electrophoretic components when subjected to subtle fractionation techniques, resolve themselves into a number of closely related entities. While there is no a priori reason why the glucuronidases should not act in like manner, certain observations favour the theory of a carrier molecule associated with independent functional prosthetic groupings.

In the course of electrophoresis on filter paper or in the Tiselius apparatus, a radical transformation in enzyme activity from multiple peaks to a single, sharp optimum at pH 5.2 is invariably apparent. This effect is not due to instability of the enzyme at the pH of electrophoresis (pH 8.0) as there is no comparable transition to the 5.2 enzyme at this pH in the absence of applied current, and it seems not unlikely that electrostatic forces play some part in binding the prosthetic groups to the parent molecule with attendant dissociation of all but the functional 5.2 grouping when current is applied.

Extending this theory further, the inhibitory effect of

salts on β-glucuronidase activity may well be explicable in like terms. $(NH_4)_2SO_4$ and K_2SO_4 have been shown to inhibit enzyme activity specifically at pH 3.4 with no attendant alteration in activity at either pH 4.5, 5.2 or 6.5. While the degree of inhibition is proportional to the ionic concentration of each constituent, $(NH_4)_2SO_4$ is more efficient in this respect than K_2SO_4. Increases in salt concentration throughout a certain range are well known to alter surface forces in such a manner as to favour dissociation. Burk and Greenberg (1930) observed a dissociation of haemoglobin in concentrated urea solutions to a molecule of molecular weight 34,000, one half the size of the molecule in water, while ultracentrifugal studies on serum proteins (McFarlane 1935) also revealed a remarkable series of association and dissociation reactions depending on the concentration of salt present. It is conceivable that dissociation of the active 3.4 grouping could occur in this manner, with reassociation of the group on dialysis. While this necessarily implies that the prosthetic group associated with activity at this pH is more subject to dissociation than the 4.5 or 5.2 entities, the inference is reasonable in view of the extreme difficulty encountered in obtaining a completely separated enzyme of this pH optimum. The alterations in pH activity curve obtained during controlled dialysis of the 1.4 M. Celite liver fraction (Figure 25) may also imply different degrees of dissociation of the active enzyme groups from the parent molecule as the salt concentration varies.

Under the terms of such a hypothesis, successful fraction-

ation is a measure of the ability to remove the prosthetic groupings from the parent molecule, the group so removed being inactive in the absence of the carrier. The different affinity constants and activation energies of the individual enzymes implies some structural difference in the active enzyme centres, but until such time as ultracentrifugal studies are possible, the actual physical nature of the enzyme system and the full significance of phenomena such as salt effects and transition of activity under applied current must remain a matter of conjecture.

Functional Studies on β-Glucuronidase.

Reference has already been made to the work of Levvy, Kerr and Campbell (1948) citing the usefulness of β-glucuronidase as an index of biochemical growth. The initial step towards formulating this hypothesis was the observation by the above authors that the glucuronidase activity in liver, kidney and spleen of young mice was much higher than that of normal adults. The data of Mills, Smith, Stary and Leslie (1950) however, indicates no similar correlation between growth processes in the rat and β-glucuronidase activity. These authors made a study of the glucuronidase concentration in the liver of growing rats from before birth to maturity, and it was found that the β-glucuronidase concentration of this organ was low in foetal rats, with a rise to maximum around 20 - 40 days post-partum, followed by a slight decline to an adult level above that for animals at birth. Analysis of the data by means of the allometric method of Huxley (1924) indicated that the accumulation rate of β-glucuronidase was greater than that for liver tissue during the growth of the animal. Moreover by using the DNA content of liver tissue as an indication of cell number along with the allometric method of analysis, it was found that the β-glucuronidase content per cell increased up to 18 days post-partum, thereafter remaining constant, after which time the growth rate was maximal.

The original observations of Levvy et al (1948) concerning mice were nevertheless confirmed by Paul (1951), who showed that

the glucuronidase content of liver tissue of infant mice was higher than in the adult. It would thus appear that the experimental findings of Levvy et al (1948) are substantially correct for this species, but the interpretation of such findings by these authors appears to be somewhat fortuitous. Quantitative differences are known to exist in the glucuronidase content of organs not only of different species, but also of different strains (Morrow et al. 1949, 1950), and it is more probable that the difference in behaviour of liver glucuronidase during growth in rats and mice is a species difference. Inasmuch as the process of cell proliferation is common to both animals, any generalisation relating β-glucuronidase to cell proliferation cannot be accepted as valid in terms of the above experiment.

Liver tissue regenerating after partial hepatectomy is a useful basis for comparative studies, and inducement of cell division in this manner is preferable to the administration of toxic agents causing initial damage to the liver tissue, although variations in liver constituents associated with a general derangement of the metabolic pattern of the animal are also operative in this instance. A comprehensive chemical analysis of the tissue is probably a more critical assessment of regeneration than the purely histological techniques employed by Levvy et al (1948), and little measure of evidence is forthcoming by such means to show that β-glucuronidase is in any way related to cellular proliferation. While little doubt exists concerning the increased β-glucuronidase activity

reported by Levvy et al (1948) for liver tissue regenerating after partial hepatectomy, the experimental findings of the present author cannot permit the conclusion that such elevations are in any way associated with growth processes in that organ. That glucuronidase activity in vivo is influenced by factors other than immediate damage to an organ is shown by the laparatomy experiments, although the full significance of such results is not obvious, particularly as the shock syndrome induced by adrenalectomy causes no alteration in the enzyme levels of kidney, liver or spleen. The profound metabolic disturbances of liver function associated with partial hepatectomy are legion however, and it would be unwise to predict that the elevated glucuronidase levels following such a radical operative procedure are reflections of the degree of mitosis occurring in that organ.

Striking changes in the liver glucuronidase activity of mice receiving subcutaneous injection of a variety of toxic agents were also observed by Levvy et al (1948), and a close parallelism was again drawn between the degree of cellular proliferation induced by such agents and the increase in β-glucuronidase activity. Assessment of the degree of regeneration in this organ was again confined to purely histological techniques, and in many instances the results quoted by the above authors barely substantiate their own hypothesis. Indeed it would appear that maximum glucuronidase activity is more readily coincident with maximum tissue damage, and the following table, adapted from the data of Levvy et al (1948) illustrates this point more specifically:

Day after administration of toxic substance showing:

Substance administered	Max. glucuronidase activity	Max. tissue damage	Max. cell division	Max. tissue repair
Carbon tetrachloride	1	1	7	7
Chloroform	1	1	1 & 8	8
Yellow phosphorus	5	2	5	10

The effects produced by a single injection of laevo-menthol (Levvy et al 1948) were equally inconclusive in this respect. After administration of this agent, liver glucuronidase activity increased greatly within 24 hours, the increase being coincident with maximum liver damage. No cell proliferation was perceptible at this time, and was not reported as such until the third day after administration of the toxic agent. While glucuronidase activity was still considerably higher than normal at three days, the point most worthy of comment is that such values were initially raised before cell proliferation had commenced - a very critical point in constructing any relationship between glucuronidase activity and mitotic processes.

A very important feature of the present work is the rise in liver β-glucuronidase activity observed after the subcutaneous administration of arachis oil to rats. Why this effect should not be apparent in mice (Levvy et al 1948) is a provocative point.

The latter authors administered arachis oil intraperitoneally in control studies, but when injecting the toxic agent in arachis oil, subcutaneous administration was chosen. This implies either that the mode of administration of the "non-toxic" agent can materially affect the tissue response of the animal, or that a species difference in response to such agents is operative. If the former is the controlling factor, the control experiments of Levvy et al (1948) are sufficiently inadequate to invalidate any conclusions drawn. The fact remains that in rats, injection of arachis oil alone produces profound and unquestionable increases in liver glucuronidase activity associated with no histological abmormality of the liver, and the results obtained by the present author suggest that damage to the liver following injection of CCl_4 in arachis oil is attended by an actual loss in glucuronidase from the liver, such losses being masked by an overall increase in glucuronidase activity related to the injection of arachis oil; such increases most probably have their origin in a general tissue response to foreign agents, emphasising the degree of caution which must be exercised in the interpretation of all in vivo studies.

While the experiments of the present author in this respect contribute little towards determining the functional significance of β-glucuronidase in vivo, they serve to illustrate the dangers inherent in proposing a too facile correlation between isolated observations. Much more evidence indeed is required to substantiate the hypothesis that β-glucuronidase activity is a measure of the degree of cell proliferation in a tissue - evidence which

is not forthcoming from the present work.

Such criticisms apply even more forcibly to the claim that β-glucuronidase activity is greater in tumour tissues than in unaffected tissues, and the fact that abnormally high glucuronidase values have been proposed as a reliable diagnostic aid in the detection of malignant neoplasms (Fishman, Kasdon and Homburger 1950) is a matter of considerable concern. From a superficial investigation of the relevant literature it would indeed appear that malignant tumours are characterised by increased β-glucuronidase values relative to the normal tissue, but a very serious criticism must be made of the type of control tissue used in the majority of investigations. This fundamental point has been adequately stressed by Paul (1951), and the following quotation from his work summarises the situation admirably: "..... in a case of metastatic ovarian carcinoma (Fishman, Anlyan and Gordon 1947) the control tissue consisted of uninvolved rectal mucosa and uninvolved vaginal mucosa, there being 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 use of unrelated control tissues 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, particularly muscle, fibrous tissue and blood cells, have a lower glucuronidase activity than those of ectodermal or entodermal origin. 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....."

The rather perfunctory assessment of malignant growth in terms of increased β-glucuronidase activity is further reflected in certain observations of Fishman and Bigelow (1950). In a large number of gastro-intestinal lymph nodes examined it was found that elevated β-glucuronidase values were not accompanied by evidence of malignant disease, while secretions of apparently uninvolved gastric mucosa frequently gave high enzyme titres. Such anomalies were dismissed with the somewhat facile assertion that these high values were characteristic of a pre-cancerous state.

It is of relatable interest to note that while the incidence of spontaneous hepatomas in C_3H mice is greater than in other strains of this species (Andervont and McEleney 1941; Edwards and Dalton 1942), Morrow, Greenspan and Carroll (1949) have reported much lower β-glucuronidase activities (7 fold) in the livers of such mice relative to other strains, nor was any close correlation found by the above authors between β-glucuronidase activity and the incidence of spontaneous mammary tumours.

Odell, Burt and Bethea (1949) have further claimed that abnormally high β-glucuronidase activity in vaginal fluid is a reliable diagnostic acid in the detection of carcinoma of the uterine cervix. Whether any prognostic significance can be attached thereto is doubtful however, as little or no account was taken of the possible sources from which the enzyme could be derived. Such sources include the vaginal mucosa, uterine cervix, ovary and fallopian tubes, benign inflammations, blood, lymph and tissue fluid, and bacterial flora. While the last factor appears to contribute little towards the overall glucuronidase activity of vaginal fluid (Kasdon et al (1951)), it is by no means a matter of certainty that abnormally high glucuronidase values in such fluid are dependent on secretions from malignant cells of the neoplastic cervix. The fact that 34% of 500 non-pregnant, healthy women exhibited high enzyme values commensurate with the levels obtained in genital malignancy (Fishman, Kasdon and Homburger 1950 c) indicates that factors other than malignancy are implicated in the explanation of vaginal fluid activity.

In a study of the serum glucuronidase and esterase activities of breast cancer patients on oestrogen therapy, it was shown by Cohen and Huseby (1951 b) that non-treated breast cancer patients showed a just significantly higher level of β-glucuronidase as compared with that of healthy women. Following endocrine therapy, such values were shown to increase with an attendant decrease in serum esterase levels; reduced esterase levels also accompanied increased β-glucuronidase in mammary tumours in mice. While no obvious correlation was apparent to the above authors between processes of malignancy and serum β-glucuronidase levels during oestrogen therapy, the inverse relationship between β-glucuronidase and esterase activities under such circumstances provoked the tenuous and unwarranted conclusion that esterase serves as a precursor for the more specific enzyme β-glucuronidase, or alternatively that some tissue involved in the production of esterase may preferentially produce the more specific enzymes.

As has already been stressed, few tissues lend themselves so admirably to a study of neoplastic growth as do the liver and hepatomas, and control tissue of a comparable cell type is more readily obtained for this type of tumour than from any other. The question arises whether such chemically induced growths are comparable to those which arise spontaneously at the same site. Many workers have fallen back upon such vague terms as "non-specific irritation" or "injury" to describe the mechanism of carcinogenesis, but to describe a reaction as non-specific is a contradiction in terms and has no scientific meaning. The mere fact that not all chemicals, but only a few, will produce cancer, is sufficient to

establish the carcinogenic process as being due to relatively unique chemical reactions. It has been asserted by Greenstein (1947) that no matter how a tumour starts, it ends by possessing properties in common with other tumours, and experimentally induced neoplastic growths themselves are nearly indistinguishable from those which arise spontaneously at the same site. High β-glucuronidase values are professed to be a characteristic of malignant processes, and it may well be argued that chemically induced rat hepatoma are not necessarily confined to this category. To draw a dividing line between the two extremes is no easy matter; benignancy and malignancy have clinical connotations and are largely based as much on clinical experience as on histological criteria, and the fact that DAB induced tumours are invariably fatal to the animal encourages classification under the general term of malignancy.

The present work on chemically induced rat hepatoma definitely indicates no correlation between β-glucuronidase activity and such neoplastic processes. The glucuronidase activity of the hepatomas was unquestionably and significantly lower than that of the control livers, while the lowering of the glucuronidase/DNA ratio suggests that the enzyme activity per cell is less in the tumour than in the normal liver. Such results render dubious the assertion that increased glucuronidase values are characteristic of neoplastic growths, while the correlation of data from the four homologous tissues - normal resting liver, hepatoma, foetal liver and liver regenerating after partial hepatectomy likewise provide no evidence that β-glucuronidase is in any way

a reflection of the degree of mitosis occurring in that tissue.

While the biological studies of the present author provide little scope for formulating a hypothesis on the function of β-glucuronidase *in vivo*, they at least serve to emphasise the extreme caution which should be applied to all such functional studies. The manner in which an experiment is controlled determines the whole pattern of that experiment, and no matter how logical the reasoning, the resultant deductions are worthless if insufficient account is taken of all operative factors. Such factors are very often obscure and it is for this reason that functional studies on the living animal should be treated with the maximum of reserve.

While the significance of β-glucuronidase *in vivo* is still problematical, the most recent work of Meyer, Linker and Rapport (1951) provides the first definite and constructive piece of experimental evidence towards solution of the problem. These authors have shown that the end product of the hydrolysis of hyaluronate by purified testicular hyaluronidases is a mixture of di- and tetrasaccharides. Meyer (1951) has further shown that when such end products are acted on by a mixture of β-glucuronidase and β-glucosaminidase, glucuronic acid and N - acetylglucosamine are liberated. On hydrolysis of the oligosaccharide end products with purified β-glucuronidase free from β-glucosaminidase however, only glucuronic acid appears with a trisaccharide composed of two equivalents of N - acetyl-glucosamine and one equivalent of uronic acid. It was thus concluded that the substrate of the β-glucuronidase is a

tetrasaccharide with N - acetylglucosamine as the reducing group and glucuronic acid on the other end. This tetrasaccharide appears to be the main material which appears after exhaustive hydrolysis with purified testicular hyaluronidase. It would thus appear that β-glucuronidase may be intimately involved in the metabolism of mucopolysaccharides of connective tissue. The wide distribution of β-glucuronidase throughout the body tissues certainly infers that the enzyme is involved in a function more general than specific "detoxication" reactions, and further studies on mucopolysaccharide degradation and synthesis in relation to β-glucuronidase may well provide the answer to the problem of 40 years standing.

Bacterial Glucuronidase.

The glucuronidase of Esch. coli is an enzyme physically distinct from the complex system operative in animal tissues, and kinetic studies indicate that the former enzyme is simple in nature with none of the reaction characteristics of the animal glucuronidases. The importance of the enzyme to the bacterial cell depends greatly on whether the snzyme is intrinsically "adaptive" or "constitutive". To determine this one must deal with substrates which the cell cannot synthesise, or one must be able to prevent the appearance of some substrate normally formed by the cell. Inasmuch as bacterial glucuronidase hydrolyses both menthylglucuronide and phenolphthalein glucuronide with equal facility, there is no reason to preclude the supposition that it may also be involved in oligosaccharide degradation; it is interesting in this connection to note however, that Meyer (1951) has shown that crude pneumococcal, staphylococcal and streptococcal hyaluronidase contain no β-glucuronidase, although β-glucosaminidase is present. If β-glucuronidase is purely "adaptive" in the sense that it is produced as a specific response to the presence of menthylglucuronide, the subsequent hydrolysis of the substrate may be a means of providing the organism with a source of readily available carbon. Indeed in one experiment it was observed that 50% of the total glucuronic acid in the culture medium had disappeared over a period of two days growth, but whether the organism had actually metabolised the glucuronic acid enzymically liberated is difficult to say.

The theory that increased β-glucuronidase activity is an intimate reflection of growth processes again recurs with the somewhat naive declaration of Green, Burt, Hesseltine and Odell (1951) that "the association between the initial increase in the number of bacteria and the liberation of enzyme ally this enzyme with the processes of growth". It is perhaps unfortunate that such deductions should have been made from growth studies in the presence of the adaptive substrate. In the majority of such instances bacterial cultures do not adapt unless active division is occurring, and relatively few instances are known of enzymic adaptation with "resting" suspensions. From a kinetic point of view it is generally assumed that the enzyme and its precursor are essentially in equilibrium with the position far over on the side of the precursor. The substrate, it is presumed, by combining with the enzyme, shifts the reaction towards further enzyme formation. This hypothesis makes possible a rather definite prediction with respect to the kinetics of the appearance of enzyme in the presence of substrate. The time - activity curve should be one essentially exponential in character and concave to the time axis. The use of growing cultures in enzymic adaption experiments however, complicates this kinetic relationship. It is difficult under such circumstances to dissociate the growth of a new cell from the appearance of enzyme activity, and the time - activity curves are <u>invariably</u> determined by the growth characteristics. It is hardly surprising therefore that β-glucuronidase should be allied with growth processes in bacteria - a phenomenon which is shared by innumerable enzymes

under similar circumstances, and by no means singular to β-glucuronidase.

SUMMARY.

1). A method has been devised for the purification of ox liver β-glucuronidase utilising the interactions of proteins with metallic ions and organic solvents. An 800 fold purification has been achieved by such means with a 5% recovery of the enzyme.

2). The properties of the purified material indicate that β-glucuronidase is a complex enzyme system with at least three and possibly four independent centres of activity.

3). Virtually complete separation of two of the liver glucuronidase fractions with pH optima at pH 4.5 and 5.2 has been achieved by means of organic solvent fractionation, while preparations overwhelmingly rich in activity at pH 3.4 can also be obtained by such means.

4). Kinetic studies on the fractionated material emphasise the independent nature of the separated entities. Significantly different values for the energies of activation and enzyme substrate dissociation constants have been obtained for such fractions from both liver and spleen, and their behaviour towards various inhibitors also show marked differences.

5). A comparative study of the fractionated liver and spleen glucuronidases indicates that the liver glucuronidases are catalytically more efficient than those of the spleen.

6). No evidence can be adduced to show that deoxyribonucleic acid functions as the coenzyme of either liver or spleen

β-glucuronidase as has previously been suggested.

7). A study of the relationship between liver glucuronidase activity and tissue damage and growth has shown no direct correlation between these factors as has been postulated by earlier workers.

8). Studies on chemically induced rat hepatoma indicate that the glucuronidase activity is lower in hepatoma than in normal liver and this in no way permits the conclusion that increased β-glucuronidase activities are characteristic of malignant neoplasms.

9). The glucuronide decomposing enzyme of Esch. coli has been identified as a β-glucuronidase, and a study has been made of a few of the reaction characteristics of this enzyme.

10). The β-glucuronidase of Esch. coli is physically distinct from the complex enzyme system operative in animal tissues.

11). These results have been discussed in relation to previous work in this field.

REFERENCES.

1. Allen R.J.L., (1940), Biochem.J.,34, 858.
2. Andervont H.B. & McEleney W.J.,(1941),J.Natl.Canc.Inst.,1.737.
3. Barker M.,Brooksbank B.W.L. & Haslewood G.A.D.,(1948),Nature, Lond.,162, 701.
4. Bass R.,(1911), Zeit.f.Exper.Path.u.Ther.,10, 120
5. Bergmann F.,(1933),Biochem.Z.,267, 296.
6. Bernfeld P. & Fishman W.H.,(1950a),Archives Biochem.,27, 475.
7. - do - (1950b),Science, 112, 653.
8. - do - (1950c),Fed.Proc., 9, 150.
9. Bucher N.L.R. & Geschickter C.F.,(1940),Endocrinology,27, 727.
10. Buehler H.J.,Katzman P.A. & Doisy E.A.,(1949a),Proc.Soc.exp. Biol.,N.Y.,72, 297.
11. - do - (1949b),Fed.Proc.,8, 189.
12. - do - (1951),Proc.Soc.exp.Biol., N.Y. 76, 672
13. Burk N.F. & Greenberg D.M.,(1930),J.biol.Chem.,87, 197.
14. Campbell J.G.,(1949),Brit.J.exp.Path.,30, 548.
15. Channon H.J.,Mills G.T. & Platt A.P.,(1943),Biochem.J.,37, 483.
16. Channon H.J. & Wilkinson H.,(1935), Biochem.J.,29, 350.
17. Cohen S.L. & Huseby R.A.,(1951a), Cancer Res.,II, 52.
18. - do - (1951b), Proc.Soc.exp.Biol.,N.Y.,76, 304.
19. Cohn E.J.,(1925), Physiol.Rev.,5, 349.
20. Cohn E.J.,Gurd F.R.N.,Surgenor D.M.,Barnes B.A.,Brown R.K., Derouaux R.K.,Gillespie J.M.,Kahnt F.W.,Liu C.H.,Mittleman D., Mouton R.F.,Schmidt K. & Uroma E.,(1950), J.Amer.Chem.Soc., 72, 465.
21. Cohn E.J.,Strong L.E.,Hughes W.L.,Mulford D.J.,Ashworth J.N., Melin M. & Taylor H.L.,(1946), J.Amer.Chem.Soc.,68, 459.

22. Cremer M. & Seuffert R.,(1912),Khem.Ber.,45,2565.

23. Cremer H.D. & Tiselius A.,(1950), Biochem.Z.,320, 273.

24. Davidson J.N. & Leslie I.,(1950a), Nature,Lond.,165, 49.

25. - do - (1950b), Cancer Res.,10,587.

26. Durrum E.L.,(1950),J.Amer.Chem.Soc.,72, 2943.

27. Edsall J.T.,(1947), Advances in Protein Chemistry, 3, 383.

28. Edwards J.E. & Dalton A.J.,(1942),J.Natl.Canc.Inst.,3, 19.

29. Falconer J.S. & Taylor D.B.,(1946), Biochem.J.,40, 835.

30. Fishman W.H.,(1939a), J.biol.Chem., 127, 367.

31. - do - (1939b), J.biol.Chem., 131, 225.

32. - do - (1940), J.biol.Chem., 136, 229.

33. - do - (1947a), J.biol.Chem., 169, 7.

34. - do - (1947b), Science, 105, 646.

35. - do - (1950), Private communication to Dr. Mills.

36. - do - (1951), Bulletin New England Medical Centre,13, 12.

37. Fishman & Anlyan A.J., (1947a), Science, 106, 66.

38. - do - (1947b), J.biol.Chem.,169, 449.

39. Fishman W.H.,Anlyan A.J. & Gordon E.,(1947), Cancer Res.,7, 808.

40. Fishman W.H.,Altman K.I. & Springer B.,(1948),Fed.Proc.,7, 154

41. Fishman W.H. & Bigelow R.,(1950),J.Natl.Canc.Inst.,10, 1115.

42. Fishman W.H. & Fishman L.W.,(1944), J.biol.Chem.,152, 487.

43. Fishman W.H.,Kasdon S.C. & Homburger F.,(1950a),J.Amer.Med. Assoc. 142, 125.

44. - do - (1950b),J.Amer.Med. Assoc. 143, 350.

45. - do - (1950c), Cancer Res., 10, 216.

46. Fishman W.H.,Springer B & Brunetti R.,(1948),J.biol.Chem.,173,449.

47. Flynn F.V. & de Mayo P.,(1951), Lancet, 2, 235.

48. Folin O. & Ciocalteau V.,(1927),J.biol.Chem., 73, 627

49. Folley S.J. & Kay H.D.,(1935), Biochem.J., 29, 1837.

50. Garton G.A.,Robinson D. & Williams R.T.,(1949),Biochem.J.,45,65.

51. Gilbert G.A. & Swallow A.J.,(1950), Biochem.J., 47, 506.

52. Graham A.F., (1946), Biochem.J., 40, 603.

53. Green L.W.,Burt J.C.,Hesseltine H.C. & Odell L.D., (1951), Amer.J.Obstet.Gynae. 61, 446.

54. Greenstein J.P., (1947), Biochemistry of Cancer, New York, Academic Press.

55. Griffin A.C.,Nye W.N.,Noda L. & Luck J.M.,(1948),J.biol. Chem., 176, 1225.

56. Hämäläinen J.,(1909),Skand.Arch.Physiol., 23, 86, 97, 297.

57. Hanson S.W.F.,Mills G.T. & Williams R.T.,(1944),Biochem.J., 38, 274.

58. Helferich B. & Sparmberg G.,(1933), Zeit.f.Physiol.Chem., 221, 92.

59. Higgins G.M. & Anderson R.M.,(1931),Arch.Path, 12, 186.

60. Hildebrandt H.,(1905), Beitr.chem.Physiol.Path.,7, 438.

61. Hofmann E.,(1935), Biochem.Z., 281, 438.

62. Houet R.,Duchateau G. & Florkin M.,(1941),Comptes Rend.de la Soc.de Biol., 135, 412.

63. Huxley J.S.,(1924), Nature, Lond.,114, 895.

64. Ishidate M.,(1929), J.Pharm.Soc.,Japan, 49, 336.

65. Karunairatnam M.C.,Kerr L.M.H. & Levvy G.A.,(1949), Biochem. J., 45, 496.

66. Karunairatnam M.C. & Levvy G.A.,(1949),Biochem.J., 44, 599.

67. - do - (1951),Biochem.J., 49, 210.

68. Kasdon S.C.,Fishman W.H. & Homburger F.,(1950), J.Amer.Med. Assoc., 144, 892.

69. Kasdon S.C.,McGowan J.,Fishman W.H. & Homburger F.,(1951) Amer.J.Obstet.Gynaec.,61, 647.

70. Kendal L.P.,(1949), Biochem.J.,44, 442.

71. Kerr L.M.H.,Campbell J.G. & Levvy G.A.,(1949),Biochem.J.,44,487.

72. Kerr L.M.H.,Graham A.F. & Levvy G.A.,(1948),Biochem.J., 42, 191.

73. Kerr L.M.H. & Levvy G.A.,(1947), Nature, Lond.,160, 572.

74. - do - (1951), Biochem.J.,48, 209.

75. Kerr L.M.H.,Levvy G.A. & Campbell J.G.,(1947),Nature,Lond., 160, 572.

76. King E.J. & Armstrong A.R.,(1934), Canad.med.Assoc.J., 31, 376.

77. Levvy G.A.,(1946), Biochem.J., 40, 396.

78. - do -(1948), Biochem.J., 42, 2.

79. Levvy G.A.,Kerr L.M.H. & Campbell J.G.,(1948), Biochem.J., 42, 462.

80. Lineweaver H. & Burk D.,(1934),J.Amer.Chem.Soc., 56, 658.

81. Lipschitz W.L. & Bueding E.,(1939), J.biol.Chem., 129, 333.

82. Lohmar R.,Dimler R.J.,Moore S. & Link K.P.,(1942), J.biol. Chem., 143, 551.

83. Lorinez M.D.,Novelli J.,McGoogan M.D. & Odell L.D., (1951), Amer.J.Obstet.Gynaec., 61, 527

84. Ma T.S. & Zuazaga G.,(1942), Industr.Eng.Chem.,14, 280.

85. Markham R.,(1942),Biochem.J.,36, 790.

86. Marrian G.F.,(1934), Biochem.J.,28, 1603.

87. Masamune H.,(1933), J.Biochem.,Japan, 18, 259.

88. - do - (1934), J.Biochem.,Japan, 19, 353.

89. McFarlane A.S.,(1935), Biochem.J.,29, 407, 660, 1175.

90. de Meio R.H. & Arnolt R.I.,(1944), J.biol.Chem., 156, 577.

91. Meyer K.,(1951), Private communication to Dr. Mills.

92. Meyer K.,Linker A. & Rapport M.M.(1951), J.biol.Chem.,192, 275.

93. Michaelis L.,(1931), Biochem.Z., 234, 139.

94. Michaelis L. & Menten M.L.,(1913), Biochem.Z., 49, 1333.

95. Miller B.F. & Van Slyke D.D.,(1936), J.biol.Chem., 114, 583.

96. Mills G.T.,(1946), Biochem.J., 40, 283.

97. - do - (1948), Biochem.J., 43, 125.

98. Mills G.T.,Paul J. & Smith E.E.B.,(1949),Abstr.1st.Intern. Congr.Biochem., 597.

99. - do - (1952a),Biochem.J.,in press.

100. - do - (1952b),Biochem.J.,in press.

101. Mills G.T. & Smith E.E.B.,(1951),Biochem.J.,49, Proc. vi.

102. Mills G.T.,Smith E.E.B.,Stary B. & Leslie I.,(1950),Biochem.J., 47,Proc. xlviii.

103. Mirsky A.E. & Pollister A.W.,(1946),J.Gen.Physiol., 30, 117.

104. Mitsuba K.,(1927), Z.Physiol.Chem.,164, 236.

105. Miwa T.,(1935), Acta Phyta Chemica, 8, 231.

106. -do- (1936), Acta Phyta Chemica, 9, 89.

107. Morrow A.G.,Carroll D.M. & Greenspan E.M.,(1951),J.Natl.Cancer Inst., II, 663.

108. Morrow A.G.,Greenspan E.M. & Carroll D.M.,(1949),J.Natl.Cancer Inst., 10, 657.

109. - do - (1950),J.Natl.Cancer Inst., 10,1199.

110. Neuberg C. & Niemann W.,(1905),Z.Physiol.Chem.,44, 114.

111. Odell L.D. & Burt J.C.,(1949), Cancer Res.,9, 362.

112. - do - (1950), J.Amer.Med.Assoc., 142, 226.

113. Odell L.D.,Burt J.C. & Bethea R.,(1949), Science, 109, 564.

114. Odell L.D.,Fishman W.H. & Hepner W.R.,(1948), Science, 108, 355.

115. Odell L.D. & McDonald D.F.,(1947),J.clin.Endocrinol. 7, 535.

116. - do - (1948),Amer.J.Obstet.Gynaec.,56, 74.

117. Odell L.D.,Priddle M.D. & Burt J.C.,(1950),Amer.J.Clin. Path., 20, 133.

118. Oshima G.,(1934),J.Biochem.,Japan, 20, 361.

119. -do- (1936),J.Biochem.,Japan, 23, 305.

120. Patridge S.M. & Westall R.G.,(1948),Biochem.J., 42, 238.

121. Patterson J.,(1937),Brit.Med.J., 2, 522.

122. Paul J.,(1951), Ph.D. Thesis, University of Glasgow.

123. Potter V.R. & Elvehjem C.A., (1936),J.biol.Chem., 114, 495.

124. Putnam F.W.,(1948), Advances in Protein Chemistry, 4, 79.

125. Quick A.J.,(1928), J.biol.Chem., 80, 515, 535.

126. Röhmann F.,(1908), Biochemie, Springer, Berlin.

127. Rossiter R.J. & Wong E., (1950), Canad.J.Res., 28, 69.

128. Sarkar N.K. & Sumner J.B.,(1950), Archives Biochem. 27, 453.

129. Schmidt G. & Thannhauser S.J.,(1945), J.biol.Chem.,161, 83.

130. Sera Y.,(1913), Z.Physiol.Chem., 88, 460.

131. -do- (1914a),Z.Physiol.Chem., 90, 258.

132. -do- (1914b),Z.Physiol.Chem., 92, 261.

133. Shibata K.,(1933), Acta Phytochim, Japan, 1, 105.

134. -do- (1930), Acta Phytochim, Japan, 5, 117.

135. Sizer I.W.,(1937), J.Cell.comp.Physiol.,10, 61.

136. -do- (1943), Advances in Enzymology, 3, 35.

137. Spencer B. & Williams R.T.,(1951),Biochem.J., 48, 537.

138. Svensson H.,(1939), Kolloidzschr., 87, 181.

139. Talalay P.,Fishman W.H. & Huggins C.,(1946),J.biol.Chem.,166,757.

140. Tiselius A.,(1937), Trans.Faraday Soc., 33, 524.

141. Turba F. & Enenkel H.J., (1950), Naturwissenschaften, 37, 93.

142. Venning E.H., (1938), J.biol.Chem., 126, 595.

143. Walker P.G. & Levvy G.A.,(1951), 49, 620.

144. Williams R.T.,(1947), Detoxication Mechanisms, Chapman & Hall Ltd., London.

145. Wills E.D. & Wormall A.,(1950), Biochem.J., 47, 158.

146. - do - (1952), Biochem.J., 50, 421.

DEPARTMENT OF BIOCHEMISTRY
University of Oxford
South Parks Road
Oxford

With the Compliments of
the Whitley Professor

R. A. Peters.