THE PHYSIOLOGICAL SIGNIFICANCE OF CHANGES IN β-GLUCURONIDASE ACTIVITY.

by

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INDEX.

INTRODUCTION.

β-Glucuronidase, an enzyme present in mammalian tissue, was discovered at the beginning of this century. It is only recently that it has been extensively examined and even yet its function in the body is not known. β-d-Glucuronides, formed in the body by conjugation of alcohols and phenols with glucuronic acid, are hydrolysed in vitro by glucuronidase. It therefore seems likely that glucuronidase must play some part in the metabolism of glucuronides *in vivo*.

In 1908 F.Roehmann found that a chloroform-water extract of dog liver was capable of destroying menthol β-d-glucuronide. This observation was extended to other glucuronides by Sera(1915) who used other animals as well as the dog and other organs besides liver. Both workers simply measured changes in the rotation of the glucuronides and they were therefore not able to show the nature of the reaction.

It was not until some twenty years later that two Japanese workers demonstrated the presence in tissue extracts of an enzyme system catalysing the hydrolysis of glucuronides. In 1934 Masamune found that β-glucuronides were hydrolysed, presumably at the oside link, by an alcohol-water extract

of ox-kidney. The enzyme," β-glucuronidase"or " β -glucuronosidaes",was found to be highly specific with respect to the hydrolysis of β -d-glucuronides. All β -d-glucuronides tested were found to be hydrolysed by the enzyme. α -d-Glucuronides and α - or β -glucosides were unaffected, ~~by the enzyme~~ with the exception of phenol β -d-glucoside. Later workers have,however,found that phenol β -d-glucoside is unaffected by pure preparations of glucuronidase (Graham,1946). Oshima (1934,1936) characterised the enzyme further and studied methods of extraction and purification. He also examined the kinetics of hydrolysis of several bio-synthetic glucuronides and the distribution of the enzyme in the tissues of the dog and the ox. β -Glucuronidase was found to be widely distributed throughout the body, the highest concentration being in spleen and liver.

Masamune prepared the enzyme by incubating minced tissue with saline for three days. This was followed by extraction with alcohol and water. The solution thus obtained was very impure and low in activity. Oshima (1936) obtained a slightly purer preparation by adsorbing the enzyme on kaolim and eluting with sodium phosphate. In 1939 Fishman described a method involving ammonium sulphate precipitation of a partially purified mince,giving over 100-fold purification of the enzyme. Although he obtained a concentrated enzyme solution he recovered only 20% of the original activity in the

tissue extract. Graham (1946),after removal of inactive protein from an acetone washed mince,also used ammonium sulphate fractionation,to obtain a pure and concentrated enzyme solution. He pointed out the pH of the solution affected the separation of active from inactive protein and that preliminary incubation of the mince caused an increase in the enzyme activity. This improved method retained about 80% of the original activity in the tissue extract.

In nearly all the early work on glucuronidase the activity of the enzyme was estimated by measuring,by means of its reducing power for ferricyanide,glucuronic acid liberated from menthol-glucuronide. Levvy (1946) has shown that while very small quantities of glucuronic acid can be detected in this way the reaction is not specific enough for reliability in the enzyme assay. During incubation of the enzyme itself there is an increase in reducing power,as was pointed out by Graham (1946). Levvy (1946) has shown that this is not due to hydrolysis of endogenous glucuronide. He has also shown (Levvy, 1948) that the action of the enzyme is,in fact, confined to the rupture of the oside link,as had been assumed previously. Fishman (1939),in kinetic studies estimated oestriol,by means of the Kober colour reaction,after hydrolysis of oestriol-glucuronide. While this method avoids the errors inherent in the reducing method,the scarcity of oestriol -glucuronide

renders it unsuitable for routine estimations. Using phenolphthalein-glucuronide as substrate, Talalay et al.(1946) were able to measure the aglycone liberated colorimetrically. As this method is specific and the substrate fairly easily prepared, it is to be preferred to any of the earlier methods of estimation. Prior to the appearance of the work of Talalay et al. a similar method, using phenol-glucuronide, was developed in Edinburgh as described below.

It was assumed by the early workers that, in vivo, β-glucuronidase catalysed the condensation of glucuronic acid with hydroxy compounds to form β-d-glucuronides. Formation of conjugated glucuronides is known to occur in many animals and in man, but the enzyme system involved in the process has not yet been identified. Florkin, Crismer and Duchateau (1942) claimed to have observed conjugation when borneol in saturated solution was incubated for several days with glucuronic acid in high concentration in the presence of beef spleen glucuronidase. Conjugated glucuronic acid was estimated by the Tollens reaction after the removal of free glucuronic acid by copper lime. Only a very small amount of conjugated acid was detected and these authors conclude that such a process could hardly be responsible for the bio-synthesis of glucuronides. The work of Lipschitz and Bueding (1939) on glucuronide synthesis by surviving liver slices suggested that glucuronidase was not involved since the process

was stimulated by certain C3 compounds, but not by free glucuronic acid. De Meio and Arnolt, also using surviving slices, found that glucuronic acid could reverse the inhibition of phenol conjugation by iodoacetic acid and that feeding borneol or phenol to rats increased in vitro conjugation by liver and kidney. They consider that glucuronides are formed by direct condensation of glucuronic acid with the aglycone. Conjugation was estimated by measuring the phenol liberated by acid hydrolysis from the conjugates. Phenol-glucuronide, however, is not hydrolysed under their conditions, so it may be assumed that whatever they were measuring it was not conjugation with glucuronic acid. (Levvy and Storey, private communication).

The first in vivo experiments with β-glucuronidase were carried out in 1940 by Fishman who considered the enzyme to be responsible for glucuronide synthesis. He believed that he could measure, by means of an in vitro hydrolysis, the adaptation in the body of β-glucuronidase to excess substrate, in the form of glucuronidogenic compounds. Following repeated feeding of menthol to mice he obtained an increase in glucuronidase activity in liver, kidney and spleen compared with organs from normal animals. Similar were obtained in the organs of a few dogs fed with borneol. Glucuronidase activity in uterus and other sex organs showed no change in either species.

Oestrogens were,however,found to cause an increase in activity in the uterus of ovariectomised mice, but not in liver,kidney or spleen. Fishman and Fishman (1944) then postulated a specificity of the enzyme in its synthetic action,according to its location,for different groups of substrates. No such specificity was,however,observed in its hydrolytic action in vitro,menthol-glucuronide being used in the assay of uterine glucuronidase under conditions found optimal for hydrolysis by spleen preparations. There were many other inconsistencies in the theory. In these experiments Fishman determined the activity of the enzyme by cerimetric estimation of glucuronic acid,a method open to many sourses of error,as shown below.

The present work was started with a view to elucidating more fully the function of β-glucuronidase in the body. It was found possible to adapt the method of King and Armstrong (1934) for phosphatase determinations to the assay of β-glucuronidase using bio-synthetic phenol-glucuronide (Williams, private communication)as substrate. The developement of this method and its application to routine assays is described.

For routine estimations a simply prepared enzyme was required which reflected as accurately as possible the activity of the enzyme in the tissue. This was obtained by using a slight modification of the method of Talalay et al.(1946),no loss in activity

being noted during the purification of the enzyme. The kinetics of hydrolysis by the enzyme prepared in this way from mouse liver, kidney, spleen and uterus were fully studied using phenol-glucuronide as substrate.

An attempt was made to repeat Fishman's (1940) findings following the administration of menthol to mice. The effects of menthol and other agents, some definitely nonglucuronidogenic, on the β-glucuronidase activity of mouse liver, kidney, and spleen ~~and uterus~~ are reported. A comparative study was also made of the action of steroid hormones and other agents on glucuronidase in uterus and other organs. From the results obtained it was possible to deduce a close correlation between the glucuronidase activity and the degree of cell proliferation in a tissue. Some interesting observations, incidental to the main work, were made regarding the actions of oestrogens.

The report is divided into the following sections;-

A. Preparation of the enzyme, the method of estimation and the kinetics of hydrolysis of phenol-glucuronide.

B. The effects of various agents on β-glucuronidase activity in liver, kidney and spleen.

C. The effects of various agents on β-glucuronidase activity in uterus and other organs.

D. The effect of various growth inhibitors on β-glucuronidase activity.

E. The β-glucuronidase activity of several tissues from various species.

SECTION A.

Preparation of the enzyme, the method of estimation and the kinetics of hydrolysis of phenol-glucuronide.

Preparation and Assay of β-Glucuronidase.

The method of King and Armstrong (1934) for phosphatase determination was adapted to the estimation of free phenol liberated during the hydrolysis of bio-synthetic phenol-glucuronide by β-glucuronidase. Preparation of phenol-β-d-glucuronide. Dr.Williams kindly supplied details of the technique for obtaining this compound from rabbit urine. The glucuronide was obtained from the urine of rabbits fed with phenol by the general procedure described by Williams (1943) for the isolation of the aminophenol-glucuronides. Before use,it was dried at 80' for 10hrs.over P_2O_5, in vacuo. Analysis showed it to be free from solvent of crystallisation. (One sample:-theoretical C-52.9%,H-5.9%;found C-52.7%,H-5.6%.M.P.162'corr.)

As bio-synthetic methods of preparation were tedious it was decided to investigate the possibility of oxidising phenol-glucoside to the glucuronide with N_2O_4 . Maurer and Drefahl (1942) state that,under the appropriate conditions,N_2O_4 preferentially oxidáses the primary alcoholic group in glucosides.

Phenol β-d-glucoside,(2g.),from the laboratory store,was suspended in 100ml.dry chloroform and the mixture saturated with dry N_2O_4. The whole was then shaken for 20 hrs.at room temperature. At the end of this period the chloroform was distilled off and the residual solid well washed with clean

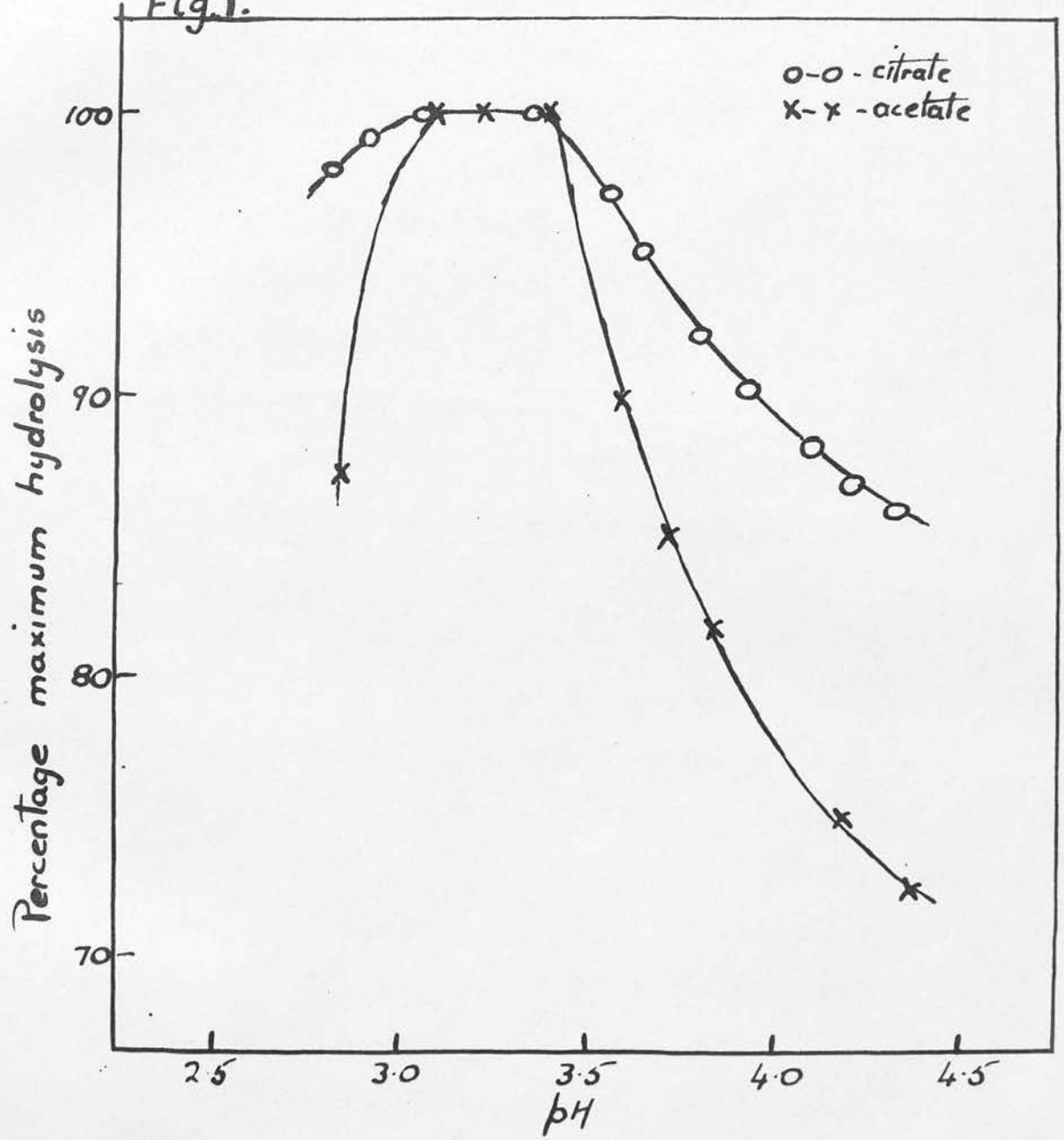
solvent. The residue was recrystallised from water and purified further, as for bio-synthetic glucuronide, with alcohol and benzene. A yield of 0.46g. was obtained of a white crystaline solid (M.P. 161° corr.) which was hydrolysed by β-glucuronidase. Repetition of this method, using phenol-glucuronide prepared from glucose by the method of Fisher (1916) and Helferich (1933) resulted in failure in a great many attempts. In one instance only was any phenol-glucuronide isolated (10 mg.). Time was not available for further investigation of the conditions for this reaction and it was decided to continue the use of rabbit urine as the source of phenol-glucuronide for assay purposes, despite obvious disadvantages.

The colour reaction for free phenol. Dr. A.F. Graham showed that free phenol could be determined, in the presence of excess phenol-glucuronide, with the reagent of Folin and Ciocalteu (1927). The colour intensity was measured with a Spekkar photoelectric absorptiometer, using Ilford No. 602 blue filters. Free glucuronic acid did not interfere in the reaction. Phenol-glucuronidein high concentration gave a faint colour, probably due to traces of free phenol (less than 0.2% of the total weight), since it varied from one sample to another.

Preparation of the enzyme. Preparations from mouse liver, kidney, spleen and uterus were studied separately in these experiments. The organs were broken up in glass homogenizers (Umbreit, Burris and Stauffer, 1945).

Unless stated to the contrary, the enzyme was partially purified following a modification of the method of Talalay et al.(1946). The homogenate was brought to pH 5.0 or 5.2 with acetate or citrate buffer. Protein which separated after 30 mins.at 37' was removed by centrifuging and the volumes of the supernatant liquid and the precipitate noted. The enzyme in the supernatant was precipitated by adding a suitable volume of saturated ammonium sulphate (S.A.S.) and the precipitate dissolved in a convenient volume of water. It was assumed that the enzyme was equally distributed between the inactive protein precipitate and the supernatant liquid. No change in the total activity of the enzyme was noted after purification in this way, when correction was made for the fraction in the inactive protein. The initial high enzyme blank was considerably reduced by this treatment. The process whereby substances giving the colour reaction on incubation of enzyme ~~alone~~ in absence of substrate was reduced to small proportions. The quantity of substances liberated in this way was found to vary with pH, as illustrated in Fig.1. Inactive protein, which coagulated at 37' and pH 5.2, was removed from the homogenate by centrifuging and the supernatant used in the next stage without further treatment. 0.4ml of this solution was incubated for 1hr.at 37' with 0.2ml.citrate or acetate buffer, ranging from pH 2.6 to 4.5, and 0.2ml. water. Protein was removed and the colour developed

Fig. 1.


o-o - citrate
x-x - acetate
Percentage maximum hydrolysis
100
90
80
70
2·5
3·0
3·5
4·0
4·5
pH

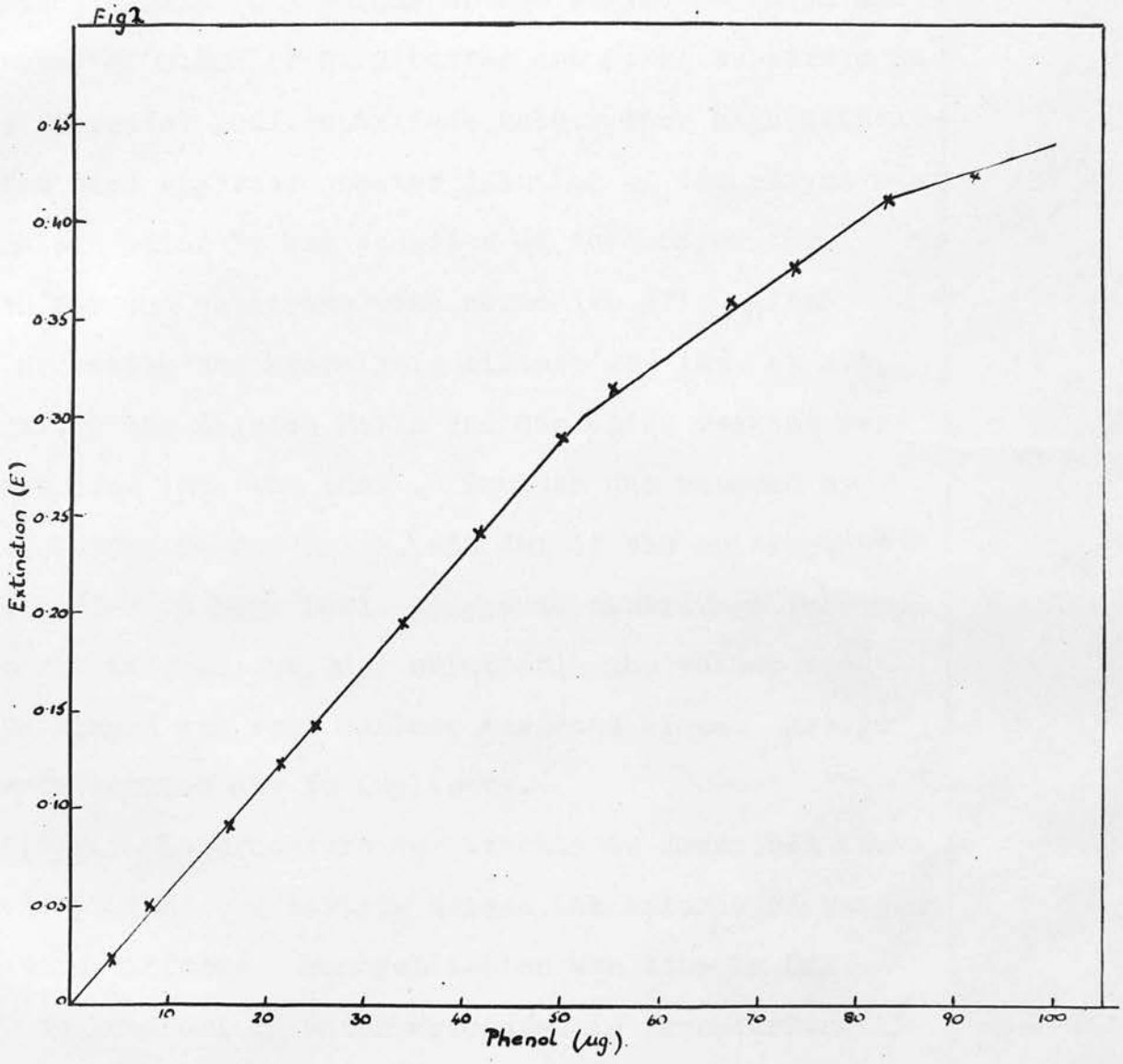
as described below. Since the reaction varies with pH it is probably enzymatic.

Procedure.

Macro-method. During the preliminary work on the kinetics of hydrolysis of phenol-glucuronide by the enzyme and in much of the subsequent work on liver, kidney and spleen it was found convenient to use this procedure. In constructing the calibration curve for this method,(see Fig.2),0.2ml.citrate buffer,pH 5.2,was added to 0.6ml.standard phenol solution followed by 2ml.of a 1:5 dilution of Folin and Ciocalteu reagent. After mixing,2ml. of the solution were transferred to a tube containing 4ml. N Na_2CO_3 solution. The contents of the tube were mixed and the colour developed by incubating for 20 mins.at 37'. Citrate,acetate or formate had no effect on the colour reaction,and the colour produced was stable for a considerable period.

Spleen and kidney. The dissected ~~was~~ organ was weighed in a chilled homogenizer tube and homogenised in 3ml.water. The cell free homogenate was transferred to a graduated tube using 1ml.wash water. After the addition of 0.5ml.0.3M citrate buffer,pH 5.2,the tube was maintained at 37' for 30mins. Coagulated protein was separated by centrifuging for 15mins.,the volume of precipitate and supernatant noted,and the latter transferred to a second graduated tube. For total enzyme precipitation,an equal volume of S.A.S.was added and the tube centrifuged for 30mins. The supernatant was discarded and the residue dissolved in water and made up to 2ml. This

Fig 2


Extinction (E')
0·45
0·40
0·35
0·30
0·25
0·20
0·15
0·10
0·05
0
10
20
30
40
50
60
70
80
90
100
Phenol (µg.)

concentration of enzyme was such that,in normal animals,0.4ml.of the solution gave readings of 20 to 40ug phenol after correcting for enzyme and substrate blanks. This volume of the enzyme solution was added to 0.2ml.of 0.1M buffer and 0.2ml.substrate in a stoppered 10ml.centrifuge tube. When high activities were expected greater dilution of the enzyme was made. Prior to the addition of the enzyme,the buffer and substrate were warmed to 37'. After incubating the hydrolysis mixture for 1hr. at 37', 2ml.of the diluted Folin and Ciocalteu reagent were measured into the tube . Protein was removed by centrifuging for 5mins.,and 2ml.of the supernatant transferred to a 10ml. stoppered centrifuge tube containing 4ml.Na_2CO_3 solution. The colour was developed and read against reagents alone. Assays were carried out in duplicate.

Liver. The procedure was exactly as described above except that ,at certain stages,the volumes of reagent used differed. Homogenization was done in 5ml. water and 3ml.of water were used in transferring the homogenate to the first graduated tube. In adjusting the pH,1ml.0.3M buffer was used. The enzyme solution, after S.A.S.precipitation,was made up to 4ml.,this volume giving readings of 20 to 40ug.phenol,with livers from normal animals,after correction for blanks

Micro-method. In order to avoid lengthy incubation periods ~~with~~ during the estimation of β-glucuronidase

activity in a single mouse uterus, the above method of estimation was modified for use with the micro-cells of the Spekkar absorptiometer. This procedure was used for all estimations on uterus and was extended to other organs, the enzyme solution being prepared as above and diluted appropriately.

Uterus. Before weighing, the dissected organ was freed from intrauterine fluid by pressing between pieces of filter paper. The figure thus obtained was found to bear a constant relation to the weight after drying at 110° for 2hrs. for all states of the uterus. (Result see table.) No error was thus introduced into the assay since intrauterine fluid contained no detectable amounts of glucuronidase.

Table 1.

Treatment.	Moist weight.	Dry weight.	Dry wt. / Moist wt. %
Normal female.	221.2 (6)	47.6(6)	21.5
Ovariectomised female.	32.7 (6)	7.8(6)	23.8
Ovariectomised + oestrone(1.7mg/kg)	228.8 (6)	50.3(6)	22.0
Ovariectomised + oestrone(0.3mg/kg)	98.3 (6)	21.8(6)	22.4
Ovariectomised + testosterone(3.3mg/kg.)	106.2 (3)	24.1(3)	22.7
Ovariectomised, 8dys. after partial hepatectomy(see text).	112.6 (3)	20.3(3)	18.2

(No.of animals in group shown in brackets.)

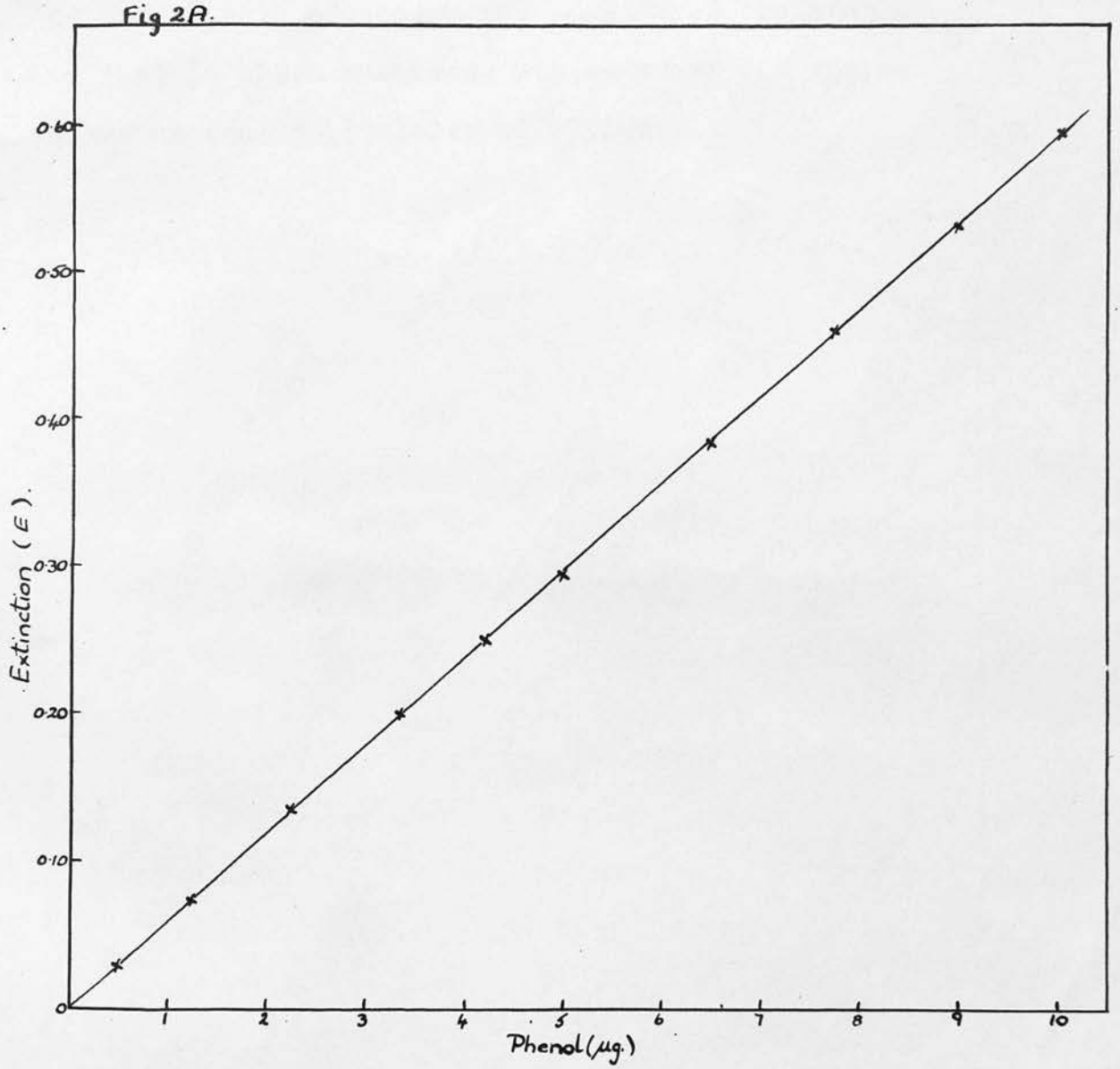
The uterus was homogenized in 1.5ml.water and 0.5ml. used in transferring the homogenate to a graduated

tube. 0.5ml.of 0.3M buffer was added to the mixture and it was incubated at 37'. After maintaining at this temperature for 30mins. coagulated protein was removed by centrifuging and the enzyme precipitated with S.A.S. The precipitate was made up to a suitable volume in water so that 0.2ml.of this solution gave readings of 2 to 4ug.phenol after correction for blanks. This volume of enzyme solution was added to 0.1ml.substrate and 0.1ml.0.1M citrate buffer at the appropriate pH. After incubation for 1hr. at 37', 0.5ml.of a 1:5 dilution of Folin and Ciocalteu reagent was added. Protein was removed by centrifugation and 0.5ml.of the supernatant transferred to a tube containing 0.5ml.1.33N Na_2CO_3. The colour was developed as described above and the result read against a graph constructed with standard phenol solution put through the same procedure.Fig.2A.

Controls. Every assay was controlled by incubating mixtures of enzyme and buffer with water in place of substrate,in the ratio of 2:1:1. The purified enzyme showed a small rise in blank during incubation. No mention is made by Mills (1948) of an incubated enzyme blank in his work on the hydrolysis of phenolglucuronide by the two enzyme fractions in beef spleen His results may therefore have to be corrected for the increase in enzyme blank during incubation.

Controls for free phenol in the substrate were also carried out at frequent intervals the solut-

Fig 2A.



ution being incubated with buffer and water in the ratio of 1:1:2. Since there appeared to be a small but perceptible increase when substrate was incubated in buffer, this procedure was considered preferable to that in which substrate was added to the enzyme after incubation, (Talalay et al. 1946).

Recovery of phenol from the incubation mixture.

The recoveries obtained in a series of experiments in which 0.2ml.standard phenol solution was added to 0.2ml.0.1M buffer and 0.4ml.enzyme solution (spleen and liver mixed) are shown in Table 2. In one case the mixture was incubated,while in the other Folin and Ciocalteu reagent was added immediately. Each figure is the average of two determinations after correction for the enzyme blank (5.2ug unincubated and 7.7ug.incubated).

In a series of 30 determinations in which 31.4ug.phenol was addedto enzyme and buffer as above and determined at once,the mean recovery was 31.5ug. (100.3%) and the standard deviation of a single determination from the mean was 0.19ug.(0.6%).

Table 2.

Phenol added. (ug).	Phenol recovered (unincubated).		Phenol recovered (incubated).	
	ug.	%	ug.	%
3.9	3.7	95	4.1	105
7.9	8.0	101	8.0	101
15.7	15.6	99	15.8	101
31.4	31.1	99	31.2	99
62.9	63.1	100	63.0	100
105.0	105.0	100	104.9	100

Kinetics of the Hydrolysis of Phenol-glucuronide.

In order to find the pptimal conditions for the assay of β-glucuronidase from liver,kidney, spleen and uterus the kinetics of the hydrolysis of phenol-glucuronide were studied for each organ,using the method of estimation just described. It was found that the pH-activity curve for mouse liver, kidney and spleen showed two distinct optima,suggesting the presence of two enzyme fractions. This possibility had been noted by Dr.Graham in the hydrolysis of menthol-glucuronide by glucuronidase from ox spleen (private communication). The presence of two distinct fractions in ox spleen glucuronidase was also observed by Mills (1947) with menthol-glucuronide. Mills succeeded in separating the fractions in ox spleen preparations,and his procedure was subsequently applied to the separation of the fractions in mouse liver and kidney. The effect of pH and of substrate concentration on the initial rate of hydrolysis of phenol-glucuronide was studied with total enzyme and with each fraction separately.

Results.

The effect of pH

The pH for optimal hydrolysis was determined over the range 3.0 to 6.5 at a substrate concentration of 0.01M in citrate buffer. To 0.2ml.0.1M buffer and 0.2ml.0.04Msubstrate,warmed to 37°,0.4ml.enzyme solution was added and the mixture incubated at 37° for 1hr. The initial effect of substrate and enzyme on the pH of the buffer was determined electrometrically. No change in pH occured during incubation.

Liver and spleen. The curves for variation in hydrolysis with pH using purified enzyme are shown in Fig.3,after correction for blanks which were of the order of 200ug.phenol/g.with spleen and 100ug/g.with liver,compared with hydrolysis values at the peak of 843ug.and 602 ug/g.respectively. Homogenates from five mice were pooled for each organ so that all points on the curve could be determined simultaneously.

Kidney. Neither crude nor purified kidney-enzyme gave a blank which varied with pH. The pH-activity curve for purified enzyme from both male and female mice is given in Fig.4,after correction for blanks. Using the micro-method of estimation,it was found possible to use enzyme preparations from a single animal for a complete curve. No sex difference was found in the average composition of the enzyme.

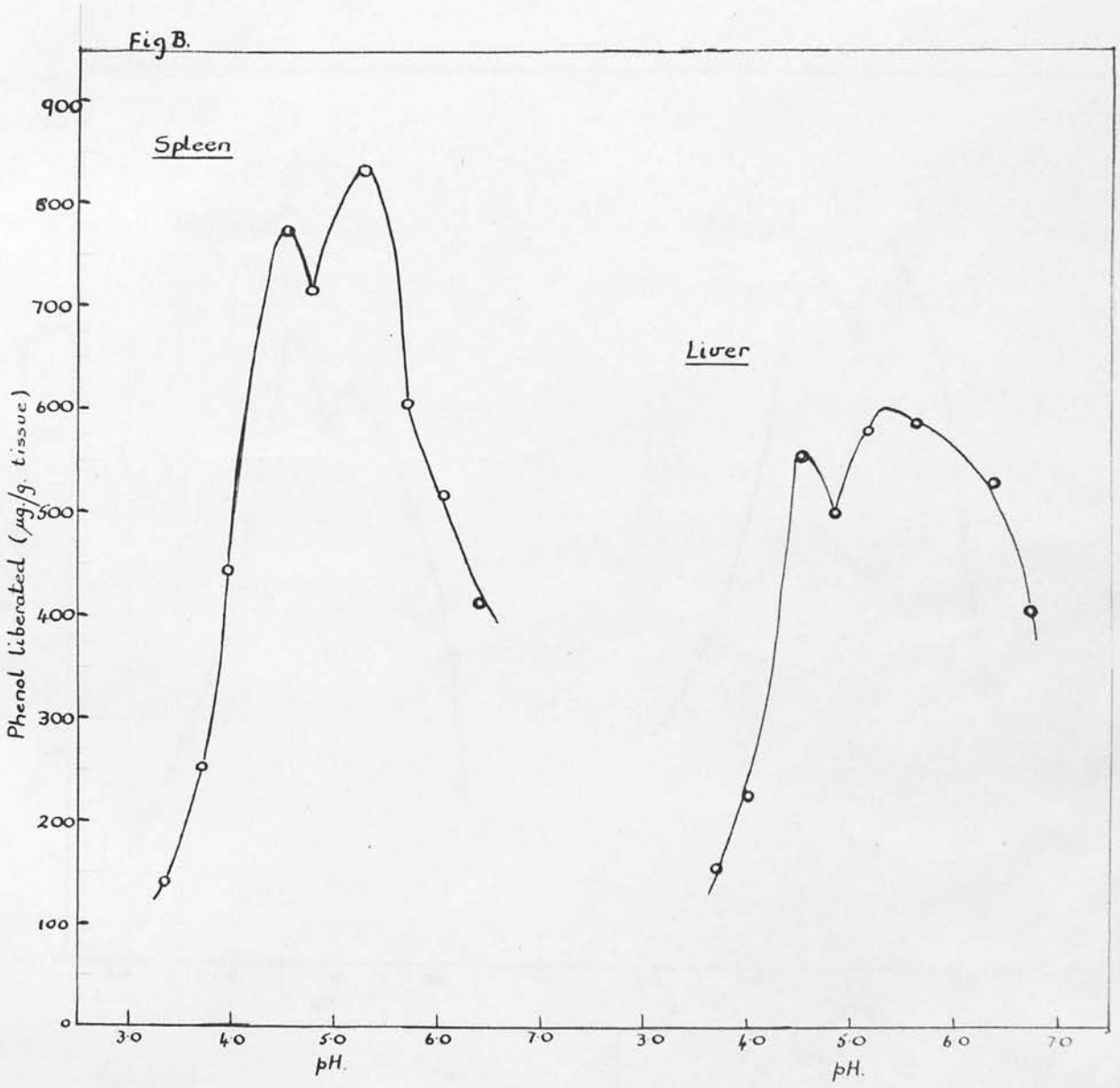
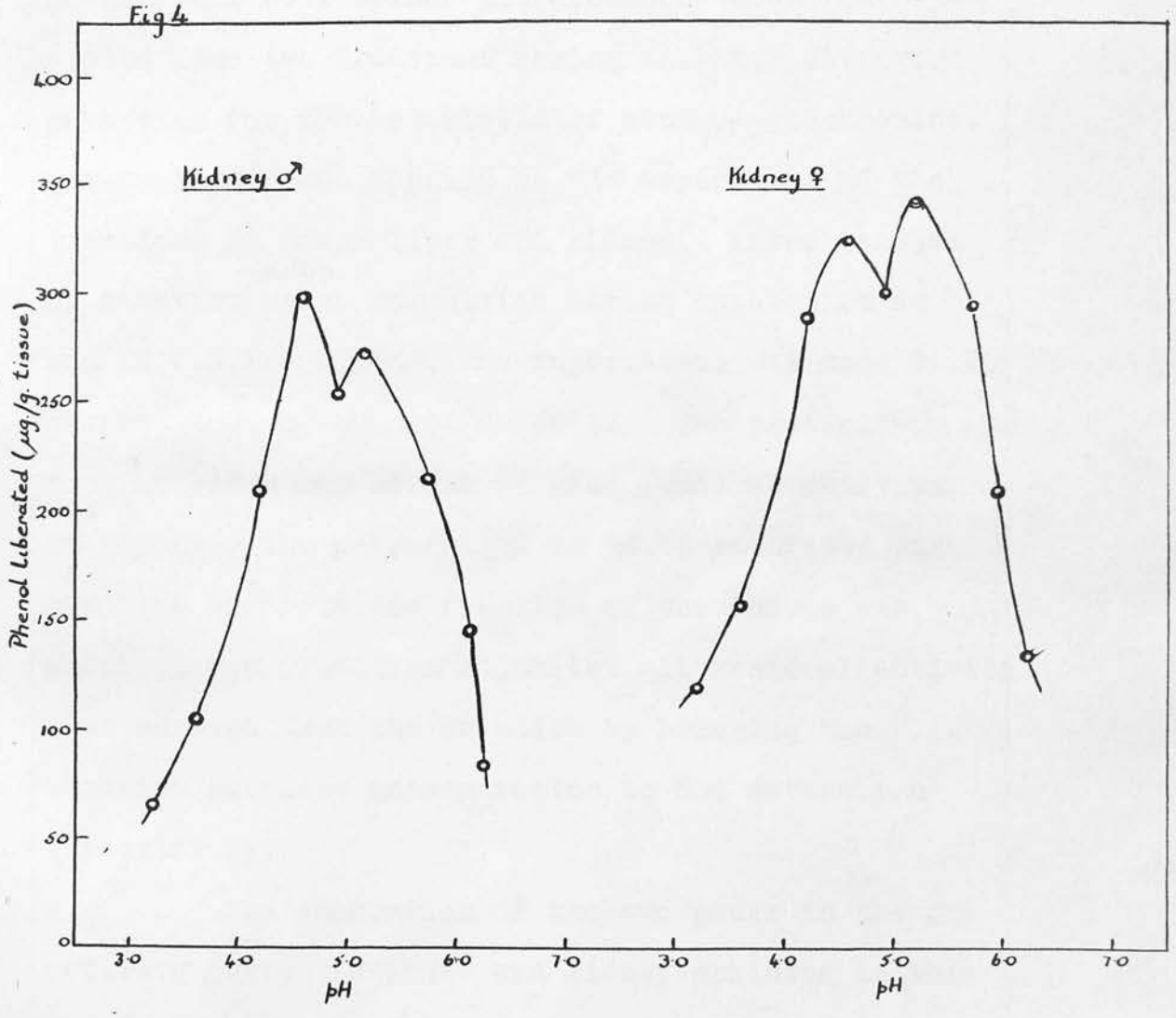


Fig B.
Spleen
Liver
Phenol liberated (µg/g. tissue)
900
800
700
600
500
400
300
200
100
0
3·0
4·0
5·0
6·0
7·0
pH.

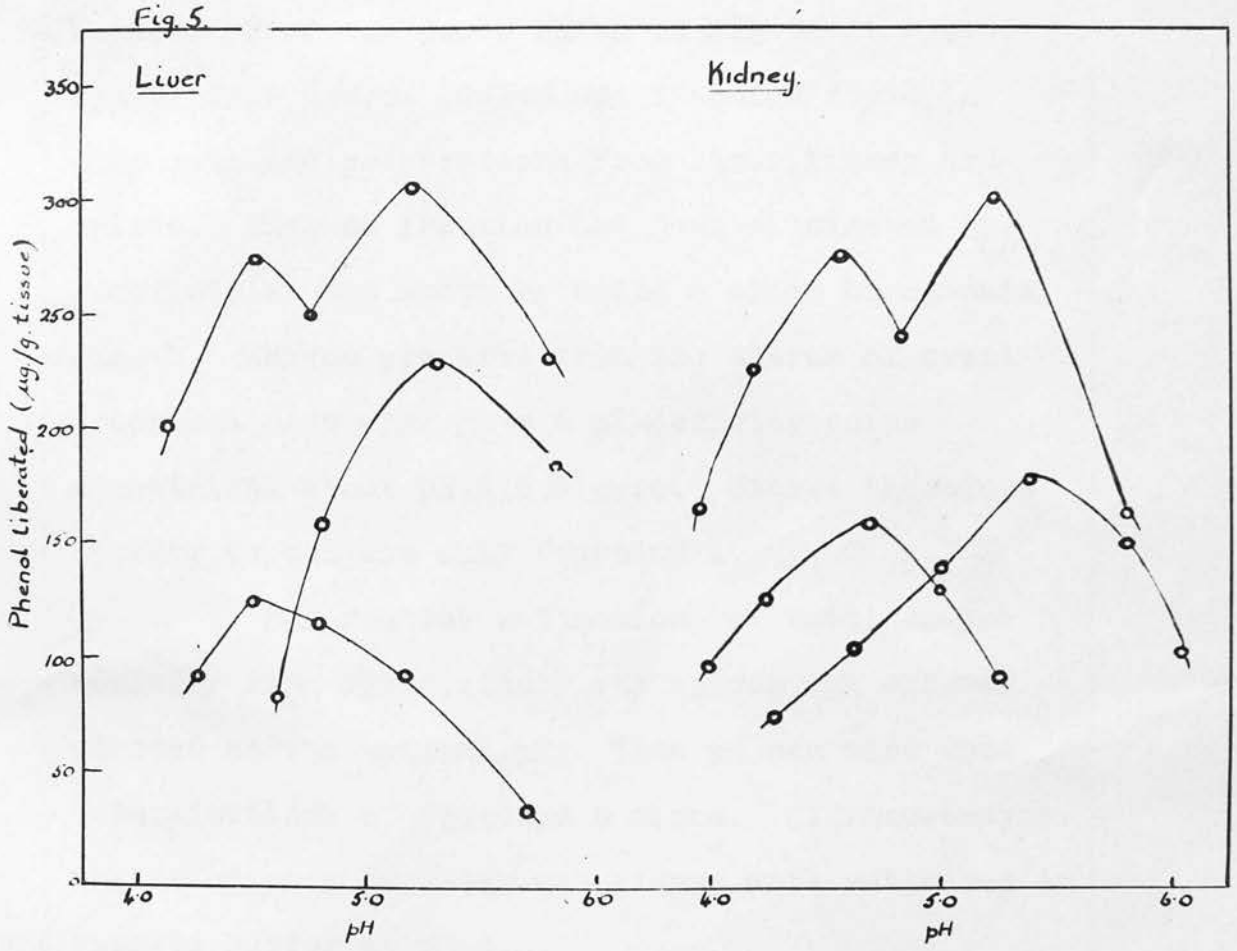
Fig 4


Kidney ♂
Kidney ♀
Phenol Liberated (µg./g. tissue)
400
350
300
250
200
150
100
50
0
3·0
4·0
5·0
6·0
7·0
pH

In all three organs the hydrolysis curve will be seen to rise to a peak at pH 4.5, fall again at about 4.75 and rise to a second peak at pH 5.2, suggesting the presence of two fractions in the enzyme preparation. At this time Mills (1947) reported that beef spleen glucuronidase could be separated into two fractions having slightly different pH optima for the hydrolysis of menthol-glucuronide. His technique was applied to the separation of the fractions in mouse liver and kidney. After removal of inactive protein which coagulated during incubation at 37° and pH 5.2, for 30mins., the supernatant was made 31.5% saturated with ammonium sulphate. The precipitate thus obtained was devoid of glucuronidase activity. On bringing the preparation to 38.5% saturated with ammonium sulphate, one fraction of the enzyme was precipitated (fraction A), whilst all residual activity was removed from the solution by bringing the ammonium sulphate concentration to 50% saturation (fraction B).

The separation of the two peaks in the pH-activity curve for liver and kidney achieved in this way is illustrated in Fig.5. In the case of liver a single animal was used and the homogenate halved, one part being brought to 50% saturation with ammonium sulphate, and the other to 38.5 and then 50% saturation. For kidney itwas necessary to pool homogenates from two animals.

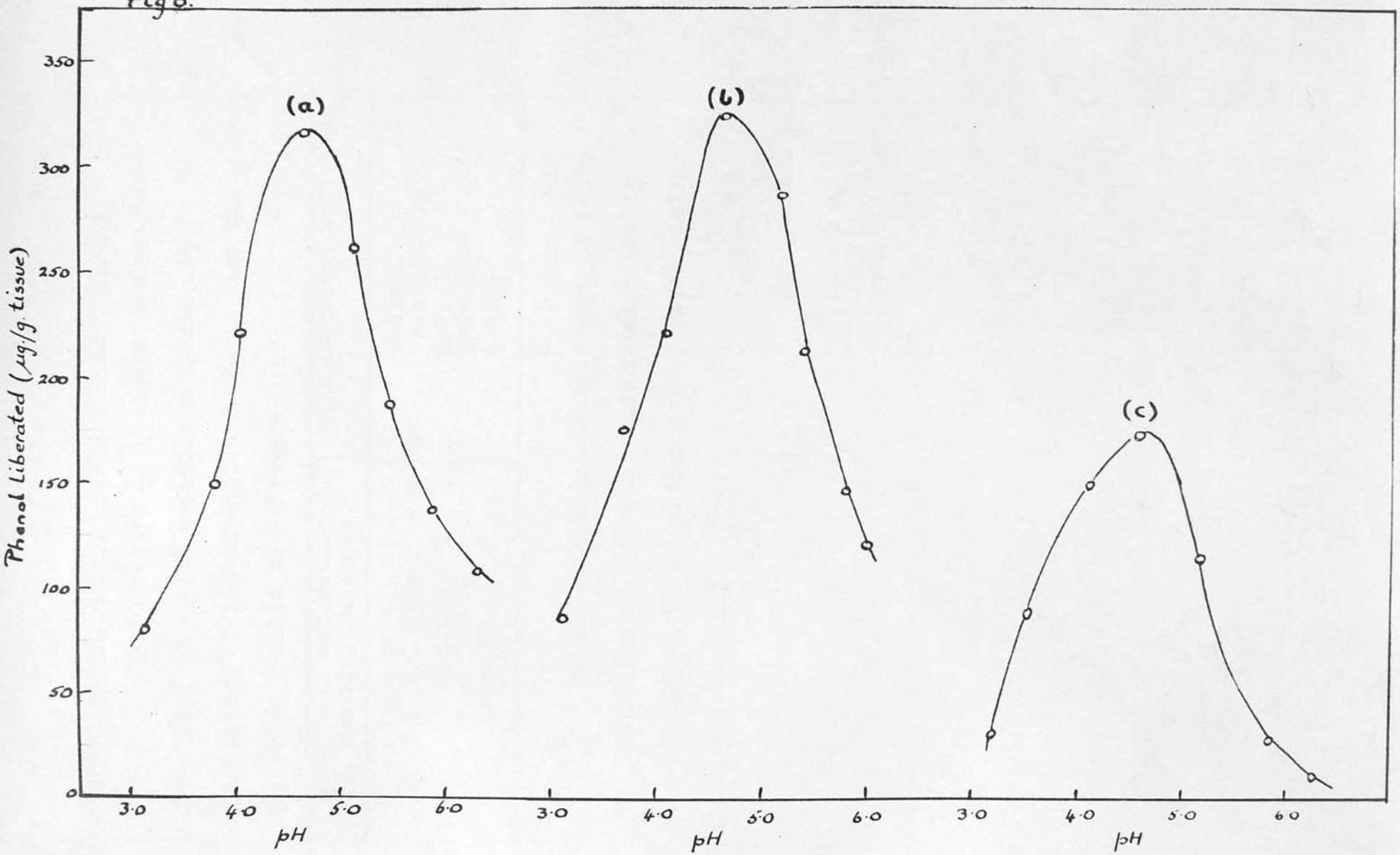
Fig. 5.


Liver
Kidney.
Phenol liberated (µg/g. tissue)
350
300
250
200
150
100
50
0
4·0
5·0
6·0
pH
4·0
5·0
6·0
pH

Uterus. Using the micro-method of estimation the variation in activity with pH was measured for uterine enzyme. Organs from four or more mice were pooled in order that all points on the curve might be determined simultaneously. A low and constant blank was obtained with both crude and purified enzyme. From the curve shown in Fig.6a it would appear that uterus lacked one fraction found in glucuronidase preparations from liver,kidney and spleen. That no fraction had been eliminated by purification was shown by using a crude homogenate, Fig.6b. Enzyme prepared from the uterus of ovariectomised mice also gave a pH-activity curve symmetrical about pH 4.5,Fig.6c. Uterus therefore appears to contain only fraction A.

For routine estimations of total enzyme activity from liver,kidney and spleen, pH 5.2 was adopted as the optimal pH. This pH was also used in determinations of fraction B alone. Uterine-enzyme and fraction A in liver and kidney were estimated in citrate buffer at pH 4.5.

Fig 6.


Phenol Liberated (µg/g. tissue)
350
300
250
200
150
100
50
0
(a)
(b)
(c)
3·0
4·0
5·0
6·0
pH

Fractionation of glucuronidase preparations.

Fractionation of homogenates from mouse liver or kidney with ammonium sulphate, after coagulation of inactive tissue debris, gave the following results in citrate buffer.

Percentage saturation with ammonium sulphate.	Glucuronidase activity,
0.0 - 31.5	Absent.
31.5 - 38.5	Present.
38.5 - 44.0	Present
44.0 - 50.0	Absent
above 50.0	Absent

Recent repetition of this work by Levvy and Karuniaratnam (private communication), using acetate buffer in the enzyme assay, gives similar results. (cf. Mills , 1948).

The effect of substrate concentration.

The effect on the rate of hydrolysis, at constant pH, of varying concentrations of substrate was studied using enzyme preparations from liver, spleen and uterus. Hydrolysis with separated fractions A and B from liver was also studied. As the addition of enzyme had little effect on the pH of the mixture in this region, substrate and citrate buffer, 0.1M, were brought to the desired pH previously. Substrate blanks were estimated at various concentrations.

The average result for three experiments using total enzyme from liver and spleen and estimated at pH 5.2 are shown in Fig.7. Results obtained with liver fraction A, estimated at pH 4.5, and liver fraction B, at pH 5.2, are given in Fig.8, and with uterine enzyme, estimated at pH 4.5, in Fig.9. In all cases homogenates from two or more mice were pooled for each organ and all points on the curve obtained simultaneously.

Marked inhibition by excess substrate was found in all cases and an optimum velocity of reaction was reached at a substrate concentration of about 0.01M. No great difference was seen between the various preparations. Because of the high substrate inhibition the results do not lend themselves to an accurate calculation of the Michaelis and Menten constant, Km, using the appropriate formula of Lineweaver and Burk (1934). If, however, the logarithm of

Fig 7.

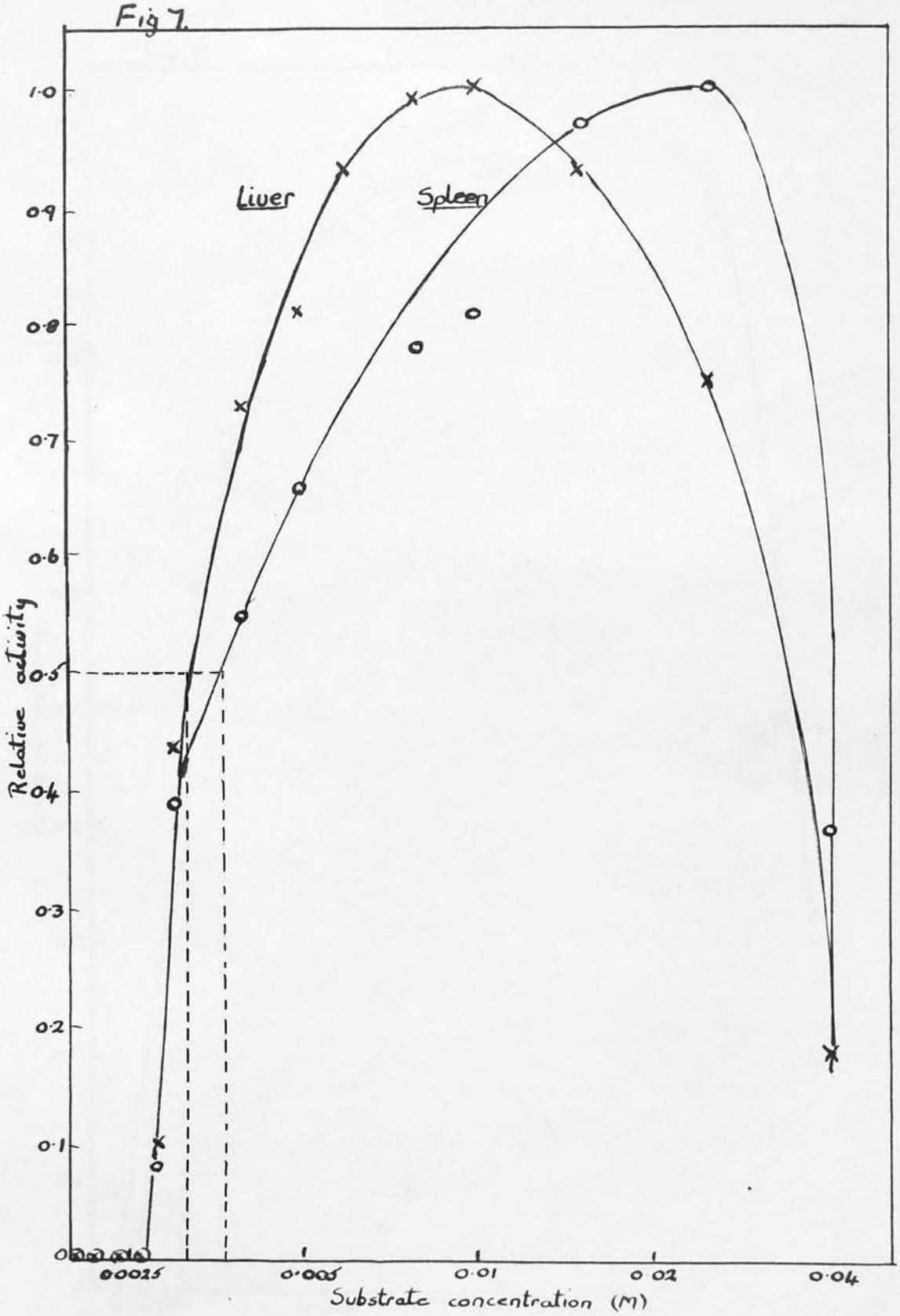

Liver
Spleen
Relative activity
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0·0025
0·005
0·01
0·02
0·04
Substrate concentration (M)

Fig. 8.

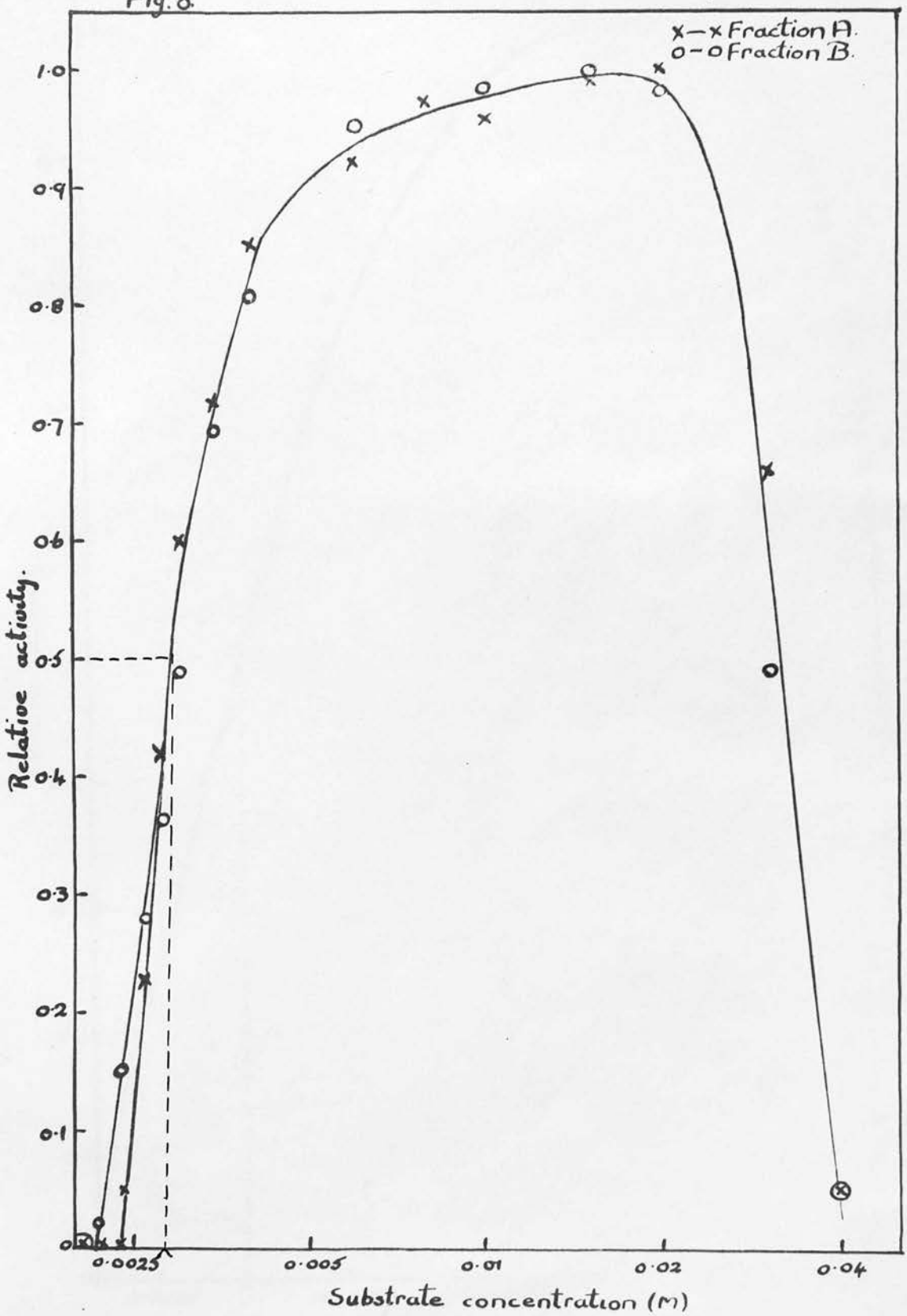
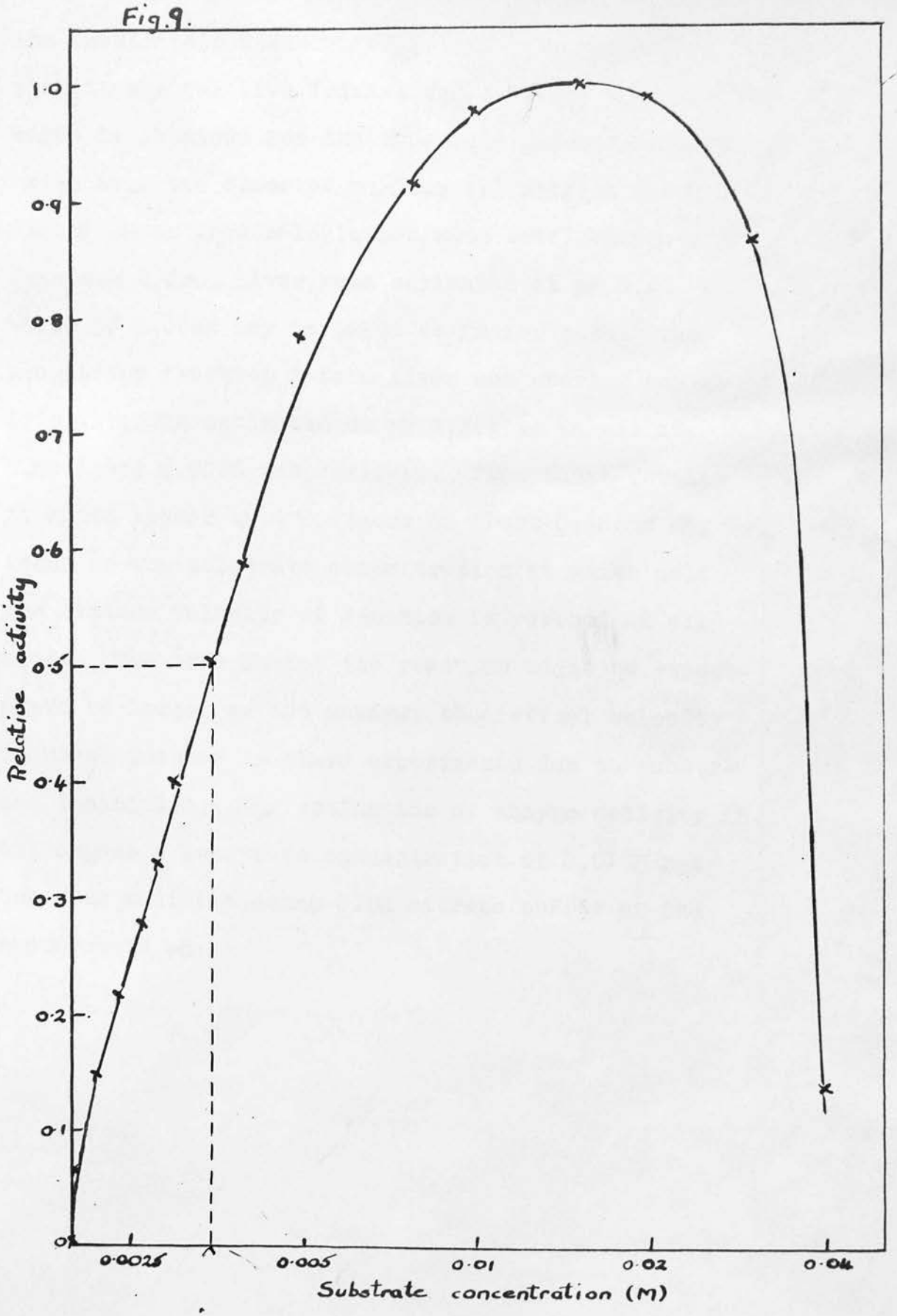

x—x Fraction A.
o—o Fraction B.
Relative activity.
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0·0025
0·005
0·01
0·02
0·04
Substrate concentration (M)

Fig. 9.


1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
Relative activity
0·0025
0·005
0·01
0·02
0·04
Substrate concentration (M)

the substrate concentration (log S) is plotted against the relative initial velocity (v),a constant value is obtained for the substrate concentration at which half the observed maximum velocity is reached. Fig.10 shows this calculation with total enzyme and fraction B,from liver,both estimated at pH 5.2. A value of 0.0032 may be taken as Km for both. The graphs for fraction A from liver and uterine enzyme (Fig.11),both estimated at pH 4.5,give values of 0.0031 and 0.0034 respectively. From these results it would appear that a figure of about 0.0035M may be taken as the substrate concentration at which half the maximum velocity of reaction is reached in all cases. The true Km for the reaction might be expected to be larger as the maximum theoretical velocity is never reached in these experiments due to substrate inhibition. For estimation of enzyme activity in all organs a substrate concentration of 0.015M was taken as suitable,using 0.1M citrate buffer at the appropriate pH.

Fig 10.

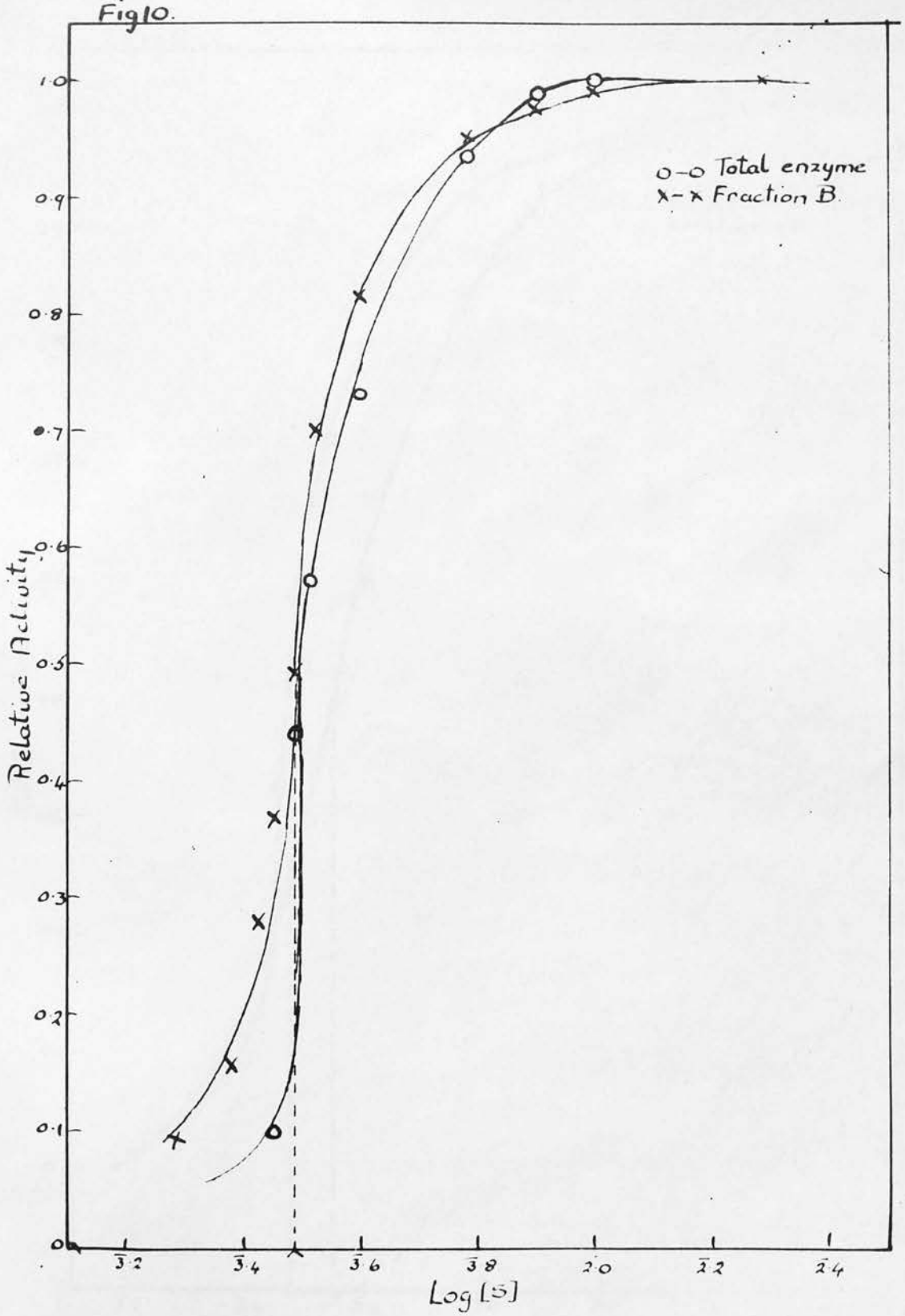
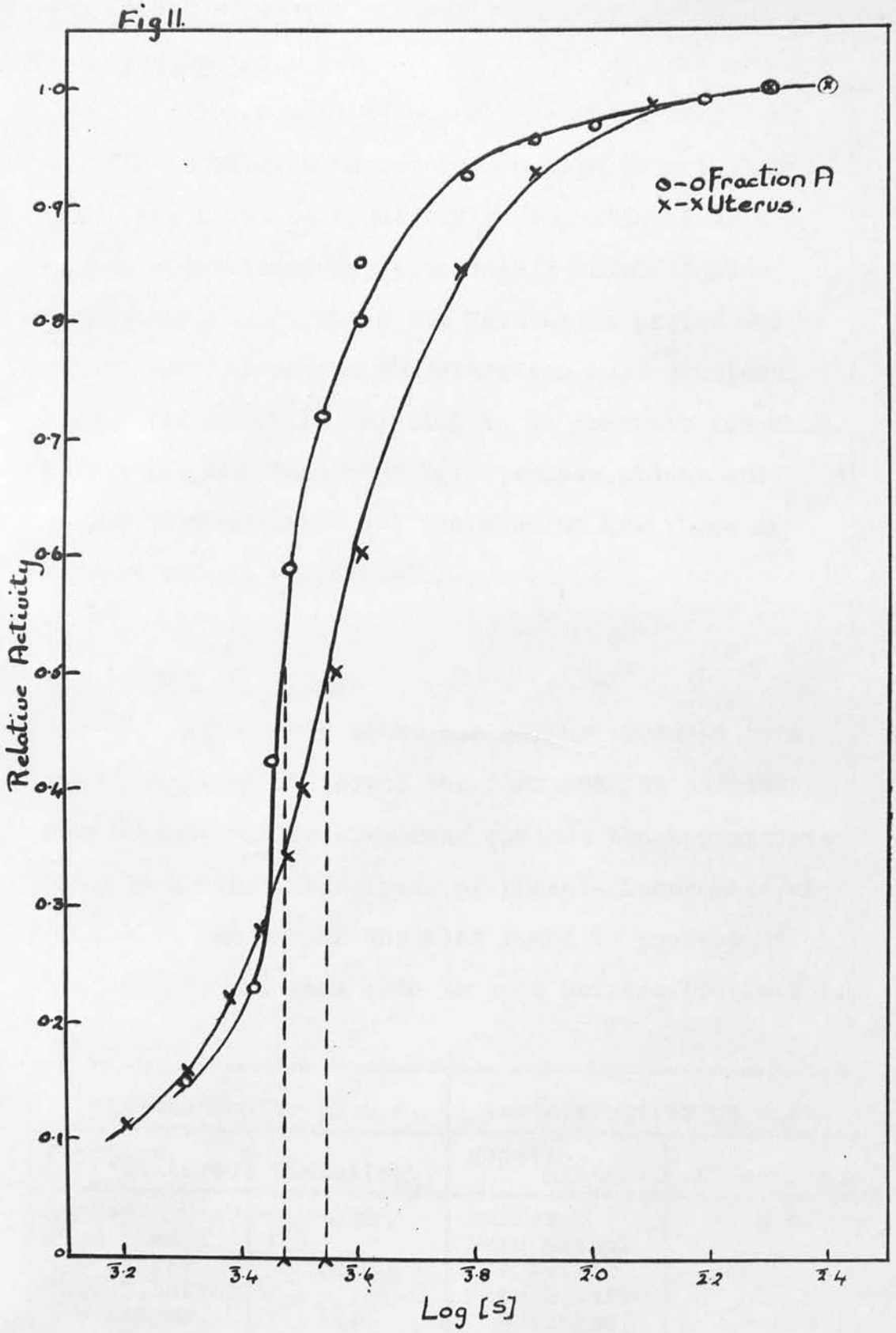

1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
Relative Activity
o–o Total enzyme
x–x Fraction B.
$\bar{3}\cdot2$
$\bar{3}\cdot4$
$\bar{3}\cdot6$
$\bar{3}\cdot8$
$\bar{2}\cdot0$
$\bar{2}\cdot2$
$\bar{2}\cdot4$
Log [S]

Fig 11.


o-o Fraction A
x-x Uterus.
Relative Activity
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
$\bar{3}\cdot2$
$\bar{3}\cdot4$
$\bar{3}\cdot6$
$\bar{3}\cdot8$
$\bar{2}\cdot0$
$\bar{2}\cdot2$
$\bar{2}\cdot4$
Log [S]

The effect of enzyme concentration and the time of incubation.

The amount of phenol liberated in 1 hour at 37'C in citrate buffer from 0.015M phenol-glucuronide was found to be directly proportional to the enzyme concentration. Experiments under similar conditions,but in which the incubation period was varied and the enzyme concentration kept constant, showed the reaction velocity to be constant for 3hrs. Enzyme preparations from liver,spleen,kidney and uterus were examined and the results are shown in Figs.12 and 13 respectively.

The effect of buffer.

As already noted,the colour produced by a known quantity of phenol was identical in citrate and acetate buffer when read against the apprppriate reagent blank. Hydrolysis of phenol-glucuronide,at pH 5.2,by the enzyme was also found to proceed at practically the same rate in both buffers.(Table 3).

Table 3.

Citrate buffer pH 5.2.		Acetate buffer pH 5.2	
Reaction mixture.	Phenol(ug.)	Reaction mixture.	Phenol(ug.)
Buffer	0.0	Buffer	0.0
Pure phenol + buffer	16.2	Pure phenol + buffer	16.2
Phenol,buffer + enzyme.	42.4	Phenol,buffer + enzyme.	42.9
Enzyme,buffer + substrate.	55.6	Enzyme,buffer + substrate.	56.6
Blanks	33.0	Blanks	33.6
Phenol by hydrolysis.	22.6	Phenol by hydrolysis	23.0

Fig. 12.

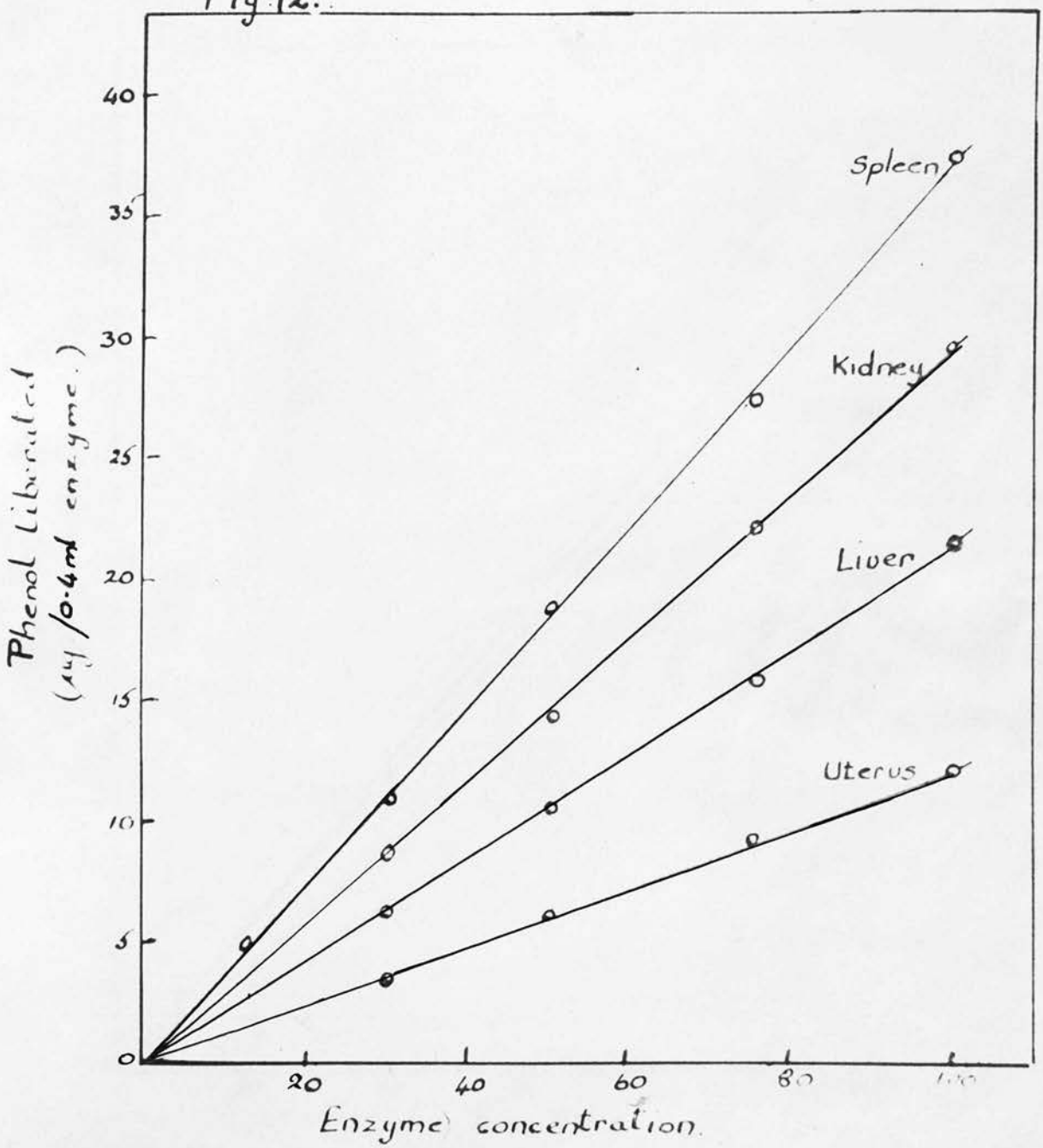
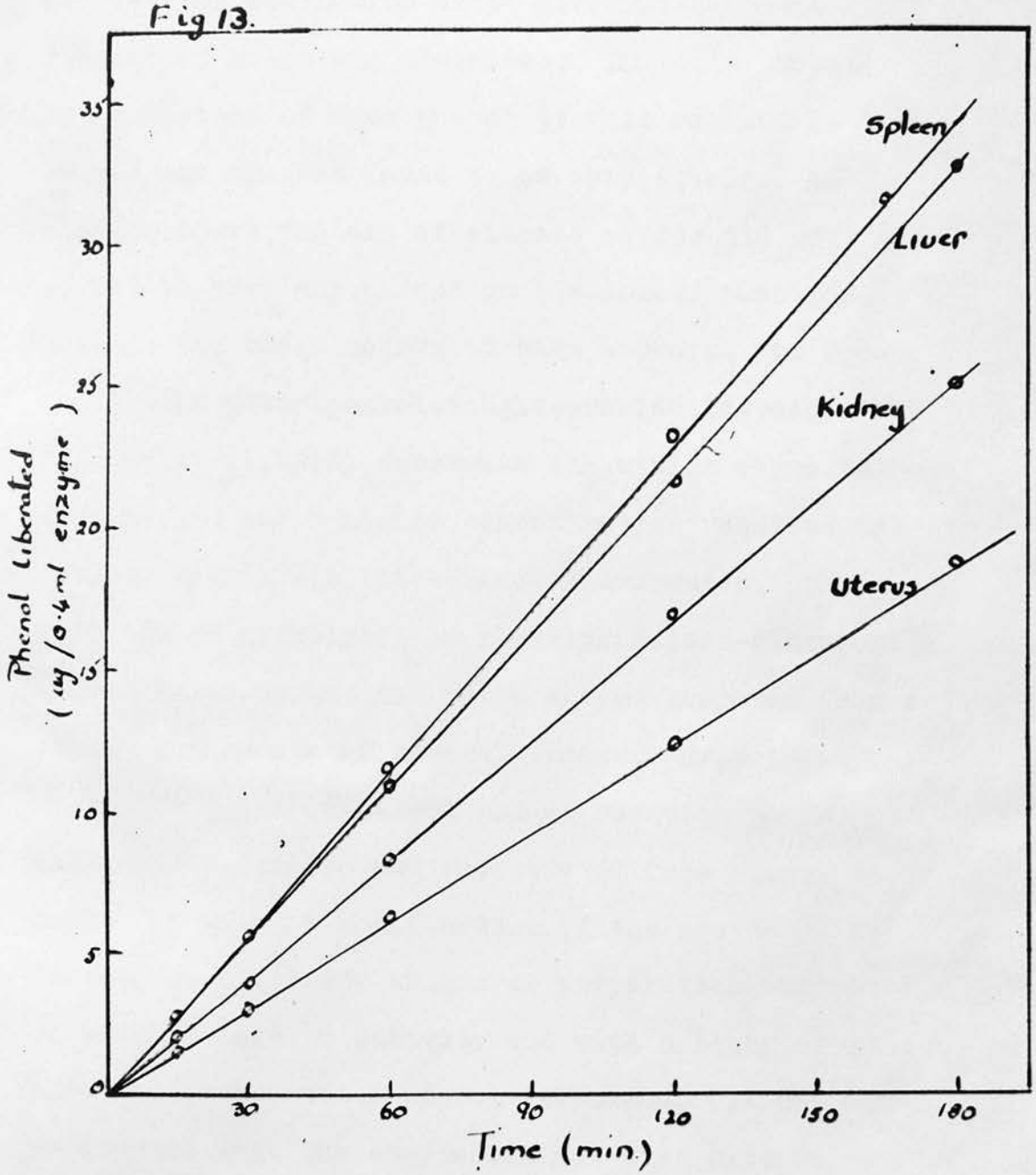

Phenol Liberated
(µg / 0·4ml enzyme.)
0
5
10
15
20
25
30
35
40
20
40
60
80
100
Enzyme concentration.
Spleen
Kidney
Liver
Uterus

Fig 13.


Phenol liberated
(µg/0·4 ml enzyme)
0
5
10
15
20
25
30
35
30
60
90
120
150
180
Time (min.)
Spleen
Liver
Kidney
Uterus

Discussion.

Phenol-glucuronide is a convenient substrate for the estimation of β-glucuronidase using the reagent of Folin and Ciocalteu. The colorimetric determination of free phenol by this reagent is simple and the colour was found to be very stable. As already noted, the use of citrate buffer did not appear to have any effect on the colour reaction, contrary to the findings of some workers. As compared with phenolphthalein-glucuronide (developed by Talalay et al., 1946), substrate inhibition seems to be more marked and a higher concentration required for optimum hydrolysis, with phenol-glucuronide. The kinetics of hydrolysis of phenolphthalein-glucuronide were, however, worked out using enzyme prepared from a pooled homogenate of several organs. Experiment has shown the Folin-Ciocalteu colour reaction to be extremely sensitive and recovery of free phenol to be 100%. By using a modification of the procedure of Talalay et al. (1946) an enzyme preparation was obtained without loss in activity and with a blank of less than 10% of the total phenol liberated. It may also be assumed that the enzyme has not been altered physiologically.

From the results obtained on the kinetics of the hydrolysis of phenol-glucuronide, it would appear that the enzymes present in liver, kidney and spleen are very similar. Uterus differs only in lack-

ing one fraction present in the other organs. It seems clear from the pH-activity curves shown above that there are two distinct enzyme fractions present in mouse liver,kidney and spleen,and that these can be separated by the difference in solubility of the protein in ammonium sulphate solution,as shown by Mills (1947) for beef spleen glucuronidase.

Despite this difference in composition of the enzyme no evidence of the specificity of action suggested by Fishman and Fishman (1944) was found. Phenol-glucuronide was hydrolysed identically by enzyme prepared from liver or from uterus. The possible bearing of the fact that liver,spleen and kidney contain both fractions A and B and uterus only fraction A on the physiological function of the enzyme was,however,investigated,and the results obtained are reported in Section B.

Good agreement with the figure obtained for the pH of optimal hydrolysis,pH 5.2,is found in the work of Masamune (1934). Estimating,by means of its reducing power,glucuronic acid liberated from phenol-glucuronide by enzyme prepared from ox spleen by more vigorous methods,he obtained a maximim between pH 5.3 and 5.6,in citrate buffer. Recent results by Mills (1948) also give pH optima for hydrolysis of phenol-glucuronide by his two fractions from beef spleen which correspond exactly with those for mouse liver, kidney and spleen found above.

A table collecting the data so far available on the hydrolysis of various substrates by β-glucuronidase prepared from the organs of several species, may be useful.

Table 4.

Substrate.	Organ.	pH Optimum.	Km.	Author.
Menthol-glucuronide.	Ox kidney	5.3 C†	-	Masamune(1934)
	Ox spleen	5.0-5.2A†	-	Oshima(1936)
	Ox spleen	5.0 A	0.004	Fishman(1939)
	Ox spleen	4.5,5.0A	-	Mills(1947)
Borneol-glucuronide.	Ox spleen	4.4 A	0.01	Fishman(1939)
Oestriol-glucuronide.	Ox spleen	4.3 A	0.0005	Fishman(1939)
Phenolphthalein-glucuronide.	Mouseliver, kidney & spleen.	4.5 A	0.00005	Talalay et al. (1946)
	Ox spleen	4.5,5.2A	-	Mills(1948)
Phenol-glucuronide.	Ox kidney	5.3-5.6C	-	Masamune(1934)
	Ox spleen	4.5,5.2A	-	Mills(1948)

It will be seen that phenolphthalein-glucuronide is the most satisfactory substrate for glucuronidase assay so far tested, having the greatest affinity for the enzyme, oestriol-glucuronide, a "physiological glucuronide", coming second. Many of the above values may, however, have to be reinvestigated in view of the most recent work of Mills.

†C -incubation carried out in citrate buffer.
A -incubation carried out in acetate buffer.

SECTION B.

The effect of various agents on β-glucuronidase activity in liver, kidney and spleen.

Introduction.

It was decided to try to determine, as far as possible, the exact function of β-glucuronidase in the body. All previous work on the enzyme suggested that it acted hydrolytically. As already pointed out, there appeared to be no reason to believe that β-glucuronidase could catalyse the synthesis of glucuronides either *in vivo* or *in vitro*. It is becoming increasingly obvious that, in general, the reversibility of a hydrolase is possible only in theory and in very "unphysiological" *in vitro* experiments.

An attempt was made to repeat Fishman's (1940) findings following repeated feeding of menthol to mice. Much larger increases in activity were obtained in liver-enzyme after a single injection than after repeated feeding, and by varying the time interval a rise in kidney-enzyme was also found. In no instance, however, was any change in spleen-glucuronidase detected. It was noted that the livers of menthol treated mice were abnormal in their gross appearance and histological examination revealed extensive damage to that organ and at a later stage to kidney also. Various agents, toxic to either or both liver and kidney, were administered and it was found that increases in enzyme activity were confined to the organ damaged. A rise in glucuronidase activity appeared to be associated with repair follow-

ing damage and to have no direct connection with the compound administered, many of which were nonglucuronidogenic.

The close correlation of enzyme activity and the degree of cell proliferation in an organ was further studied in livers of mice subjected to partial hepatectomy and in the organs of young animals High activities were obtained in all cases. In young animals the glucuronidase activity in liver was found to follow roughly the growth curve as described by Medawar (1941).

During earlier work only changes in the total enzyme activity were measured, using a citrate buffer at pH 5.2. A possible specificity of response to extrinsic agents of either fraction A or B was then investigated since uterus was later found (section A) to contain only A and the distribution of the different fractions might in some way influence the response by the glucuronidase activity of an organ. The enzyme was separated using the method described by Mills (1947) and the activity of each fraction and the total enzyme determined at their respective pH optima. No specificity in response was found with any of the compounds administered.

Results are expressed as G.U./g. moist tissue, where one G.U. (glucuronidase unit) liberates 1ug. phenol from 0.015M phenol-glucuronide in 1 hour at the appropriate pH. Wherever possible in the

following tables of results, the standard error is given. Although in many cases it is based on too small a group of animals to have any statistical significance it shows individual variation in a convenient form. The number of animals in the group is shown in brackets after each result.

Histology. All histology reported was done by Dr.J. G.Campbell,the Poultry Research Centre,Edinburgh.

Portions of organs from animals used for enzyme assay or whole organs from other animals treated similarly were fixed immediately in Susa and taken in the usual way through the ethanols to a mixture of chloroform and cedarwood oil and finally cleared in pure cedarwood oil. After embedding in paraffin wax, sections were cut at 8u and stained with Mayers haemotoxylin and eosin. The distribution of fat was studied in frozen sections prepared from tissue rapidly fixed by heat in formal saline and stained with haemotoxylin and Sudan III.

Damage,repair and cell division,as reported to us by Dr.Campbell,are shown in the following tables by an arbitrary system of ✕ signes. In the case of damage, ✕ indicates that while present it was neither extensive nor severe and XXX that it was at its maximum for the agent in question. The course of repair is measured likewise, XXX indicating that the replacement of damaged cells by normal cells was practically complete. Under cell division an estimate is given of the number of mitotic and amitotic figures and hyperchromatic nuclei in excess of normal. Since a deviation from normal could never be distinguished in spleen no histological findings are reported for that organ.

Results.

Total enzyme activity at pH 5.2.

Normal animals and controls.

Average values for β-glucuronidase activity in liver kidney and spleen were the same, within experimental error, for normal adult mice (25 to 35g.) of both sexes and drawn from several different colonies. In the course of the work pure strain A, CBA, and C57 mice, inbred mice from Schofield Mousery and mice from our own heterozygous colony were used, each experiment being controlled, as far as possible, with mice from the same colony. All results are grouped together in the normal values given in the following tables. Spleen showed a wider deviation in its normal enzyme activity than did either liver or kidney. Intraperitoneal or subcutaneous injection of relatively large amounts of 0.9% saline, olive oil or nut oil, the vehicles used for administration of the toxic agents, had no effect on the glucuronidase level in any organ examined after an interval of 1 to 7 days. The results are shown in Table 5.

Table 5.

Agent.	Dose.	Days.	G.U./g. moist tissue.		
			Liver.	Spleen.	Kidney.
None.	1 ml.Ipi.	-	273±13 (23)	636±70 (23)	363±24 (11)
0.9% saline	1 ml.Ipi.	2	203±31 (3)	385±61 (3)	325±26 (3)
	0.2ml.Sci.	7	298±11 (3)	-	355±17 (3)
Nut oil	1 ml.Ipi.	2	253±15 (3)	557±40 (3)	353±14 (3)
Olive oil	1 ml.Ipi.	2	249±19 (3)	526±47 (3)	301±16 (3)
	0.2ml.Sci.	7	288±17 (3)	672±52 (3)	382±20 (3)

Changes in β-glucuronidase activity and histological findings after administration of L-menthol.

Intra peritoneal injection of 333mg. L-menthol/kg. caused a rapid rise in the total liver-glucuronidase activity, reaching a maximum after 24 hours and persisting for 7 days. Greatest liver damage was observed after 24 hours, but repair processes were not perceptible at this time. After 14 days, when repair was almost complete, the enzyme activity had returned to its original value, although cell division still seemed to be slightly in excess of normal. During the first 2 days kidney was normal in enzyme activity and structure, but after 3 days damage was evident. The figure for glucuronidase activity had risen after 7 days. Kidney was normal in all respects at the end of 14 days. No effect on spleen-glucuronidase was observed at any stage and no deviation from ~~the~~ normal could be detected in the structure of this organ. The results obtained with liver and kidney were not influenced by the sex of the animals used. Table 6 gives figures for enzyme activity, with histological findings when available, for liver, kidney and spleen.

Table 6.
Changes in glucuronidase activity and histological findings after injection of L-menthol.

Interval (days).	Spleen enzyme.	Liver.				Kidney.			
		Enzyme.	Damage.	Cell division.	Repair.	Enzyme.	Damage.	Cell division.	Repair.
Normal.	636±70(23)	273±13(23)	-	-	-	363±24(11)	-	-	-
0.125-0.5	720±63(9)	467±24(9)	-	-	-	381±40(9)	-	-	-
1.	690±41(3)	825±135(3)	XXX	0	0	285±46(3)	0	0	0
2.	738±86(3)	884±74(6)	-	-	-	344±71(3)	-	-	-
3.	902±208(5)	953±39(5)	XX	XXX	X	-	XX	0	0
7.	646±86(6)	775±46(7)	X	XX	XX	603±52(7)	0	X	XXX
14.	600±14(3)	318±17(3)	X	X	XX	337±23(3)	0	0	XXX

Average enzyme activity and standard error expressed as G.U./g. moist tissue(see text). Number of animals in the group shown by figures in brackets.

As no details of a toxic action of L-menthol could be found in the literature a brief description of the changes found by Dr.J.G.Campbell in liver and kidney may be of interest. In the liver the first deviation from normal was a cloudy swelling, followed by fatty degeneration and necrosis surrounding the central vein and extending about one third of the way into the lobule. The nuclei showed hypertrophy and hyperchromatism. Many binucleate cells appeared (amitotic division) and at a later stage mitotic division became evident. The Kupffer endothelial cells were swollen. In the case of kidney the damage was not severe being confined to the distal portions of the convoluted tubules and to some glomeruli, the endothelium of which was swollen and in places necrotic.

Intraperitoneal injection of mice with a large dose of menthol (700mg./kg.), caused prolonged depression of respiration and unconsciousness. No attempt was made to determine the lethal dose.

Changes in β-glucuronidase activity after repeated administration of L-menthol and L-menthol β-d-glucuronide.

Results for β-glucuronidase activity in liver, kidney and spleen after intraperitoneal injection of mice with L-menthol or its glucuronide twice or thrice daily for varying periods were similar to those described above after a single injection of menthol. The glucuronide was injected as a neutral solution in 0.9% saline and menthol as a solution in olive oil. Neutral solutions of acid compounds in 0.9% saline were prepared as described by Chance, ~~Graham~~ Crawford and Levvy(1945).

Repeated oral administration of menthol in olive oil, in doses totalling 1.2 to 2g./kg., produced increases in liver-glucuronidase of the same order as did the injection of 333mg./kg. If the dose were increased to 9.3g./kg. a barely perceptible rise was produced as seen from Table 7. The latter was, however, as great as that found by Fishman(1940), and was found to be statistically significant (P-0.01). In feeding the larger dose, the experiment was carried out exactly as described by Fishman, using the Odell, Skill and Marrian(1937) technique, except that three of the mice in the group were fed a solution of menthol in olive oil instead of as a suspension in soap solution. The change in vehicle had no effect on the enzyme response .

No histology was done on the organs of mice

receiving menthol in these experiments. Changes in liver and kidney after injection of menthol-glucuronide were found to be similar to those following a single injection of menthol. No deviation from the normal glucuronidase level or structure was observed in spleen during these experiments.

Table 7.
Changes in glucuronidase activity after repeated administration ofL-menthol and L-menthol-glucuronide.

Agent and mode of administration.	Total dose. (g./kg.)	Days after 1st.administration.	Glucuronidase activity. Spleen.	Liver.	Kidney.
Untreated.	-	-	656±70 (25)	273±13 (25)	363±24 (11)
L-menthol,orally	1.2	3	843±185(3)	741±146(3)	-
	2.0	1	-	895±77 (6)	-
	9.3	5	499±48 (6)	369±40 (6)	260±2 (2)
L-menthol,Ipi.	0.8	2	254±38 (3)	1149±136(3)	-
	1.2	3	599±42 (6)	869±58 (6)	-
L-menthol (β-d-glucuronide,Ipi.	1.5	2	-	576±31 (3)	374±15 (3)
	2.3	1.5	995 95 (3)	1104±222(3)	295±13 (3)
	2.5	2	-	604±29 (3)	363±11 (3)

Results are expressed as in Table 6.

Changes in β-glucuronidase activity in liver, kidney and spleen produced by single injection of various substances.

The changes in glucuronidase activity and histology in liver, kidney and spleen after injection of various substances, some known liver or kidney poisons, were studied. The changes in enzyme activity in spleen were relatively small with wide variation in individual figures so that no definite conclusions could be reached. As before no deviation from the normal histological picture was observed in spleen.

Subcutaneous injection of 5.3g. carbon tetrachloride/kg. in olive oil caused severe fatty degeneration and early necrosis in liver within 24 hours. After 3 days damage was extensive but repair processes had commenced, and after 7 days repair was far advanced. A marked increase in liver glucuronidase activity occured within 24 hours and was maintained for seven days, falling again to normal as repair was completed at 10 days, (Table 8). Doses of 0.5 to 2g./kg. caused similar changes in liver-glucuronidase activity. There was no change in kidney-glucuronidase activity at any stage nor was there any in the pathology of kidney.

The changes in liver after subcutaneous injection of chloroform in olive oil resembled those produced by carbon tetrachloride, increases in enzyme activity being found after injection of as little as 200mg./kg. Results with up to 2g./kg. are shown in

Table 8. Kidney,however,showed an interesting sex specificity in the response of the enzyme to chloroform. In agreement with the observation ~~that~~ of Eschenbrenner (1944) this compound was found to cause renal necrosis in male but not in female mice. The rise in glucuronidase activity which was confined to male mice,as seen from Table 8, was not found in the early stages of damage but was evident after 8 days by which time repair was extensive. Further data on the sex specificity of chloroform poisoning in kidney and the associated changes in glucuronidase activity are reported later (p 56).

Table 8.

Changes in glucuronidase activity in liver, kidney and spleen produced by a single injection of chloroform or carbontetrachloride.

Agent and dose.	Interval. days.	Sex	Spleen	Liver.				Kidney.				
			Enzyme.	Enzyme.	Damage.	Cell division.	Repair	Enzyme.	Damage.	Cell division.	Repair.
None.	-	M,F.	636±70 (23)	275±13 (23)	-	-	-	563±24 (11)	-	-	-
CCl_4 (0.5 g./kg. Ipi.)	1	F	571±42 (5)	680±65 (5)	-	-	-	558±19 (3)	-	-	-
CCl_4 (2g./kg. Ipi.)	1	M	864±59 (5)	729±70 (5)	-	-	-	585±25 (3)	-	-	-
CCl_4 (5.3g./kg. Soi.)	1	M	840±121 (5)	1138±48 (5)	XXX	0	0	139±9 (5)	X?	0	0
	4	M	-	763±65 (5)	XX	XX	0	257±19 (5)	0	0	0
	7	M	462±138 (4)	927±48 (4)	X	XXX	XX	523±45 (4)	0	0	0
	10	F	-	579±56 (5)	0	0	X	292±12 (5)	0	0	0
$CHCl_3$ (0.2 g./kg. Soi.)	3	F	-	508±16 (3)	-	-	-	-	-	-	-
$CHCl_3$ (0.5 g./kg. Soi.)	1	F	766±81 (5)	583±27 (5)	-	-	-	-	-	-	-
$CHCl_3$ (5.3 g./kg. Soi.)	1	M	994±154 (6)	939±90 (7)	XXX	0	0	194±16 (6)	XXX	0	0
	8	M	746±89 (5)	711±54 (5)	XX	XX	X	628±119 (5)	X	XX	XX
	1	F	-	-	-	-	-	251±18 (3)	0	0	0
	8	F	612±54 (5)	608±44 (5)	X	XX	XX	274±15 (5)	0	0	0

(Results expressed as in Table 6.)

Mercuric nitrate, given as a neutral solution in 0.9% saline, had no marked effect on liver-glucuronidase activity or histology but caused severe cortical necrosis, with hyaline casts, in kidney within 24 hours. Kidney enzyme showed no rise at this stage but after 3 days, by which time repair was practically complete, it was more than twice its normal value (Table 9).

Yellow phosphorus, injected subcutaneously in olive oil, had no evident effect on kidney enzyme or histology but produced extensive damage to liver in the form of congestion, fatty degeneration and necrosis. From the results shown in Table 9 it appeared that no rise in glucuronidase activity occured in an organ when damage was at its height. Not until repair was evident was the rise perceptible. In the case of phosphorus there was an unmistakable initial drop in the enzyme activity to 1/3rd. of the normal value. At the end of 5 days, when repair was well under way, the enzyme level showed the usual increase, only to fall again as repair was completed.

Of the remaining substances tested ether and pregnanediol had no effect on glucuronidase activity and produced no pathalogical changes in either liver or kidney. Pregnanediol-glucuronide also had no effect on the enzyme activity but was not examined for histological effects. Ether was given by inhilation and pregnanediol and its glucuronide were injected intraperitoneally as suspensions

in olive oil.

Sulphathiazole caused cloudy swelling in liver after subcutaneous injection of 45g./kg. as a neutral solution in 0.9% saline. There was a small but significant rise (P-0.05 to 0.02) in liver-glucuronidase activity. This compound was without action on kidney. Sodium sulphapyridine given similarly to sulphathiazole caused fatty degeneration and necrosis in liver accompanied by some cell division but there was no appreciable change in the enzyme activity. In some animals there was evidence of slight damage to kidney, again without any rise in the glucuronidase activity. Results for all the above compounds are given in Table 9.

Table 9.

Changes in glucuronidase activity in spleen, liver and kidney after administration of various agents.

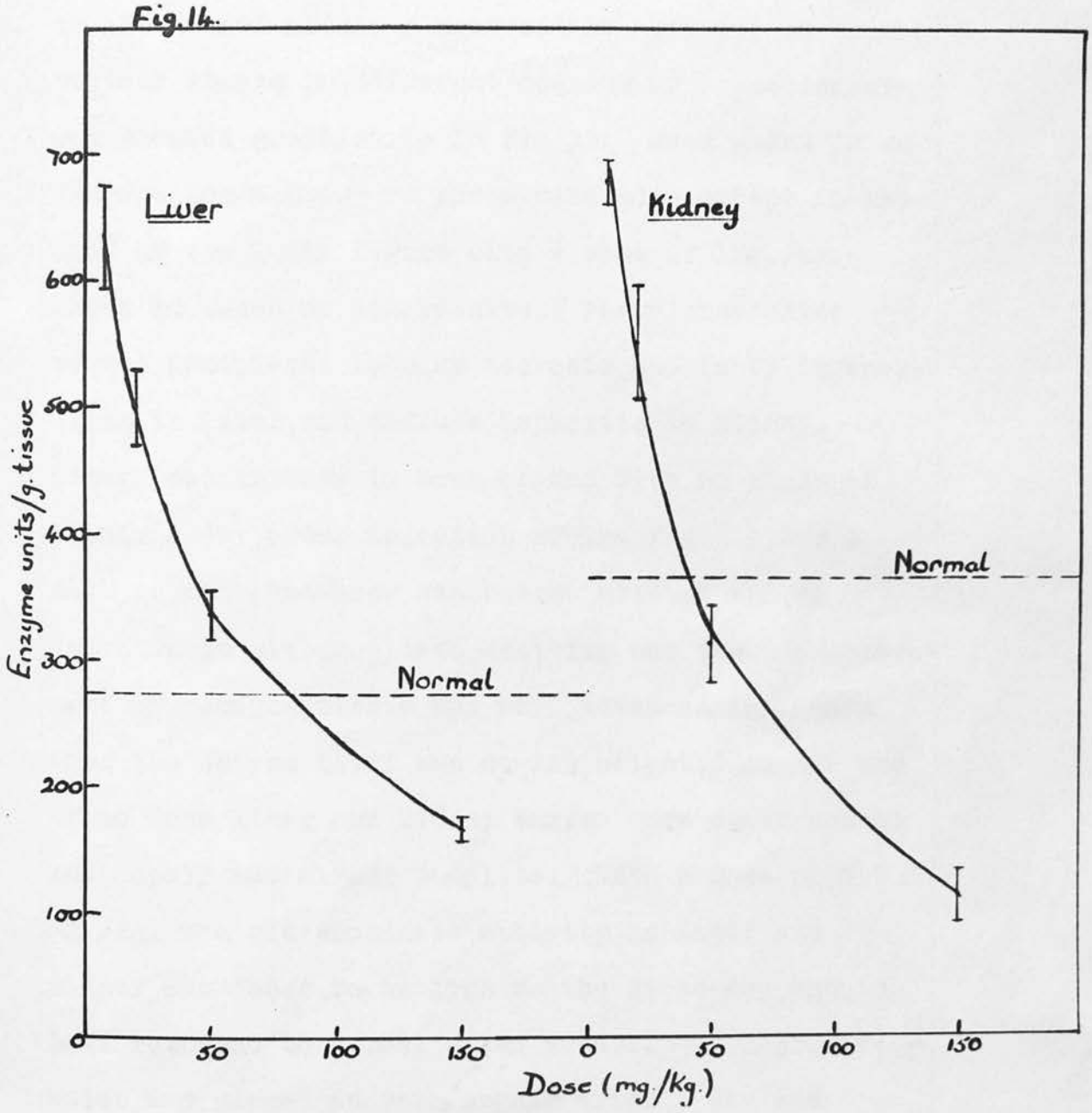
Agent and dose.	Interval. (days)	Sex	Spleen.	Liver.				Kidney.			
			Enzyme.	Enzyme.	Damage.	Cell division.	Repair.	Enzyme.	Damage.	Cell division.	Repair.
None.	-	M.F	636±70 (23)	273±13 (23)	-	-	-	363±24 (11)	-	-	-
Mercuric nitrate, (20mg./kg.).	1	M	-	436±28 (2)	X	0	0	208±45 (2)	XXX	0	0
	3	M	807±49 (4)	469±29 (5)	0	X	0	808±61 (5)	0	X	XXX
Yellow phosphorus, (7.5mg./kg.).	2	M	506±24 (3)	91±23 (3)	XXX	0	0	338±35 (3)	X?	0	0
	5	M	704±62 (3)	744±37 (3)	XX	XX	X	462±89 (3)	0	0	0
	10	M	578±29 (3)	429±80 (3)	0	0	XXX	309±32 (3)	0	0	0
Ether, (deep aneasthesia, 40mins.).	$1\frac{3}{4}$	M.F	-	265±30 (4)	0	0	0	369±63 (3)	0	0	0
Pregnanediol, (333mg./kg.).	1.7	F	1000±47 (3)	287±65 (3)	0	0	0	321±64 (3)	0	0	0
Pregnanediol-3-d glucuronide, (800mg./kg.). ##	1.7	F	627±124(3)	241±36 (3)	+	-	-	264±19 (3)	-	-	-
Sulphathiazole, (4.3g./kg.).	3	M	561±30 (3)	460±29 (3)	X	0	0	368±13 (3)	0	0	0
Na-sulphapyridine-mono-hydrate, (18-36g./kg.)	2	M.F	636±171(4)	327±17 (7)	XX	X	X	362±11 (7)	X?	0	0

Pregnane-3(α):20(α)-diol glucuronidic acid free from pregnane-3(α)-ol-20-one glucuronidic acid, (Sutherland and Marrian, 1947).

The effect of uranyl acetate on β-glucuronidase activity in liver and kidney.

Results for glucuronidase activity in liver and kidney after subcutaneous injection of uranyl acetate, as a neutral solution in 0.9% saline, illustrate further the point that an increase in the dose of the toxic agent may retard the rise in enzyme activity in the early stages of damage. A drop in activity was often observed. Each point on Fig.14 is an average of three male mice killed 2 days after injection. The enzyme activity in both liver and kidney will be seen to fall steadily from the elevated value following the injection of the smallest dose, to a figure of about half that found in normal animals, with the largest dose. In kidney severe tubular "nephrosis" was noted after 2 days with all 4 doses. Cell proliferation was observed with all but the largest dose (160mg./kg.), becoming more marked as the dose was reduced to 7mg./kg.. In the case of liver, the histological findings were more difficult to interpret as damage was transitory and rapidly succeeded by intense cell activity. Only the latter response was noted after injection of 7mg./kg. In general damage was greatest and repair processes slower to appear as the dose was increased. In both liver and kidney repair was complete 10 days after the injection of the smallest dose and the enzyme level had returned to normal.

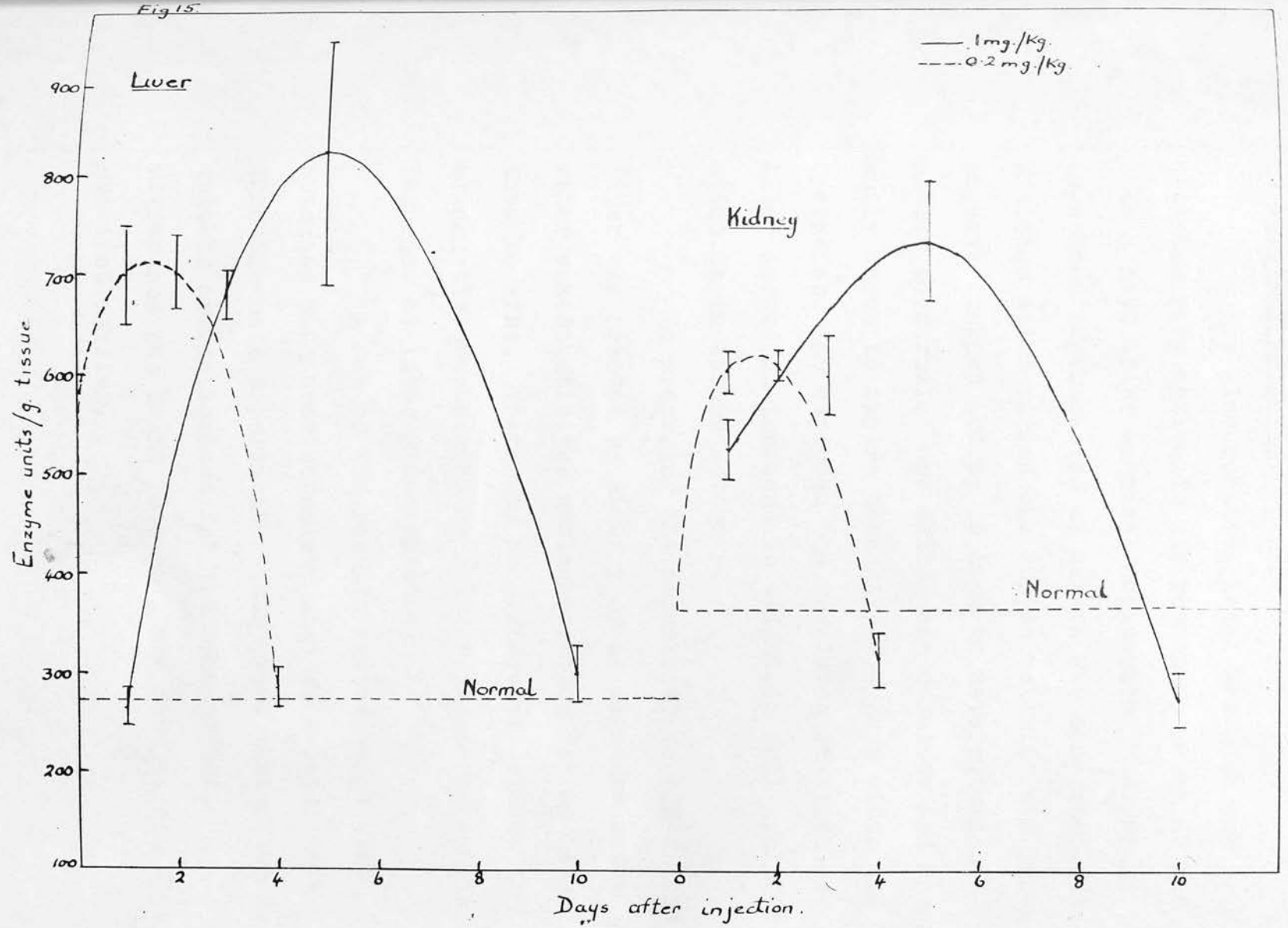
Fig. 14.


Liver
Kidney
Normal
Normal
Enzyme units/g. tissue
700
600
500
400
300
200
100
0
50
100
150
0
50
100
150
Dose (mg./Kg.)

The effect of phenylarsenoxide on β-glucuronidase activity in liver and kidney.

Phenylarsenoxide was injected as a neutral solution in 0.9% saline. Results showing the changes in liver- and kidney-glucuronidase activity at various stages in different degrees of poisoning are represented graphically in Fig.15. Each point is an average for a group of three male mice except in the case of the 1 day figure with a dose of 1mg./kg. which is based on six results. Phenylarsenoxide caused peripheral lobular necrosis and fatty degeneration in liver, and diffuse nephritis in kidney. Damage was intense in both organs with no signs of repair 1 day after injection of 1mg./kg. After 3 days, repair processes had become evident and an increase in glucuronidase activity was seen. Replacement of damaged tissue was well advanced in 5 days when the enzyme level was at its height. At the end of 10 days liver and kidney enzyme were again normal and repair was almost complete. With a dose of 0.2 mg./kg. the glucuronidase activity in liver and kidney was found to be high on the first day and to have returned to normal after 4 days. Cell proliferation was marked in both organs after 1 day and repair was well advanced by the fourth day. Phenylarsenoxide produced similar changes in both male and female mice.

Fig 15.


Liver
Kidney
1 mg./Kg.
0·2 mg./Kg.
Normal
Normal
Enzyme units/g. tissue
900
800
700
600
500
400
300
200
100
2
4
6
8
10
0
2
4
6
8
10
Days after injection.

The effect of partial hepatectomy on β-glucuronidase activity in liver.

The glucuronidase level was high and cell division very active in the remaining lobes of liver, 3 to 8 days after subtotal hepatectomy. After 10 days when regeneration of tissue was complete, but cell division still marked the enzyme activity was found to have dropped and by 12 days to have returned almost to normal. One animal was comatose and apparently about to expire when killed 3 days after the operation. As expected the remaining fraction of liver showed no increase in weight, in cell proliferation or in enzyme activity.

In preparing the animals, 40 to 60% of the liver was removed by cautery or by ligature under ether anaesthetic, the whole operation taking less than 10 mins. There was no difference between the alternative techniques or between the sexes in the response of liver-glucuronidase.

A few of the results quoted below were obtained with ovariectomised mice at a later date than the main experiment. These were controlled by animals ovariectomised for the same period. No difference was found between these results and those obtained earlier.

Table 10.
The effect of partial hepatectomy on glucuronidase activity in liver.

Days after operation.	G.U./g.moist tissue.
None.	273±13 (23)
2.	572±53 (3)
3.	951±115 (6)
5.	882±42 (3)
8.	1065±62 (9)
10.	657±35 (3)
12.	424±41 (3)

(Results expressed as in Table 6.)

The β-glucuronidase activity in liver, kidney and spleen of young animals.

Figures for the glucuronidase activity in the liver, kidney and spleen of mice ranging in age from 1 to 15 days are shown in Table 11. A much higher activity was found in all organs, including uterus (Section C), than in those of normal adult mice. From the pH-activity curve of enzyme from the liver of 6 day old mice, Fig 17, it is seen that both fractions A and B show an increase in activity. This is also true for kidney.

Glucuronidase activity in liver was studied during the period of active growth up till the age of 4 weeks. The enzyme level was found to remain fairly constant at a value 6 times that found in adults during the first week. During the second and third weeks the activity fell steeply to become constant around the thirtieth day. From Fig.16 it will be seen that the age-glucuronidase activity curve resembles a specific growth curve as described by Medawar (1941). Glucuronidase activity was found to be a function of age rather than of weight. The liver weight was also found to be a function of age, but to construct a specific rate of growth curve for this organ it would be necessary to kill a very large number of pure strain mice at various ages in order to ascertain the true weight increment. With our heterozygous mouse colony this was not possible.

Groups of pure strain mice, strain A, C57

Fig 16

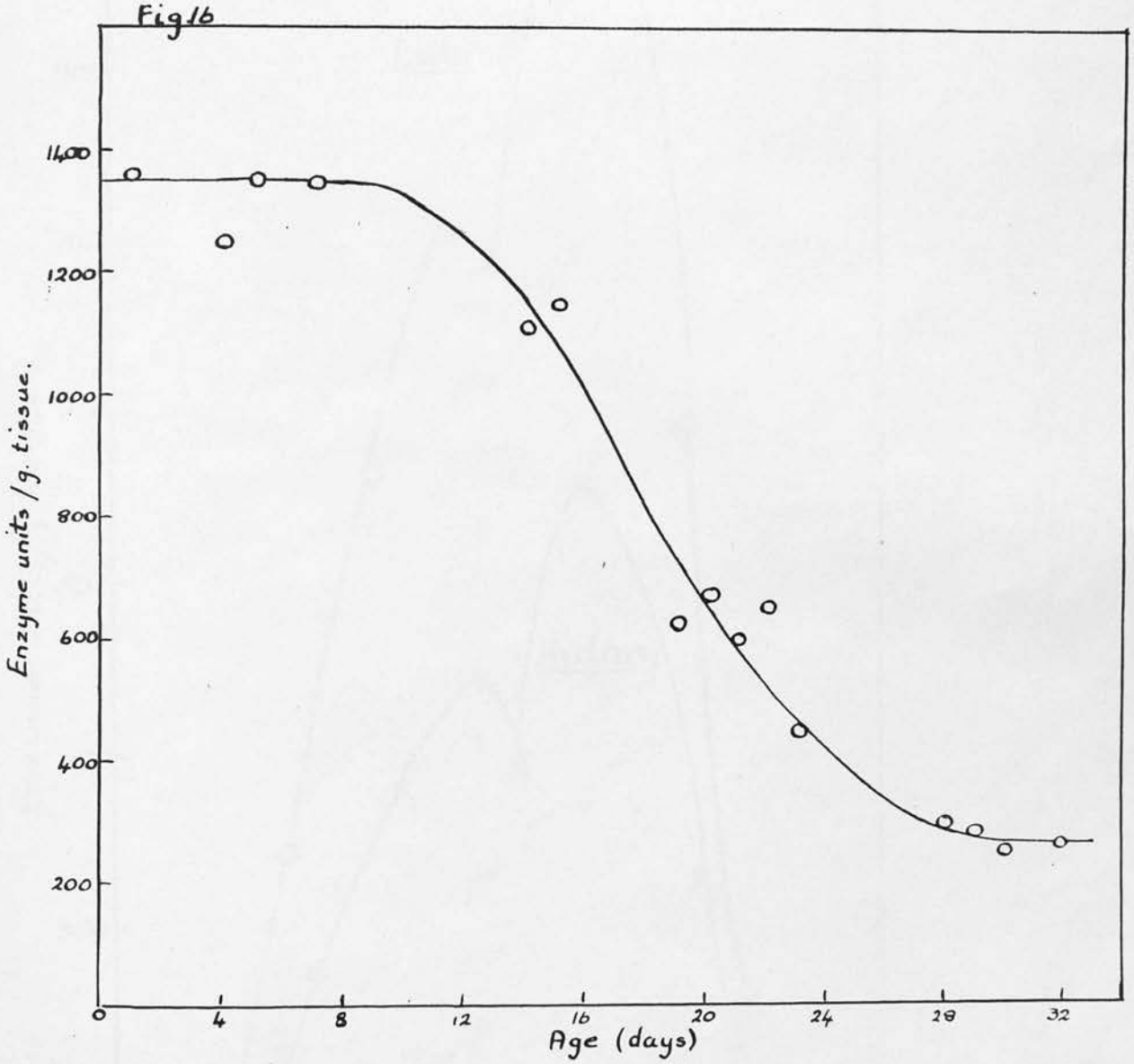
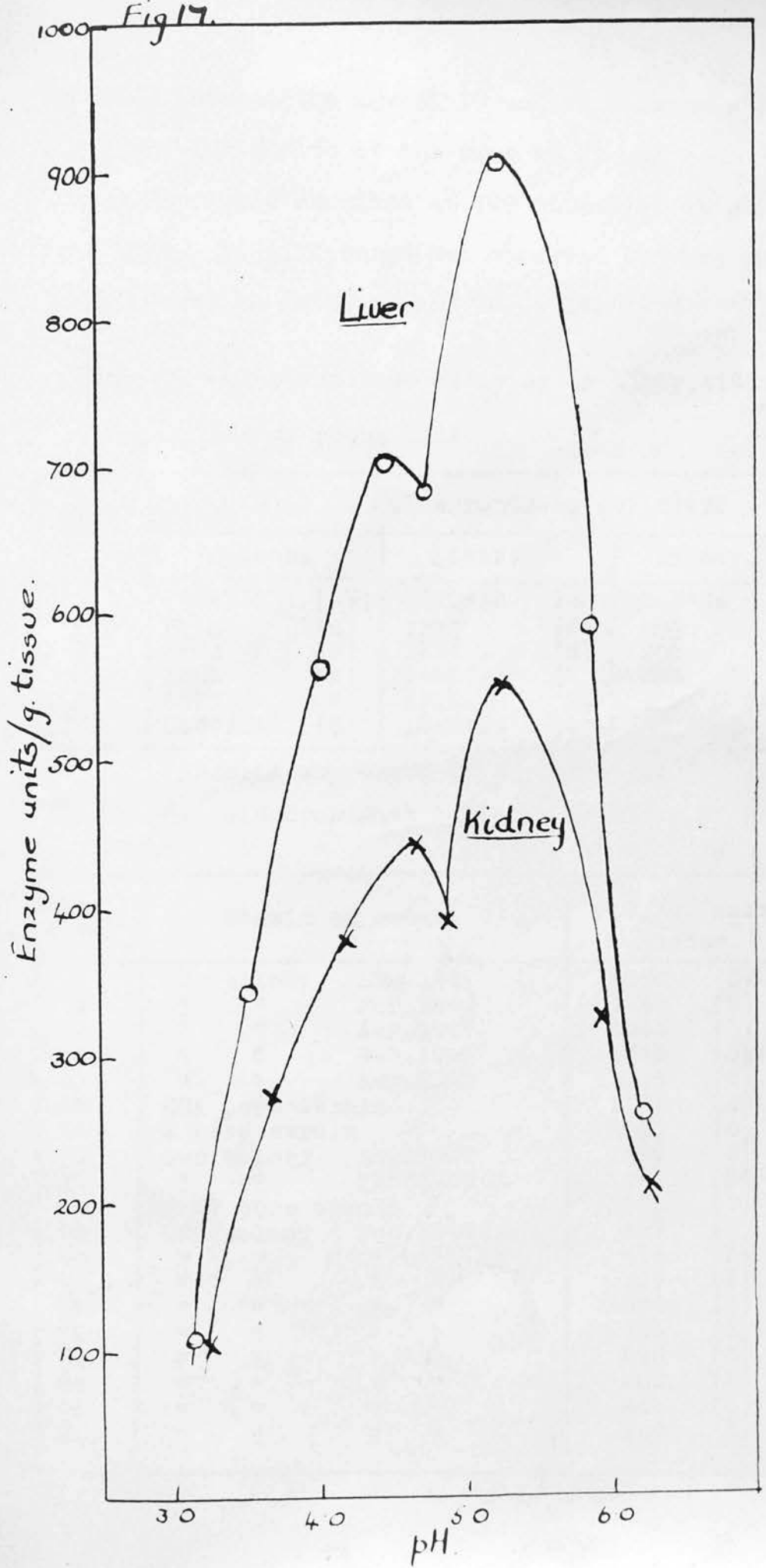

1400
1200
1000
800
600
400
200
Enzyme units / g. tissue.
0
4
8
12
16
20
24
28
32
Age (days)

Fig 17.


1000
900
800
700
600
500
400
300
200
100
Enzyme units/g. tissue.
Liver
Kidney
30
40
50
60
pH

and CBA, between the age of 14 and 15 days were compared with adults of the same strain, and our own inbred mice were examined at two different stages in this work. No difference was observed between the strains used, as is seen from Table 12.

Table 11. Glucuronidase activity in liver, kidney and spleen of young mice.

Age. (days).	Glucuronidase activity.		
	Spleen.	Liver.	Kidney.
Adult.	636±70 (23)	273±13 (23)	363±24 (11)
1	5100 (3)	1370 (3)	881 (3)
5	2670 (2)	1294 (3)	702 (2)
5	3820 (2)	1432 (2)	883 (2)
13	1521 (2)	2218 (2)	606 (2)
15	5169±2823 (3)	1259±49 (3)	727±108 (3)

(Results expressed as in Table 6).

Table 12. Glucuronidase activity in liver of young mice.

Age. (days)	Strain of mouse.	G.U./g. moist tissue.
1	Own colony Aug.1947	1370 (3)
4	" " Feb.1949	1246 (3)
5	" " Aug.1947	1363 (3)
7	" " Feb.1949	1345 (3)
13	" " Aug.1947	2218 (2)
14	CBA pure strain	1121 (4)
14	A pure strain	1090 (4)
15	Own colony Aug.1947	1239 (3)
15	" " Feb.1949	1043 (3)
15	C 57 pure strain	1171 (4)
19	Own colony Feb.1949	627 (3)
20	" " " "	673 (3)
21	" " " "	607 (3)
22	" " " "	636 (3)
23	" " " "	450 (3)
28	" " " "	298 (3)
29	" " " "	282 (3)
30	" " " "	248 (3)
32	" " " "	269 (3)

The effect of various substances on the enzyme assay in vitro.

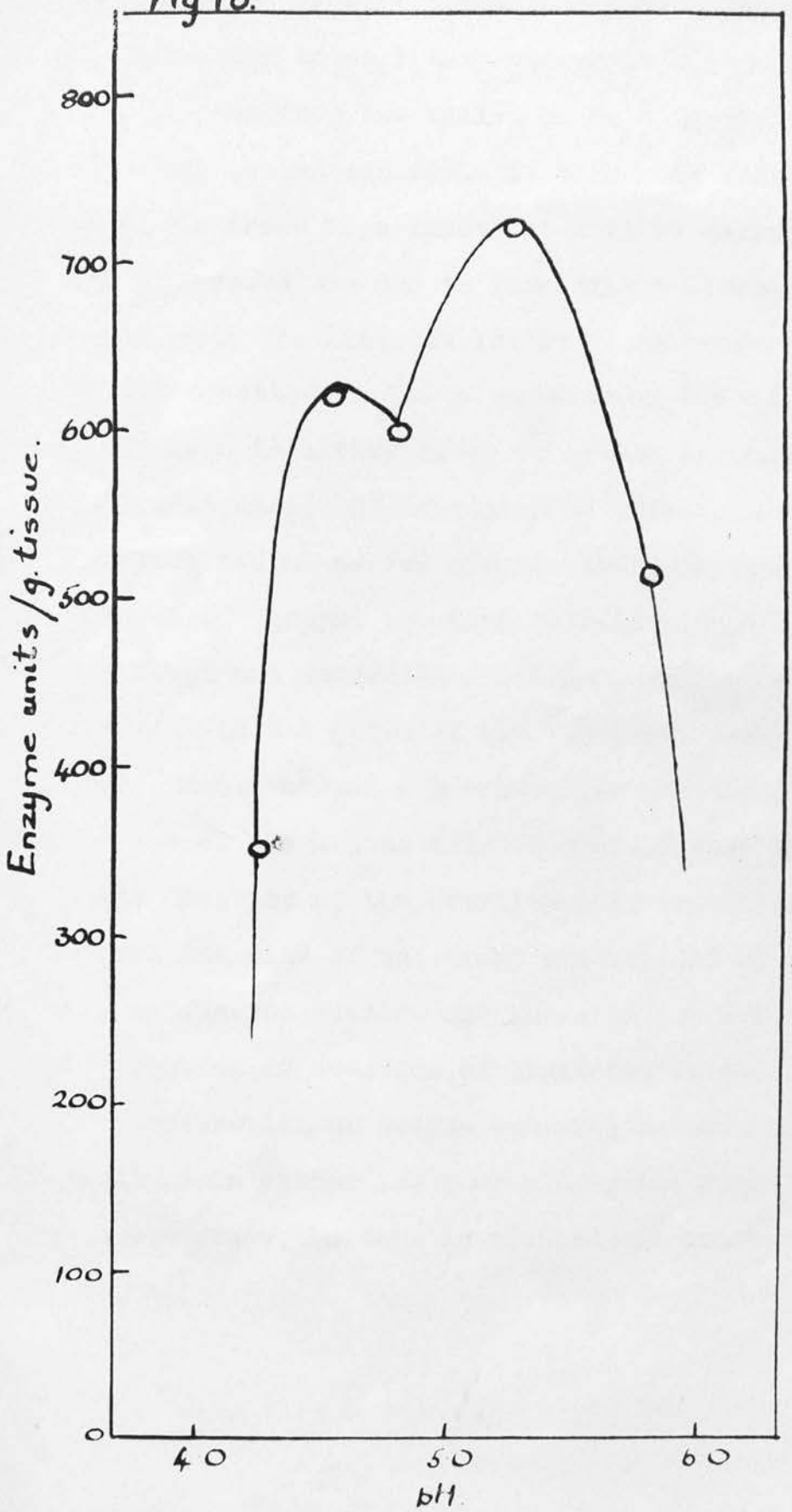
All the substances examined for their effect on β-glucuronidase in vivo, with the exception of pregnanediol-glucuronide, were also examined for their effect on the enzyme assay in vitro in a concentration of 0.1% (w/v). A solution, in the medium used for injection, was added to citrate buffer and the mixture vigorously shaken. In no case did the presence of the agent in the incubation mixture affect the activity of β-glucuronidase from normal mouse liver.

Total enzyme activity at pH 5.2 and the activity of the separated enzyme fractions A and B at pH 4.5 and 5.2 respectively.

The effect of administration of many compounds found to cause an increase in total enzyme activity in liver or kidney was also examined for their effect on the separated fractions of the enzyme from these organs. Initially it was found that the general shape of the pH-activity curve for enzyme from liver or kidney was not effected by changes in enzyme activity following the injection of carbon-tetrachloride. Both fractions A and B showed an increase in activity in approximately the same ratio as was found in normal animals, Fig. 18. The pH-activity curve has already been shown to be unchanged in shape with glucuronidase preparations from young animals, Fig. 17.

Notwithstanding these results a systematic examination was made of changes in fractions A and B. The homogenate was divided into two parts. From one fractions A and B were separated, using ammonium sulphate, and estimated at their respective pH optima, pH 4.5 and 5.2. The second part was used in determining the total activity of the organ at pH 5.2. The micro-method was used during this work to allow single organs to be fully examined. Since fraction A was determined at pH 4.5 and the total activity at pH 5.2 the figures for the latter shown in the following

Fig 18.


800
700
600
500
400
300
200
100
0
Enzyme units/g. tissue.
4 0
5 0
6 0
pH

tables do not equal the sum of the two fractions. The value for total kidney-enzyme in control animals will be seen from the tables to be slightly lower than that quoted earlier. As this was found consistently in these experiments it must be assumed that the difference was due to some slight alteration in technique. The distribution of glucuronidase activity between fractions A and B appears, on the average, to be the same in either liver or kidney for normal male and female mice. Castration 3 to 4 weeks before sacrifice had no marked effect on the results in either sex. Enzyme prepared from liver and kidney of treated and untreated animals was found to show variation in the ratio of the two fractions. Occasional animals showed a complete lack of fraction A or B in one of the organs with no compensatory increase in the activity of the fraction present. When this occured the size of the group was reduced by one in calculating the average and the standard error for the fraction in question, as indicated in the table. The distribution of enzyme activity between the two fractions in either liver or kidney was found to be, on the average, the same in treated and untreated animals.

The effect of various agents on fractions A and B in β-glucuronidase in liver and kidney.

Following the subcutaneous injection of 20mg./kg. mercuric nitrate, as a solution in 0.9% saline, fraction A and B in kidney were found to rise and fall together as repair processes became active and were completed. Liver was not studied in these experiments as mercuric nitrate had been found to be without effect on that organ.

Results obtained during the prolonged rise in glucuronidase activity in liver which follows the subcutaneous injection of carbontetrachloride showed no differentiation between fractions A or B. Kidney is not affected by carbontetrachloride. Except for a slight suggestion that fraction A returned to normal more rapidly than fraction B, similar results were obtained during the increased activity in liver after injection of chloroform, Table 13.

Many interesting results were obtained regarding the effect of chloroform on glucuronidase activity and histology of the kidney of male, female and castrated mice. Eschenbrenner (1944) found that chloroform caused renal necrosis in male but not in female mice and it was later shown by Eschenbrenner and Miller (1945) that the effect on males could be prevented by castration at an early age. The sex-linked nature of this response was associated with a difference in the structure of Bowman's capsule (Crab-

tree,1948). Chloroform necrosis involved the convoluted tubules,but did not extend to the capsule itself.

In the previous section it was seen that changes in enzyme activity and histology in kidney due to chloroform was confined to male mice. From the figures for kidney-glucuronidase activity given in Table 13 it appeared that castration 3 weeks before treatment abolished this response. There was no necrosis and Bowmans capsule had become predominantly female in character. In ovariectomised mice a small but significant rise (P-0.02 for results 1 day after injection;P-0.01,grouping all results) in kidney enzyme following injection of chloroform was found,associated with necrosis and repair. Extending the period between ovariectomy and injection from 3 to 13 weeks did not appreciably effect the results, but in the interval a change towards the male type of epithelium became more pronounced. The fact that normal mice showed no change in composition of the two enzyme fractions corresponding to changes in kidney morphology and that the rise in enzyme activity in male kidney appeared to be confined to fraction B may reflect an unequal distribution of the enzyme throughout the kidney with predominance of fraction B in the convoluted tubules. Friedenwald and Becker (1948),using histochemical techniques,showed a greater concentration of activity in rat kidney tubules than in the glomeruli when hydrolysis of a suitable

glucuronide by glucuronidase in unfixed frozen sections was allowed to proceed at pH 5.0.

Table 13.

The effects of various agents on fractions A and B in glucuronidase in liver and kidney.

Agent and dose	Inter-Ival. (days)	Sex.[x]	Liver. A.[xx]	Liver. B.[xxx]	Liver. Total.[xxx]	Kidney. A.[xx]	Kidney. B.[xxx]	Kidney. Total.[xxx]
None.	-	M	106±17 (83)	223±12 (6)	281±20 (6)	124±16 (6)	123±38 (6)	226±31 (6)
"	-	cM	115±12 (6)	247±38 (6)	334±48 (6)	109± 8 (6)	118±16 (6)	266±39 (6)
"	-	F	127±13 (6)	217±18 (6)	301±18 (6)	130±22 (6)	194±18 (6)	286±20 (6)
"	-	cF	116±12 (6)	167±47 (6)	250±49 (6)	113±10 (6)	130±22 (6)	261±43 (6)
Carbontetrachlor-ide,(5.5g./kg.)	1	M	545±18 (3)	654±40 (2)@	830±62 (3)	122±27 (3)	150±61 (3)	209±28 (3)
	4	M	364±58 (3)	537±27 (3)	763±65 (3)	118±19 (3)	138±18 (3)	257±19 (3)
	4	cF	499±109(3)	588±24 (3)	664±57 (3)	126±14 (3)	158± 6 (3)	235±23 (3)
	7	cF	517±35 (3)	568±35 (3)	693±31 (3)	1127±14 (3)	186±70 (3)	270±42 (3)
Chloroform,(2g./kg.).	1	M	343±188 (6)	766±147(6)	881±163 (6)	136±19 (6)	121±17 (6)	206±19 (6)
	4	M	260±42 (3)	561±44 (3)	766±46 (3)	128±18 (3)	234±22 (3)	302±12 (3)
	7	M	184±29 (6)	559±38 (3)	650±72 (6)	123±29 (6)	395±54 (6)	524±38 (6)
	1	F	537±64 (6)	410±194(6)	812±91 (6)	173±10 (6)	135± 9 (6)	227±23 (6)
	4	F	271±45 (3)	570±52 (3)	696±61 (3)	126±19 (3)	148±35 (3)	238±27 (3)
	7	F	151±42 (6)	559±106(6)	705±64 (6)	108±16 (6)	223±78 (6)	310±55 (6)
	1	cM	453±39 (3)	537±25 (3)	659±174(3)	119±13 (3)	141±17 (3)	221±38 (3)
	7	cM	152±20 (3)@	549±42 (3)@	673±32 (3)	132±20 (3)	124±22 (3)	238±30 (3)
	1	cF	277±22 (2)@	594±32 (2)@	575±23 (3)	187±21 (3)	256±29 (6)	398±67 (6)
	4	cF	224±25 (3)	510±20 (3)	620±26 (3)	189±15 (3)	247±18 (3)	352±24 (3)
	7	cF	359±150(5)	569±39 (5)	657±78 (5)	175±32 (7)	260±69 (7)	359±67 (7)
Mercuric nitrate, (20mg./kg.).	3	M	)-	-	-	269±35 (3)	319±55 (3)	463±59 (3)
	6	M	-	-	-	122±14 (3)	158±15 (2)@	277±19 (3)
	3	F	-	-	-	224±24 (3)	551±45 (3)	491±62 (3)
	6	F	-	-	-	122±10 (3)	124±26 (3)	237±61 (3)

x c-castrated.

xx One G.U.(glucuronidase unit) liberates 1ug.phenol from 0.015M phenol-glucuronide in 1hr.at 37' and pH 4.5.

xxx One G.U.(glucuronidase unit) liberates 1ug.phenol from 0.015M phenol-glucuronide in 1hr.at 37' and pH 5.2

@ One animal in the group devoid of this fraction.

Discussion.

In the work just described Fishman's results with menthol on liver and kidney glucuronidase in mice were confirmed. That no rise in glucuronidase activity was detected in spleen can be explained only by the wide variation normally seen in that organ, a variation which might be expected if glucuronidase activity reflects cell activity. After repeated feeding of menthol to mice Fishman obtained a rise in enzyme activity that was only a fraction of that produced in the present work by a single subcutaneous injection. In the experiments with uranyl acetate etc., reported above, normal or even reduced enzyme levels were found to be produced by an overdosage of the toxic agent. Intense poisoning thus appeared, by suppressing repair processes, to prevent an early rise in enzyme activity. Severe damage by repeated administration of menthol could therefore account for the small rises found by Fishman. Since many of the substances administered were nonglucuronidogenic, it would appear that the rise in glucuronidase activity provoked by menthol was associated with increased cellproliferation following damage by that compound rather than due to adaptation of a synthetic enzyme to excess substrate.

If glucuronidase acts synthetically in the body no effect would be expected on the enzyme activity or histology of animals after administration of

compounds already conjugated with glucuronic acid. The increase in activity, as well as the damage, in liver reported after injection of menthol-glucuronide was presumably due to hydrolysis of the glucuronide by enzyme already present in the liver. This is further upheld in the experiments with pregnanediol and its glucuronide. Pregnanediol, shown by Venning and Browne (1936) to form a glucuronide in the body, had no toxic action on either liver or kidney, nor did it cause a rise in glucuronidase activity in either organ. Sulphathiazol, found by Thorpe and Williams (1940) to give rise to a hydroxy compound which may be excreted as a glucuronide in rabbits and phenyl-arsenoxidewhich could conceivably form a derivative conjugated with glucuronic acid. Only with the latter compound which produced severe damage followed by repair in both liver and kidney was there any appreciable change in the glucuronidase activity. Sulphapyridine, also known to form a glucuronide (Scudi, 1944) was without effect on the enzyme or histological picture. It seems quite impossible for chloroform, carbontetrachloride, uranyl acetate, mercuric nitrate or yellow phosphorus to give rise to a glucuronide in the body and their action in stimulating glucuronidase activity must therefore be secondary to tissue damage and repair. The manner in which changes in glucuronidase activity parallel the response to chloroform poisoning of the kidney further substantiates this idea.

The close correlation between tissue proliferation and glucuronidase activity was also found in liver regenerating after partial hepatectomy and in the organs of young animals where no chemical stimulant was present. It is, however, impossible to say from the experiments just described whether β-glucuronidase is essential for celldivision or merely reflects an increase in cell activity. Both fractions A and B in liver and kidney were shown to respond similarly to extrinsic agents.

The possibility that the increased activity in the enzyme found by Fishman and Fishman (1944) and Fishman (1947) after treatment of mice with oestrogens could be explained on the basis of increased cell division in uterus is discussed in Section C. It was suggested by Fishman and Anlyan (1947) that in some cases of human carcinoma the tumour showed a higher glucuronidase activity than did the corresponding normal tissue. In view of the high rate of cell proliferation in tumour tissue this might be expected. The high blood-glucuronidase levels reported by Odell (1948) during pregnancy disorders may perhaps be open to a similar interpretation.

From this work it is no longer necessary to postulate a synthetic role for glucuronidase in the body to explain its response to agents such as menthol.

on the

SECTION C.

The effects of various agents on β-glucuronidase activity in uterus and other organs.

Introduction.

It has just been shown that the β-glucuronidase activity of mouse liver, kidney and spleen reflects the degree of cell proliferation in the organ and it seemed possible that the increased enzyme activity in uterus found by Fishman and Fishman (1944) after administration of oestrogens to ovariectomised mice could be explained on the basis of increased growth in that organ. In 1947 Fishman published results on the effect of testosterone propionate and oestradiol benzoate, separately and in combination, on uterine weight and glucuronidase activity. In these experiments testosterone was not found to antagonise the action of oestradiol in causing a rise in the glucuronidase activity in uterus and Fishman interpreted this as "a unique type of specificity by the oestrogen". At the dosage level of androgen used testosterone, by itself, caused an increase in uterine weight and enzyme activity. Administration of the androgen along with the oestrogen did not prevent an increase in the wet weight of the uterus. Fishman's results therefore seem entirely compatible with the view that an increase in enzyme activity in uterus, as elsewhere, reflects increased growth. His failure to observe antagonism between the oestrogen and the androgen resulted from the use of too large an excess of the latter.

A comparative study of the effects of

several of the sex hormones on the glucuronidase activity and histology of uterus, liver and kidney has been made. This has led to the discovery of new facts concerning the actions of these compounds. Four days after the injection of oestrone into ovariectomised mice, in addition to the expected rise in uterine weight and enzyme activity, a marked increase in the enzyme level and in cell division was observed in liver. Testosterone and progesterone, in doses without action on either liver or uterus, antagonised the effect of oestrone on enzyme activity and cell proliferation in both organs. Oestradiol, oestriol, and oestriol-glucuronide did not effect liver glucuronidase activity and the two former agents had only a slight stimulatory action on mitosis. The glucuronide was not examined for this effect.

One week after injection of ovariectomised mice with chloroform or carbontetrachloride, in addition to the expected changes in liver glucuronidase, an increase in the enzyme activity of uterus was observed. This was accompanied by an increase in wet weight in that organ. These results were not due to a direct toxic action of these compounds on the uterus but were secondary to liver regeneration since partial hepatectomy also stimulated uterine growth and enzyme activity. Testosterone and progesterone antagonised these changes in uterus without influencing the rise in liver glucuronidase activity associated with regeneration of this organ.

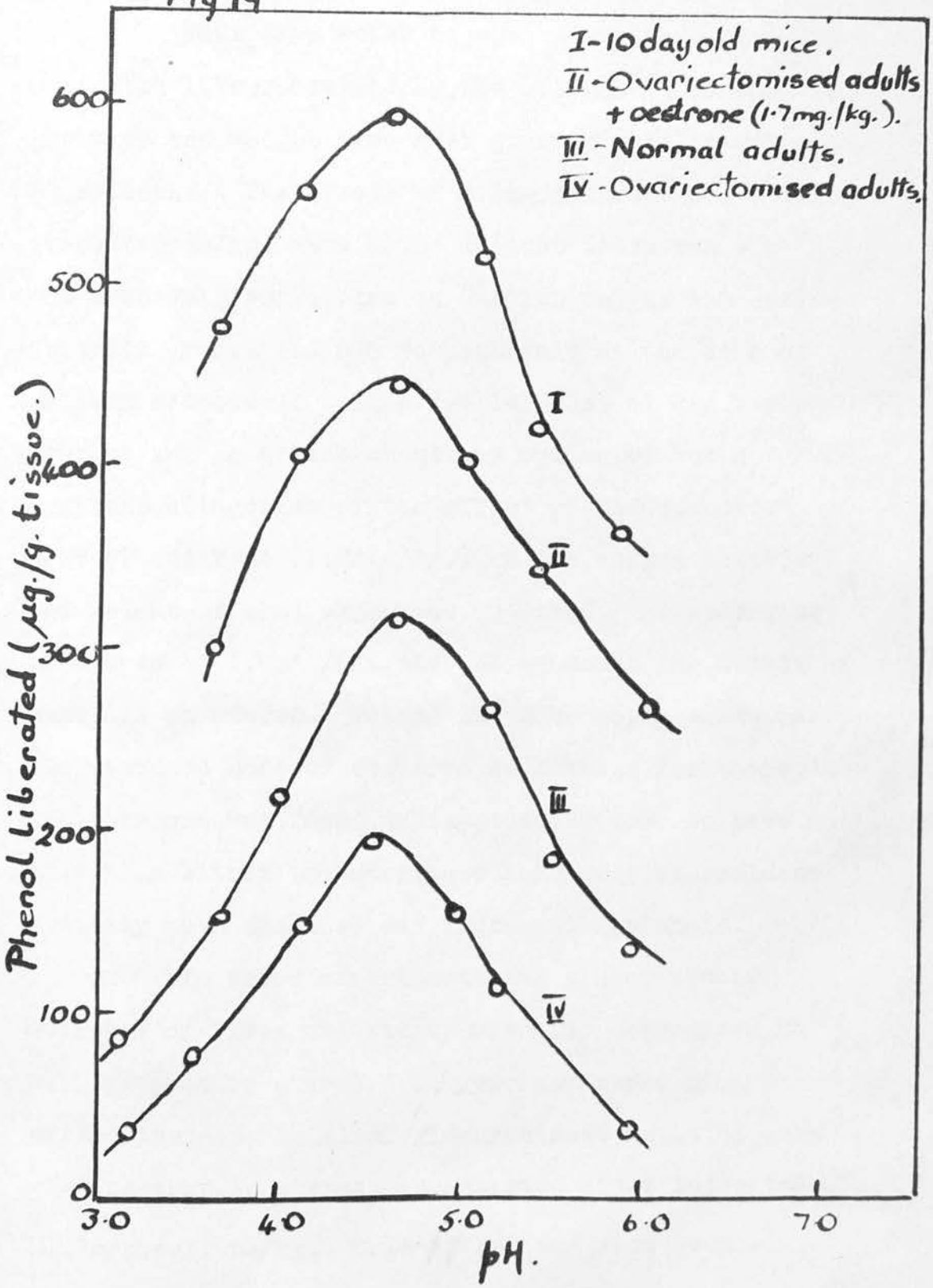
Results.

The β-glucuronidase activity of uterus.

The glucuronidase activity of uterus in normal mice has been shown to be confined to fraction A (see Section A). This suggested a difference in function of uterine glucuronidase compared with that of liver, kidney or spleen which contains both fractions A and B. The possibility of a change in composition of uterine enzyme occuring active growth was investigated. The pH-activity curve for enzyme prepared from the uteri of ovariectomised mice three days after subcutaneous injection of 1.7mg. oestrone/kg. a dose which produced full oestrus, was studied. The curve was again symmetrical about pH 4.5. This was also found to be the case with enzyme from ten day old mice. Fig. 19 compares the pH-activity curves found with uterine preparations from normal mice, mice three weeks after ovariectomy, oestrone treated mice and infant animals of ten days old. The shape of the curve will be seen to be the same in all instances. In order that all points on the curve might be determined simultaneously homogenates from three or more mice were pooled.

During the subsequent work uterine glucuronidase was precipitated at 50% saturation with ammonium sulphate and the total enzyme activity measured at pH 4.5 in citrate buffer. After each new treatment of mice estimations were also carried out at pH 5.2. In no instance was any enzyme detected other than fraction A at pH 4.5.

Fig 19


I-10 day old mice.
II-Ovariectomised adults + oestrone (1·7mg./kg.).
III-Normal adults.
IV-Ovariectomised adults.
Phenol Liberated (µg./g. tissue.)
600
500
400
300
200
100
0
I
II
III
IV
3·0
4·0
5·0
6·0
7·0
pH.

The effect of various sex hormones on β-glucuronidase activity in uterus, liver and kidney.

Four days after injection of ovariectomised mice with 1.7mg.oestrone/kg. the uterine glucuronidase activity and weight were much greater than in untreated controls. The effect of 0.3mg.oestrone and 3.3mg. testosterone/kg. were alike in that there was a comparatively small rise in uterine weight and enzyme activity (P-0.1 and 0.2 respectively at the time of maximum response). In a smaller dose of 2mg.testosterone/kg. no action on uterus was noted but a complete antagonism of the effect of the "standard" dose of oestrone (1.7mg./kg.) on the enzyme activity and weight of that organ was obtained. Progesterone, in a dose of 1.8mg./kg., also antagonised the action of oestrone on uterus. Taking the dose which antagonised the standard dose of oestrone as a basis for comparison progesterone was found, unlike testosterone, to have no effect on either the uterine weight or glucuronidase activity when the dose was increased threefold.

In these experiments the glucuronidase activity of liver and kidney was also determined as well as that of uterus. In ovariectomised mice a marked increase in liver-glucuronidase activity, preceeding that in uterus, was observed after injection of 1.7mg.oestrone/kg. This effect was also seen in normal and castrate males but was absent in normal female mice even when the dose was increased to 4.3 mg./kg. Table 14. This effect was abolished by

reducing the dose to 0.3mg./kg. Histological examination, by Dr.J.G.Campbell, revealed intense mitotic activity with little evidence of damage in the livers of castrate male and female mice. In normal males the effect was on mitosis was slight but there was a marked increase in the number of binucleate cells. Testosterone, 2mg./kg., and progesterone, 1.8mg./kg., both without action on liver, antagonised the effect of oestrone on glucuronidase activity and mitosis in that organ, Table 14.

Table 14.

The effects of oestrone, testosterone and progesterone on glucuronidase activity in liver, kidney and uterus.

Agent and dose.	Interval. (days)	Sex.[x]	G.U./g.moist tissue. Liver.[xx]	Kidney.[xx]	Uterus.[xxx]	Uterine weight. (mg.)
None.	-	F	334±48 (6)	266±39 (6)	333±53 (6)	234±56 (6)
	-	cF	250±49 (6)	261±39 (6)	174±45 (6)	34±18 (6)
	-	M	281±20 (6)	266±31 (6)	-	-
	-	cM	301±18 (6)	286±20 (6)	-	-
Oestrone, (1.7mg./kg.).	1	F	365±60 (3)	223±47 (3)	388±45 (3)	320±24 (3)
	4	F	267±26 (3)	253±41 (3)	441±87 (3)	253±31 (3)
	1	cF	431±42 (3)	313±17 (3)	300±54 (3)	26±5 (3)
	2	cF	481±34 (3)	275±67 (3)	343±47 (3)	47±8 (3)
	4	cF	569±73 (6)	281±33 (6)	548±106 (6)	211±20 (6)
	6	cF	399±67 (3)	356±52 (3)	346±41 (3)	52±10 (3)
	8	cF	315±18 (3)	299±23 (3)	223±16 (3)	46±5 (3)
	1	M	879±98 (3)	283±95 (3)	-	-
	4	M	303±12 (6)	221±50 (6)	-	-
	1	cM	359±43 (3)	324±22 (3)	-	-
	4	cM	562±58 (3)	333±52 (3)	-	-
Oestrone, (4.3mg./kg.)	1	F	258±39 (3)	370±33 (3)	463±41 (3)	167±66 (3)
	4	F	244±32 (3)	357±44 (3)	439±63 (3)	192±76 (3)
Oestrone, (0.3mg./kg.).	1	cF	271±51 (3)	359±72 (3)	181±13 (3)	51±10 (3)
	2	cF	274±34 (3)	321±33 (3)	247±38 (3)	47±2 (3)
	4	cF	269±34 (3)	327±61 (3)	226±34 (3)	102±15 (3)
	6	cF	256±38 (3)	305±22 (3)	181±36 (3)	63±9 (3)
Testosterone, (2mg./kg.).	1	CF	253±54 (3)	345±16 (3)	156±31 (3)	32±13 (3)
	4	cF	259±18 (3)	345±25 (3)	154±31 (3)	39±9 (3)
Testosterone, (3.3mg./kg.)	1	cM	271±53 (3)	172±14 (3)	-	-
	4	cM	311±36 (3)	201±23 (3)	-	-
	½	F	254±32 (3)	297±20 (3)	289±27 (3)	172±54 (3)
	4	F	272±40 (3)	343±48 (3)	326±19 (3)	193±35 (3)
	½	cF	236±62 (3)	339±38 (3)	199±20 (3)	57±12 (3)
	1	cF	289±34 (3)	319±56 (3)	260±14 (3)	101±19 (3)
	2	cF	284±25 (3)	363±46 (3)	185±35 (3)	44±6 (3)
	4	cF	251±48 (3)	326±46 (3)	189±39 (3)	35±11 (3)
Progesterone, (5.4 mg./kg.).	1	cF	277±19 (3)	379±18 (3)	172±12 (3)	33±5 (3)
	4	cF	272±19 (3)	372±18 (3)	189± 7 (3)	36±4 (3)
Progesterone, (1.8 mg./kg.)	1	cF	265±29 (3)	349±21 (3)	156±8 (3)	38±3 (3)
	4	cF	284±15 (3)	355±17 (3)	167±10 (3)	41±4 (3)
Testosterone, (2mg./kg.) and oestrone, (1.7mg./kg.).	1	cF	266±51 (6)	379±28 (6)	179±34 (6)	46±12 (6)
	4	cF	279±20 (5)	340±56 (5)	205±31 (5)	47±7 (5)
	1	M	261±20 (3)	314±51 (3)	-	-
	4	M	295±78 (3)	323±64 (3)	-	-
Progesterone, (1.8mg./kg.) and oestrone, (1.7mg./kg.).	1	cF	266±24 (3)	380±24 (3)	188±14 (3)	34±4 (3)
	4	cF	289±22 (6)	374±19 (6)	174±13 (6)	32±3 (6)

x c-castrated.

xx One G.U.(glucuronidase unit) liberates 1ug.phenol from 0.015M phenol-glucuronide in 1hr.at 37° and pH 5.2

xxx One G.U.(glucuronidase unit) liberates 1ug.phenol from 0.015M phenol-glucuronide in 1hr.at 37° and pH 4.5.

The action on liver of oestradiol and oestriol, in doses which produced effects in the uterus comparable to those shown for the "standard" dose of oestrone, was studied. Neither oestradiol nor oestriol had any effect on liver-glucuronidase activity even when the dose was increased to three-fold. The ability of these steroids to stimulate mitosis was slight compared with that of oestrone. Oestriol-glucuronide, found to cause only a small rise in enzyme activity and weight in the uterus even in a large dose (see Table 15), was also without effect on liver-glucuronidase activity. The glucuronide was not examined for histological effects on liver.

Table 15

The effect of various oesrtogens on glucuronidase activity in liver, kidney and uterus.

Agent and dose.	Interval. (days)	Sex[x]	G.U./g.moist tissue. Liver.[xx]	Kidney.[xx]	Uterus.[xxx]	Uterine weight. (mg.)
None	-	cF	250±49 (6)	261±43 (6)	174±45 (6)	34±18 (6)
	-	M	281 ±20 (6)	266±31 (6)	-	-
Oestrone, (1.7mg./kg.)	1	cF	431±42 (3)	313±17 (3)	300±54 (3)	26±5 (3)
	4	cF	569±73 (6)	281±33 (6)	548±106(6)	211±20 (6)
	1	M	879±98 (9)	283±95 (9)	-	-
	4	M	303±12 (6)	221±50 (6)	-	-
Oestradiol, (6mg./kg.)	1	cF	277±16 (3)	385±17 (3)	257±36 (3)	116±7 (3)
	4	cF	287±20 (3)	342±22 (3)	398±23 (3)	273±10 (3)
	1	M	292±19 (3)	352±13 (3)	-	-
	4	M	279±13 (3)	384±17 (3)	-	-
Oestradiol, (2mg./kg.).	1	cF	289±13 (3)	375±12 (3)	231±12 (3)	62±2 (3)
	4	cF	301±15 (3)	368±17 (3)	357±15 (3)	291±4 (3)
Oestriol, (5mg./kg.).	1	cF	289±22 (3)	338±33 (3)	451±38 (3)	275±16 (3)
	4	cF	279±26 (3)	367±26 (3)	506±41 (3)	298±21 (3)
	1	M	281±17 (3)	378±18 (3)	-	-
	4	M	295±18 (3)	368±20 (3)	-	-
Oestriol, (2.5mg./kg.).	1	cF	281±17 (3)	351±22 (3)	209±11 (3)	57±6 (3)
	4	cF	306±22 (3)	376±26 (3)	305±15 (3)	257±11 (3)
Oestriol-β-d-glucuronide, (13.8 mg./kg.).	2	cF	341±22 (3)	361±27 (3)	235±14 (3)	75±8 (3)
	6	cF	271±15 (3)	372±19 (3)	309±17 (3)	114±10 (3)
					177±20 (3)	45±6 (3)
Oestriol-β-d-glucuronide, (4.6 mg./kg.).	2	cF	268±25 (3)	354±34 (3)	299±12 (3)	89±11 (3)
	4	cF	273±17 (3)	373±30 (3)	319±9 (3)	105±7 (3)
	6	cF	279±19 (3)	376±31 (3)		

Footnote as for Table 14.

The effect of liver regeneration on β-glucuronidase activity and weight in uterus.

An enhancement in the potency of administered oestrogen has been noted by several workers in animals poisoned with carbontetrachloride or in animals after partial hepatectomy. They consider this to be due to a disturbance of the normal metabolism of the oestrogen in the liver. In these experiments the animals were treated with an oestrogen or alternatively the ovaries were still present. It was therefore with surprise that increases in glucuronidase activity and weight in the uterus were observed 7 days after treatment of ovariectomised mice with carbontetrachloride or chloroform, Table 16. At the end of 10 days the weight and enzyme activity had fallen considerably towards the normal values.

This effect was not due to a direct action of the toxic agent on the uterus but was secondary to liver regeneration. This follows from the finding that 8 days after subjecting ovariectomised mice to partial hepatectomy there was a similar rise in uterine glucuronidase activity and weight. With all methods of treatment the rise in ~~uterus~~ uterine weight at its greatest was statistically significant ($P = 0.001$). By 12 days when liver repair was well advanced the enzyme level and weight of the uterus had fallen again.

These compounds were given as single subcutaneous injections of a solution in olive oil and any variation in the strain of mouse controlled by comparison with untreated ~~controls~~ mice.

As before, all substances examined for their effects on glucuronidase activity *in vivo* were also tested for their effect on the enzyme assay *in vitro* in a concentration of 0.1% (w/v). No change in activity was observed with enzyme from normal animals.

Table 16.

The effect of liver regeneration on uterine weight and glucuronidase activity in ovariectomised mice.

Agent and dose.	Interval. (days)	G.U./g.moist tissue. Liver.xx	Kidney.xx	Uterus.xxx	Uterine weight. (mg.)
None.	-	250±49 (6)	261±43 (6)	174±45 (6)	54±18 (6)
Chloroform,(2g./kg.).	1	515±25 (3)	398±67 (5)	181±33 (5)	28±10 (5)
	4	620±26 (5)	352±24 (5)	162±51 (5(	55±7 (5)
	7	557±78 (5)	359±67 (7)	469±51 (7)	105±20 (7)
Chloroform,(6g./kg.)@.	8	605±75 (5)	455±56 (5)	386±61 (5)	99±18 (3)
	10	501±50 (5)	355±52 (5)	306±21 (5)	65±12 (5)
Carbontetrachloride, (5.5g./kg.).	1	712±55 (3)	301±42 (5)	203±48 (5)	25±6 (5)
	4	664±57 (3)	235±25 (5)	285±52 (5)	18±9 (5)
	7	715±60 (5)	290±59 (5)	496±64 (6)	77±25 (6)
	10	579±56 (5)	293±12 (5)	205±20 (5)	42±5 (5)
Partial hepatectomy.	2	572±53 (5)	284±22 (5)	225±67 (5)	75±14 (5)
	4	552±85 (5)	305±29 (5)	240±42 (5)	64±8 (5)
	6	625±85 (5)	342±54 (5)	412±51 (5)	123±14 (5)
	8	555±61 (6)	328±54 (6)	395±45 (6)	115±19 (6)
	12	424±41 (5)	329±52 (5)	277±29 (5)	55±6 (5)
Mercuric nitrate,(20 mg./kg.).	3	241±32 (3)	523±27 (5)	179±18 (3)	50±4 (5)
	6	238±26 (5)	325±24 (5)	168±26 (5)	59±6 ((5)

Footnote as for Table 14.

@-Divided into 3 daily doses;timed from first dose.

In a separate group of 6 mice the uteri were examined histologically by Dr.J.G.Campbell 9 days after partial hepatectomy. Metoestrus, pro-oestrus and, in one case, full oestrus were observed, as compared with dioestrus in ovariectomised controls.

Vaginal smears from ovariectomised mice were examined during much of the treatment described above. On the basis of the Marrian and Parkes Criteria, a 100% positive response was obtained after injection of 1.7mg.oestrone/kg. while only 75% were positive after treatment with the smaller dose of oestrone (0.3mg./kg.) and 50% after the larger dose of testosterone (3.3mg./kg.). The combined dose of oestrone and testosterone and the ~~larger~~ smaller dose of testosterone alone produced completely dioestrus smears.

By comparison with the smears from the oestrone treated mice, those taken during the eight days following partial hepatectomy or injection with chloroform or carbontetrachloride were only very weakly positive. In 60% of those examined, leukocytes were present along with cornified cells or mucus, and nucleated cells were observed. Only in one instance was a truely positive smear noted (after treatment with chloroform).

Professor Marrian kindly examined several smears from mice during liver regeneration and he has come to the following conclusion :-

"The smears are not positive on the basis of the Marrian and Parkes Criteria. In many smears there

was a lack of leukocytes and considerable mucification. In some there was a lack of leukocytes and nucleated epithelial cells were present while in others cornified cells and leukocytes were observed. The action appeared to be similar to that observed with a dose of oestrogen just below that required for a positive smear on the basis of the Marrian and Parkes Criteria".

Smears were taken from untreated control ovariectomised mice for eight days. In no case was a positive response observed.

As these changes in uterus could be explained by the action of an extra-ovarian oestrogen, it was thought that testosterone and progesterone might antagonise the effects. Testosterone,2.0mg./kg. and progesterone,1.8mg./kg., injected on the first and fourth day after partial hepatectomy,both prevented any increase in uterine weight or enzyme activity. It will be seen from Table 17 that these agents had no effect on the enhanced liver enzyme accompanying proliferation of the remaining fragment of that organ. The increase in weight of the uterus during liver regeneration is not so great as that produced by the "standard" dose of oestrone but rather of the order found with 0.5mg.oestrone/kg. This might be expected from a comparison of the vaginal smears.

Table 17.

The antagonism of the effect of liver regeneration on uterine weight and glucuronidase activity in ovariectomised mice.

Agent and dose.	Interval. (days)	G.U./g.moist tissue.		Uterine weight. (mg.)
		Liver.	Uterus.	
None.	-	250±49 (6)	174±45 (6)	34±18 (6)
Partial hepatectomy.	8	555±61 (6)	395±45 (6)	115±19 (6)
Partial hepatectomy and testosterone, (2mg./kg.on 1st. and 4th.days after).	8	572±48 (6)	247±27 (6)	45±7 (6)
Partial hepatectomy and progesterone, (1.8mg./kg.on 1st. and 4th.days after).	8	509±36 (5)	186±16 (5)	58±5 (5)

Footnote as for Table 14.

Discussion.

The glucuronidase activity in uterus as in other organs, such as liver and kidney, may be taken as an index of the state of growth of the tissue. No specificity of response of the enzyme, as suggested by Fishman (1947), is needed to explain the increases in activity observed after injection of the oestrogens An increase in enzyme activity was accompanied, in every case, by an increase in growth of the uterus, as measured by an increase in weight. By adjusting the dose of testosterone to a level itself without any oestrogenic activity, it was possible to antagonise the action of oestrone in stimulating both uterine growth and enzyme activity.

The action of oestrone in stimulating mitosis in liver in normal and castrate male and in ovariectomised mice, and the antagonism of this by testosterone, is in accordance with the finding of Bullough (1946). From a systematic study of the action of oestrone on mitosis, he concluded that the effects of such hormones were more widespread throughout the body than was generally recognised. Bullough, using normal female mice, observed no action on liver, confirming our results. This might suggest some form of control of the liver by the ovary.

The increase in weight and glucuronidase activity in uterus following partial hepatectomy or the injection of chloroform or carbontetrachloride, and the antagonism of this by testosterone and

progesterone can be explained only on the basis that the body is capable of producing an extra-ovarian oestrogen. Increased activity on the part of the pituitary is eliminated as this gland acts indirectly through a stimulation of the ovary and only ovariectomised mice were used.

Carbontetrachloride poisoning was shown by Talbot (1939) to cause an increase in the weight of the uterus in immature rats,and Pincus and Martin (1940) to enhance the effectiveness of administered oestrone. Partial hepatectomy causes a similar increase in potency of administered oestrogen , (Segaloff,1946). It appears from the work of Roberts and Szego (1947) that the increase in sensitivity to oestrogens occurs during active liver regeneration rather than during the initial stages of damage. As already stated,the animals were treated with an oestrogen or the ovaries were stillpresent in these experiments. In view of our observations in ovariectomised mice,in the absence of administered oestrogen,it would seem that thses results should be reinterpreted. The action of carbontetrachloride and chloroform on uterus and of oestrone on liver completely rules out Fishman's view of a distinction between uterine glucuronidase on the one hand and the enzyme in liver on the other.

In a study of the oestrogenic activity of many compounds Emmens (1943) found that they could be divided into two groups,"true"oestrogens and "pro-

oestrogens". Pro-oestrogens, unlike true oestrogens, were no more effective when administered locally than when given systemically. Substances with a feebly oestrogenic activity, such as pro-oestrogens, must be suspected of acting through liver mediation. The fact that a true oestrogen is excreted in the urine following administration of these compounds, does not exclude the possibility that they acted primarily on the liver. In view of the effect of oestrone in stimulating mitosis in liver, it is interesting to speculate how far its action on the uterus is of a secondary nature.

SECTION.D.

The effectof growth inhibitors on β-glucuronidase activity in liver,kidney and uterus.

Introduction.

β-Glucuronidase activity has been shown to parallel cell proliferation closely but it was not possible to say whether the action of the enzyme was essential for division of the cell or whether it merely reflected an increase in specialised activities of the cell. It was thought possible that some indication of the rôle of glucuronidase might be obtained by studying the effect on the enzyme of various substances known to inhibit growth in some manifestation or other.

Colchicine has been shown to cause inhibition of cell division in normal tissue and to prevent growth of pathological tissue, (Litz, 1938). The effect of the oestrogens in stimulating growth of the uterus can be prevented by colchicine, (Williams, 1941). In normal adults colchicine, in the dose used, did not affect the enzyme level in liver, kidney or uterus, but several interesting observations were made when this compound was administrated to animals in which mitosis had been stimulated.

In 1943 Medawar et al. isolated an unsaturated lactone, hexenolactone, from malt which they went on to show to be capable of causing a selective inhibition of growth in connective tissue. The closely related compound, sorbic acid, has been found to inhibit growth in young animals and its action on glucuronidase has been studied.

Substituted stilbenes and anilines of the

type found by Haddow and Kon (1947) to inhibit growth in pathological tissue had no effect on glucuronidase activity *in vivo* under the conditions studied. The Lederle Laboratory antifolic acid factor was also found to be without action on the enzyme. This compound is an analogue of folic acid with an extra methylene group in the linkage to the pteridyl group.

Xanthopterin, found by Lewisohn (1944) to inhibit growth in tumours, produced no change in the enzyme activity in normal adult liver but caused a rise in kidney when injected over a period of several days. The increase in kidney-glucuronidase activity could be prevented by sorbic acid. Haddow (1948) has found that xanthopterin caused hypertrophy in mouse kidney.

Saccharic acid was shown by Karunairatnam and Levvy (1949) to cause almost complete inhibition of the *in vitro* hydrolysis of phenol-glucuronide by β-glucuronidase. The possibility that this compound might inhibit the enzyme *in vivo* was investigated. In normal animals no depression of the enzyme level in liver, kidney or uterus was observed even when saccharic acid was given in a very large dose over a considerable period of time. It was, however, found to cause a depression in the elevated value of liver enzyme obtained after ~~partial hepatectomy~~ treatment of ovariectomised mice with oestrone or normal mice with menthol-glucuronide. As this was also observed when an equal quantity of glucose was substituted for

the saccharic acid it may be assumed to be due simlpy to a protective action on the part of these compounds for liver tissue.

Results.

The effect of certain growth inhibitors on β-glucuronidase activity in vitro.

It was thought possible that compounds known to cause an inhibition of mitosis might also have an inhibitory action on the *in vitro* hydrolysis of phenol-glucuronide by glucuronidase prepared from normal tissue. Of the substances tested none had any effect on glucuronidase activity *in vitro* when added to the incubation mixture in the concentrations shown. Colchicine and 2'methyl4-dimethylaminostilbene gave a colour with the phenol reagent but corrections for this were made in calculating the inhibitory effect.

Table 18.

Inhibitor.	Conc. (%)	Hydrolysis (ug.phenol)	Inhibitor blank. (ug.phenol)	Percentage inhibition.
None	-	9.35	-	-
Xanthopterin	1	9.35	-	o
Sorbic acid	1	9.35	-	0
NN-di(chloro-ethyl)aniline	0.1	9.34	-	0
2'methyl-4-di-methylamino-stilbene.	0.1	10.46	1.15	0
Colchicine	1	11.48	2.10	0

The effect of colchicine on the β-glucuronidase activity of liver, kidney and uterus.

A single subcutaneous injection of 1.5mg. colchicine/kg., as a neutral solution in 0.9% saline, was found to have no effect on the glucuronidase activity of liver, kidney or uterus in normal animals. When this dose was injected into ovariectomised mice along with our "standard" dose of oestrone no increase in uterine weight or enzyme activity was observed and the increase in liver-glucuronidase activity was much less marked than with oestrone alone (see Section C). Thus by inhibiting the growth of the uterus, colchicine had prevented any rise in enzyme activity.

A very definite increase in enzyme activity has been shown to occur in liver following partial hepatectomy, (Section B). This rise was not seen if colchicine was injected 6 hours after the operation and the animals were about to expire when killed on the fourth day. If, however, the interval between operation and injection was increased to 24 hours the enzyme level was found by the fourth day to have risen and regeneration to have commenced. Colchicine in a dose of 1mg./kg. was found by Scheifley and Higgins (1940) to arrest regeneration of rat liver after partial hepatectomy only if given in the initial stages of recovery.

Table 19

The effect of colchicine on glucuronidase activity in liver, kidney and uterus.

Agent and dose.	Interval. (days)	Sex.[x]	G.U./g.moist tissue. Liver.[xx]	Kidney.[xx]	Uterus.[xxx]	Uterine weight. (mg.)
None	-	M	281±20 (6)	266±31 (6)	-	-
	-	♂F	250±49 (6)	261±43 (6)	174±45 (6)	34±18 (6)
Colchocine,(1.5 mg./kg.).	2	M	275±22 (5)	277±42 (5)	-	-
	2	♂ F	282±16 (5)	379±51 (5)	187±22 (5)	39±8 (5)
Partial hepatectomy	4	M	888±54 (5)	375±57 (5)	-	-
Partial hepatectomy, and colchicine,(1.5 mg./kg.6hrs.after).	4	M	224±31 (5)	271±28 (5)	-	-
Partial hepatectomy and colchicine,(1.5 mg./kg.24hrs.after).	4	M	718±68 (5)	280±58 (5)	-	-
Oestrone,(1.7mg./kg	1	♂F	451±42 (5)	315±17 (5)	500±54 (5)	26±5 (5)
	4	♂F	569±75 (5)	281±55 (5)	548±106(6)	211±20 (6)
Oestrone,(1.7mg./kg and colchicine,(1.5 mg./kg.together).	1	♂F	307±9 (5)	370±23 (5)	246±52 (5)	52±2 (5)
	4	♂F	520±20 (5)	562±19 (6)	251±27 (6)	40±8 (6)

Footnote as for Table 14.

The effect of colchicine on the rate of growth of young mice was studied. Litters were removed from their mothers and divided into two groups, one receiving a dose of 1mg. colchicine/kg. by subcutaneous injection every second day and the other an equal volume of saline by the same route. Both groups were weighed daily and the percentage change in weight calculated over the period of treatment. As expected colchicine prevented any growth in the mice and they were about to expire when killed. The glucuronidase activity in the livers of the colchicine treated animals was less than half that found in the controls after about one week. Results for two separate experiments are shown in the table.

Table 20.

Treatment.	Age at start of experim. (days)	Days under treatment	%increase in body weight.	G.U./g.liver at end of treatment.
Colchicine	9	9	-1.5(4)	347
Controls	9	9	25.0(4)	673
Colchicine	10	5	-1.0(4)	310
Controls	10	5	13.5(4)	1043

(No. of animals in group given by figure in brackets)

The effect of sorbic acid on β-glucuronidase activity in liver, kidney and uterus.

Since sorbic acid might be expected to behave like hexenolactone, it was studied for its effects on glucuronidase activity *in vivo*. It was injected as a neutral solution in 0.9% saline.

Following a single subcutaneous injection of either 120mg. or 160mg. sorbic acid/kg. into normal mice no change in the glucuronidase activity of liver, kidney or uterus was observed and the animals were normal in gross appearance. Histological examination, by Dr. J.G. Campbell, of organs from animals receiving the larger dose revealed slight damage, cloudy swelling and mitosis in liver while kidney was normal. When the dose was increased still further, to 240mg./kg., there was a drop in the enzyme level in liver and kidney, in some cases to zero. This was not seen until the fourth day. Uterine glucuronidase was unaffected at any time. Histological examination showed slight damage in liver with little sign of repair while in kidney there was no evidence of damage. When a dose of 360mg./kg. was used the animals did not survive the second day. Estimation of the glucuronidase activity in such animals, killed during the first two days, showed normal enzyme levels in liver, kidney and uterus but histological examination showed severe necrosis without repair in liver and very slight nephritis in kidney. There was evidence of an arrest of mitosis

throughout the body. It should be noted that the dose of 240mg./kg. had no effect on the enzyme level in any organ after 2 days.

We have previously seen (Section B) how an over dosage of the toxic agent may prevent a rise ~~in~~ or may even cause a fall in the glucuronidase activity of the organ affected. Overdosage of the toxic agent could explain the effect of 240mg. sorbic acid/kg. on the liver enzyme but the effect on kidney can be explained only on the basis of an arrest of mitosis .

Table 21.

The effect of a single injection of sorbic acid on glucuronidase activity in liver, kidney and uterus

Dose.	Interval. (days)	Sex.	G.U./g.moist tissue. Liver.	Kidney.	Uterus.	Uterine weight. (mg.)
None.	-	M	281±20 (6)	266±51 (6)	-	-
	-	cF	250±48 (6)	261±43 (6)	174±45 (6)	54±18 (6)
	-	F	554±48 (6)	266±59 (6)	355±53 (6)	234±56 (6)
120mg./kg.	2	M	282±45 (5)	267±53 (5)	-	-
	4	F	250±42 (5)	551±25 (5)	257±17 (5)	209±9 (5)
160mg./kg.	2	M	279±37 (5)	299±29 (5)	-	-
	4	M	282±40 (5)	537±27 (5)	-	-
	6	M	269±58 (5)	282±55 (5)	-	-
	2	cF	277±26 (5)	298±32 (5)	179±8 (5)	56±7 (5)
	4	cF	291±21 (5)	546±27 (5)	185±11 (5)	51±9 (5)
240mg./kg.	2	M	284±37 (5)	361±27 (5)	-	-
	4	M	27±20 (12)	31±25(12)	-	-
	5	M	135±21 (5)	254±19 (5)	-	-
	6	M	240±29 (5)	345±26 (5)	-	-
	9	M	287±27 (5)	389±24 (5)	-	-
	11	M	327±18 (5)	394±51 (5)	-	-
	2	F	275±21 (5)	360±27 (6)	275±19 (6)	247±17 (6)
	4	F	17±9 (12)	34±28 (12)	301±20(12)	202±14(12)
360mg./kg.	1	M	259±26 (5)	370±36 (5)	-	-
	2	F	245±31 (5)	345±28 (5)	265±19 (5)	209±17 (5)

Footnote as for Table 14.

When given as a single injection 240mg. sorbic acid/kg. was found to be without effect on the changes in liver-glucuronidase activity provoked by oestrone or carbontetrachloride but the effect on the normal kidney level was sometimes seen ,Table 22. The rise in liver enzyme secondary to partial hepatectomy was,however,arrested.

Table 22.

The effect of a single injection of 240mg./kg. sorbic acid on changes in glucuronidase activity in liver,kidney and uterus.

Other treatment.	Interval. (days)	Sex.[x]	G.U./g.moist tissue. Liver.[xx]	Kidney.[xx]	Uterus.[xxx]	Uterine weight. (mg.)
Carbontetrachloride, (5.5g./kg.) 6 days before sorbic acid.	10	M	324±37 (3)	371±27 (3)	-	-
Carbontetrachloride, (5.5g./kg.) with sorbic acid.	4	M	576±49 (3)	62±28 (3)	-	-
Carbontetrachloride, (5.5g./kg.) 4 days after sorbic acid.	6	M	790±52 (3)	324±29 (3)	-	-
Partial hepatectomy with sorbic acid.	4	M	298±32 (6)	78±25 (6)	-	-
Oestrone, (1.7mg./kg.) with sorbic acid.	4	cF	503±25 (3)	358±26 (3)	329±27 (3)	401±29(3)
	4	♂	428±32 (3)	367±35 (3)	-	-

Footnotes as for Table 14.

The effect of a cumulative dose of sorbic acid was tried. In normal adult mice 160mg. sorbic acid/kg. produced no effect on the glucuronidase activity of liver, kidney or uterus when injected daily for periods up to 10 days. This dose did, however, prevent growth in young animals. Litter mates, 10 days old, were removed from their mother and divided into two groups, one receiving sorbic acid and the other saline for 12 days. At the end of this period the sorbic acid treated animals showed only a 9% increase in weight as compared with 66% in the control animals. The glucuronidase activity in liver was one third of that in the control animals. Several of the sorbic acid injected mice were moribund when killed. Only slight damage with no cell division was observed histologically in liver, and the kidney was undamaged. Increasing the dose of sorbic acid to 240mg./kg., but injecting this dose only every third day, caused no alteration in the growth rate of two weeks old mice over a further period of two weeks.

Table 23.

The effect of sorbic acid on the growth rate of young mice.

Treatment.	Age at start of expt (days)	Days under treatment.	% increase in body weight.	G.U./g.liver at end of treatment.
Sorbic acid, (240mg./kg.) every 3 days.	14	14	9	156 (4)
Controls.	14	14	9	298 (4)
Sorbic acid, (160mg./kg.) daily.	10	12	9	286 (8)
Controls.	10	12	66	605 (8)

Repeated daily injection of 160mg. sorbic acid/kg. was also found to be without effect on the high glucuronidase activity seen in liver after injection of carbontetrachloride or in liver and uterus after oestrone. A definite depression in liver enzyme was noted with this dose of sorbic acid after partial hepatectomy. The rise in uterine weight and enzyme activity associated with liver regeneration (Section C) was also absent in ovariectomised partially hepatectomised mice.

A daily injection of 240mg. sorbic acid/kg. killed the animals. No reduction in glucuronidase activity was observed when this dose of sorbic acid was injected every second day and the animals partially hepatectomised or injected with oestrone or carbontetrachloride on the third day of treatment. As with colchicine, the time interval between sorbic acid administration and the other treatment seems to be important.

It is apparent that sorbic acid acts like colchicine but that the dose cannot be increased to a level necessary to inhibit all the stimulatory actions studied without death supervening due to the toxicity for liver and other organs.

Table 25.

The effect of repeated administration of sorbic acid on changes in glucuronidase activity in liver, kidney and uterus.

Other treatment.	Interval. (days)	Sex.	G.U./g.moist tissue. Liver.xx	Kidney.xx	Uterus.xxx	Uterine weight. (mg.)
Sorbic acid,(160mg./kg.)daily.	6	cF	258±26 (5)	582±36 (5)	175±20 (5)	41±8 (5)
	10	cF	265±24 (5)	559±25 (5)	168±16 (5)	35±7 (5)
Carbontetrachloride, (5.5g./kg.)5 days after 1st.sorbic acid.	10	M	858±48 (5)	378±25 (5)	-	-
Partial hepatectomy, 5 days after 1st sorbic acid.	10	cF	525±36 (5)	547±26 (5)	142±18 (3)	46±9 (5)
	10	M	307±29 (5)	507±32 (5)	-	-
Oestrone,(1.7mg./kg) 5 days after 1st sorbic acid.	7	cF	468±25 (5)	562±29 (5)	377±25 (5)	197±8 (5)
Sorbic acid,(240mg./kg.)every 2nd.day.						
Carbontetrachloride, (5.5g./kg.)3 days after 1st.sorbic acid.	10	cF	822±39 (5)	549±26 (5)	272±19 (5)	88±10(3)
Partial hepatectomy, 3 days after 1st sorbic acid.	10	cF	646±27 (5)	578±36 (5)	505±19 (5)	130±19(5)
Oestrone,(1.7mg./kg) 3 days after 1st. sorbic acid.	7	cF	407±28 (5)	548±29 (5)	270±18 (5)	173±18(5)

Footnote as for Table 14.

The effect of other "growth inhibitors" on β-glucuronidase activity in liver and kidney.

The effect of certain other growth inhibitors on the glucuronidase activity of liver and kidney in vivo was studied. The inhibitory action on tissue growth of such compounds as 2¹-methyl-4-dimethylaminostilbene and NN-di(chloroethyl)aniline was described by Haddow (1947). Saturated solutions of these compounds in 0.9% saline were injected daily into adult mice and the glucuronidase activity of liver and kidney estimated after 6 and 12 days. No change in the enzyme activity was observed after either period.

The important part played by folic acid in normal growth is now well known. In 1948 Lederle Laboratory produced an antagonist to the vitamin which was found to produce liver necrosis, a decrease in body weight and eventually death in animals fed a diet containing this substance (Franklin et al.1948). Mice were fed 1% antifolic acid factor (kindly supplied by the Lederle Laboratory) in the diet for periods up to 5 weeks. The livers and kidneys of these animals were normal in gross appearance and enzyme activity, as is shown in Table 26.

Table 26.

The effect of other "growth inhibitors" on glucuronidase activity in liver and kidney.

Treatment.	Days.	G.U./g.moist tissue.	
		Liver.	Kidney.
None.	-	275±13 (25)	365±24 (11)
2-methyl-4-dimethylamino-stilbene, (100mg./kg.daily)	6	254±36 (5)	337±31 (5)
	12	296±16 (5)	557±42 (5)
NN-di(chloroethyl)aniline, (134mg./kg.daily)	6	269±25 (5)	355±36 (5)
	12	282±37 (5)	369±23 (5)
Antifolic acid factor, (1% fed in diet).	21	279±19 (5)	337±25 (3)
	56	276±22 (3)	359±12 (3)

Results expressed as in Table 6.

The effect of xanthopterin on β-glucuronidase activity in liver and kidney.

According to Lewisohn et al.(1944) this compound inhibits tumour growth. Haddow (1948) has observed that after repeated administration it causes hypertrophy of the mouse kidney. Daily subcutaneous injection of 200ug. xanthopterin/kg.,as a neutral solution in 0.9% saline,for 14 days had no effect on liver glucuronidase activity but caused an increase in the activity of the enzyme in kidney. Sorbic acid (160mg./kg.daily) counteracted the action of xanthopterin on the kidney enzyme activity,Table 27.

Table 27.

Treatment.	Days.	G.U./g.moist tissue	
		Liver.	Kidney.
Xanthopterin(200ug./kg.Sci.daily)	14	280±13 (6)	490±33 (6)
Xanthopterin(200ug./kg.Sci.daily) and sorbic acid(160mg./kg.Sci.daily)	14	117±30 (3)	267±51 (3)

The effect of saccharic acid on β-glucuronidase activity in liver, kidney and uterus.

In view of the inhibitory action for glucuronidase activity described by Karunairatnam and Levvy (1949) many experiments were carried out by the author and Mr. Karunairatnam in an attempt to show an action of this compound on glucuronidase activity in vivo. Some of the more recent and most interesting experiments carried out by the author are described in this section.

Saccharic acid was given by subcutaneous injection of a neutral solution of the mono-potassium salt in 0.9% saline. Injection of 2.5gm. K-H-saccharate/kg. thrice daily for periods up to 10 days had no effect on normal glucuronidase activity in liver, kidney or uterus. An earlier dosage level of 0.3gm. K-H-saccharate/kg. thrice daily was also without effect

Antagonism of measures stimulating glucuronidase activity in liver, and in some cases uterus, by saccharate was studied. These measures were partial hepatectomy or administration of carbontetrachloride or oestrone, as previously described. The response of glucuronidase activity in liver and uterus to carbontetrachloride or partial hepatectomy was unaffected by either of the above dosage levels of saccharate. The effect of oestrone on the glucuronidase activity in the liver of ovariectomised mice was antagonised by the smaller dose of saccharate while the uterine response and enzyme activity was unaffected. It was

with surprise that the higher dose of saccharate was found not to prevent the rise in glucuronidase activity after oestrone administration, and no explanation can be offered for this anomolous result.

Glucose and KCl, in equivalent dose to the saccharate, (0.3g./kg. thrice daily), also antagonised the effect of oestrone on liver glucuronidase without affecting the uterine response to the steroid hormone.

On the assumption that hydrolysis by glucuronidase initially present in the body is necessary for any change in the activity of the enzyme after administration of a glucuronide saccharate might be expected to prevent the rise in liver glucuronidase activity produced by menthol-glucuronide (Section B). With a dose of saccharate of 2.5g./kg. thrice daily this was found to be the case. The substitution of an equivalent amount of glucose and KCl for the saccharate had, however, the same effect.

It would appear that the two antagonistic actions of saccharate noted against the stimulation of glucuronidase activity in liver can be explained as a simple protective action for the organ rather than an inhibition of glucuronidase, since glucose behaved similarly. Glucose has a slight inhibitory effect on glucuronidase activity <u>in vitro</u> but this is of a much lower order than that of saccharate. A 10^{-4} M solution of saccharate causes 50% inhibition of hydrolysis while the same concentration of glucose only 20%.

inhibition. By increasing the concentration of the saccharate to 10^{-2}M almost complete inhibition was noted but glucose, at this strength, only reduced the hydrolysis by 30%.

Menthol-glucuronide has been shown to cause necrosis in liver. Histological examination, by Dr.J.G.Campbell, of thelivers from animals receiving saccharate or glucose-KCl after menthol-glucuronide revealed little evidence of damage.

Table 27.

The protection of liver by saccharate and glucose

Agent and dose.	Interval. (days)	Sex[X]	G.U./g.moist tissue		Uterine weight. (mg.)
			Liver.[XX]	Uterus.[XX]	
L-menthol-glucuronide, (2.5g./kg.)	2	cF	704±61(3)	191±26 (3)	52±9 (3)
and saccharate, (2.5g./kg.thrice daily).	4	cF	326±38 (3)	189±21 (3)	48±6 (3)
and glucose-KCl	4	M	334±41 (3)	-	-
Oestrone, (1.7mg./kg.)	4	cF	569±73 (5)	548±106(5)	211±20(5)
and saccharate, (0.3g./kg.thrice daily).	6	cF	333±20 (6)	286±47 (5)	203±9(5)
and glucose-KCl.	6	cF	343±32 (5)	371±41 (5)	209±8 (5)

Footnote as for Table 14.

Table 28.
The effect of saccharic acid on glucuronidase activity in liver, kidney and uterus.

Agent and dose.	Interval. (days)	Sex.[x]	G.U./g. moist tissue. Liver.[xx]	Kidney.[xx]	Uterus.[xxx]	Uterine weight. (mg.)
None.	-	F.	334±48 (6)	266±39 (6)	353±53 (6)	234±56 (6)
	-	cF	250±49 (6)	261±43 (6)	174±45 (6)	34±18 (6)
Saccharate@(0.3g./kg.).	1.5	F	267±47 (3)	213±19 (3)	501±27 (3)	221±30 (3)
Saccharate,(2.5g./kg.).	4	cF	258±10 (3)	390±20 (3)	184±24 (3)	36±12 (3)
Saccharate,(2.5g./kg.), and partial hepatectomy on 2nd.day.	10	cF	573±56 (3)	353±27 (3)	344±34 (3)	104±16 (3)
Saccharate,(2.5g./kg.), and carbontetrachloride, (5.3g./kg.)on 2nd.day.	9	cF	758±49 (3)	359±40 (3)	337±45 (3)	111±19 (3)
Saccharate,(0.3g./kg.), and oestrone,(1.7mg./kg.) on 2nd.day.	6	cF	333±20 (3)	352±46 (6)	286±47 (6)	203±15 (6)
Saccharate,(2.5g./kg.), and oestrone,(1.7mg./kg.) on 2nd.day.	6	cF	459±41 (3)	348±26 (3)	493±19 (3)	215±8 (3)
Saccharate,(2.5g./kg.), and L-menthol-glucuronide,(1.5g./kg.),on 2nd. day.	4	cF	380±54 (6)	350±22 (6)	241±38 (6)	56±6 (6)
Saccharate,(2.5g./kg.), and L-menthol-glucuronide,(2.5g./kg.),on 2nd. day. @@	4	cF	326±38 (3)	357±32 (3)	187±17 (3)	48±10 (3)

Footnote as for Table 14.
@-All injections of saccharate given thrice daily.
@@ -Dose divided into two injections.

The inability to detect any inhibition of glucuronidase activity *in vivo* by saccharate might be due to loss of the inhibitor during the preparation and purification of the enzyme. That this was not so was shown by two experiments with crude homogenate of livers from mice on the higher dosage level of saccharate (2.5g./kg. thrice daily). The results are shown in Table 29. In one case the animals were treated with saccharate only and in the other they were also treated with oestrone. In both cases the enzyme activity was identical to that seen when the assay was carried out in the usual way.

Table 29.

Treatment.	Liver enzyme activity. G.U./g.moist tissue
None (nommal assay)	273±13 (23)
Saccharate(2.5g./kg.thrice daily. Crude homogenate)	278±11 (3)
Oestrone(1.7mg./kg.)	431±42 (3)
Oestrone(1.7mg./kg)and saccharate(2.5g./kg.thrice daily. Crude homogenate).	422±31 (3)

Discussion.

Taking the results reported above as a whole it appears to be the case that a fall in glucuronidase activity in an organ is secondary to an inhibition of mitosis. Repair was arrested when the enzyme activity was reduced to normal by doses of a growth inhibitor which sometimes had no effect on the enzyme level in normal animals. Colchicine was without effect on the organs of untreated animals but prevented an increase in activity in regenerating tissue by arresting mitosis. The very low enzyme activity found in liver and kidney after sorbic acid treatment can be explained ~~only~~ on the basis of an arresting of normal mitosis rather than of a direct inhibition of the enzyme itself. It seems unlikely that changes in the enzyme activity could give rise to a depression of mitotic activity. Changes in glucuronidase activity may therefore be the result of changes in cell proliferation and not the cause.

One might imagine that the state of nutrition of the liver would affect its response to endogenous and exogenous poisons and the findings with glucuronidase activity are in accordance with this view. The fact that saccharate and glucose were apparently able to protect the liver against the effect of oestrone suggests that the nutritional state of the animal may modify the endocrine balance. This might be expected if the rôle of glucuronidase is one of regulating a balance between the free

active steriod hormone and the inactive conjugate with glucuronic acid.

Williams (1949) has suggested that animals on a diet too low in protein content may be more susceptible to external hazards such as benzene poisoning. In an experiment with animals on low and high protein diet there was no evidence that the glucuronidase activity in liver was effected by the change in diet. One group of mice was fed a diet containing 7.5% protein and the other 25% protein.for a period of four weeks. No change from the normal glucuronidase level in liver was observed at any time with either group. The number of animals was,however, too small for any definite conclusion to be drawn.

Xanthopterin was found,under the conditions employed,to stimulate mitosis in kidney. It is interesting to speculate how widespread throughout the body this action may be in view of the fact that xanthopterin occurs in milk.

The action of saccharate and glucose could have an alternative explanation. During cell proliferation one might expect the supply of carbohydrate required for some aspect of carbohydrate metabolism in the dividing cell to be increased. The liberation of glucuronic acid from endogenous glucuronides by glucuronidase could be a source of this carbohydrate which was bypassed if the tissue was flooded with compounds such as glucose. As against this view there is the fact that the livers from animals on the glucose

treatment after administration of menthol-glucuronide showed less evidence of damage than did controls receiving only menthol-glucuronide.

Such effects as could be observed with saccharate *in vivo* could be explained by a protective action for liver arrising out of the similarity in structure to glucose. It has been shown (Carr 1947) that prolonged administration of this compound to rats for several generations caused no change in the structure of any tissue nor in the growth of the animals. The failure in this work to observe any effect on the glucuronidase activity *in vivo* may be due to the fact that saccharic acid will be rapidly metabolised or excreted in the body and a sufficiently high concentration therefore never obtained. An inhibition of the enzyme might be observed using a substituted acid less easily metabolised.

SECTION E.

The β-glucuronidase activity of several tissues from various species.

The β-glucuronidase activity of several tissues from various species.

In addition to the organs of the mouse examined in the course of the present work several people have asked for glucuronidase estimations to be carried out on various tissues from different animals. The tissues were treated as described for routine enzyme assay, the estimation being carried out in citrate buffer at pH 5.2 except in a few instances when an additional estimation at pH 4.5 was also made. In few cases was the number of animals in the group large enough for any stress to be laid on the results but a collected table may be of interest.

The high figure for enzyme activity in the livers of young rats confirms the above findings for young mice. Tumour tissue also showed a high enzyme activity as was first suggested by Fishman (1948).

Table 30.
The average glucuronidase activity in several tissues from various species.

Animal.	Source.	Tissue.	G.U./g.tissue.	
			pH 4.5	pH 5.2.
Mouse.	Own colony.	Liver.(adult)	-	273(23)
"	"	Kidney. "	-	363 "
"	"	Spleen "	-	636 "
"	"	Uterus(normal)	333	- (6)
"	"	Uterus(castrate)	174	- (6)
"	"	Breast.	-	78x
"	"	Lung (adult).	-	185(3)
"	"	Lung (infant).	-	316(3)
Mouse	Mr.Riley,	Breast tumour.	-	630x
"	(Surg.Dpt.)	Carcinoma 2146.	697	751(6)
"	"	Crocker sarcoma No.180.	596	429(6)
Rat.	Dr.Walpole, (I.C.I.)	Liver(adult).	-	1380(4)
"	Pharmacol, Dept.	Liver(adult).	-	1471(4)
		Liver(infant).	-	2977(4)
Horse.	Dr.Alexander,(R.V.C.)	Intestine.	46	64(2)
Fowl.	Dr.Campbell	Liver.	-	228(1)
"	(P.R.C.)	Liver tumour G.R.C.H.	-	785(1)
"	"	Liver carcinoma, (virus.).	-	312(1)

Number of tissues shown by figure in brackets.

x-Only a very small portion of tissue from one animal was available for this estimation and the figure may therefore be erroneous.

GENERAL DISCUSSION.

As a result of the present work it is no longer necessary to say that the function of β-glucuronidase in the body is to catalyse the condensation of hydroxy compounds with glucuronic acid during the formation of conjugated glucuronides. In every instance when a change in the glucuronidase activity of an organ was observed it was possible to show a change in the amount of cell proliferation in that organ. During active cell division following the administration of such compounds as menthol, oestrone or carbontetrachloride or in the tissues of young animals a much higher enzyme activity was noted than in the corresponding adult tissues. Although the number examined was small and no accurate control was available, the glucuronidase activity of tumours was high, as might be expected from the rapid rate of growth of such tissue. Fishman (1947) published figures for the glucuronidase activity of certain sarcomas and carcinomas from human subjects and although the tissue used for comparison was, in many cases, not a strict control it was obvious that a greater activity was found in the tumour than in the control. There was also a suggestion that the enzyme activity paralleled the malignancy of the growth. The glucuronidase level in blood has been followed throughout pregnancy and it has been suggested by McDonald and Odell (1947) that it may be used in clinical diagnosis of pregnancy disorders. During

pre-eclampsia and hypertension they have shown that the glucuronidase level rises to a value well above the normal range. The increase may be accounted for on the relationship of enzyme activity to cell proliferation following damage to any organ.

Since a depression in the high enzyme levels of tissues in which mitosis had been stimulated was not found without a simultaneous inhibition of mitosis it may well be that a rise in glucuronidase activity is the result of an increase in cell proliferation and not the cause. This would appear to follow from the fact that mitosis inhibitors, such as colchicine,were found to prevent the rise in liver enzyme associated with regeneration after partial hepatectomy but were without action on normal glucuronidase levels.

It is interesting to speculate as to the function of glucuronidase in the dividing cell. We may assume that the enzyme acts,at least to some extent,hydrolytically in the body as the effect of free menthol could be detected in liver after the administration of menthol-glucuronide. During active cell division the concentration of nucleic acid in the organ has been found to increase. One could imagine glucuronidase hydrolysing an endogenous glucuronide with subsequent decarboxylation of the liberated glucuronic acid to give a pentose. This pentose,after rearrangement of the molecule, could be built up into the nucleic acid. Since

glucuronic acid occurs in mucoproteins the action of glucuronidase may be necessary as a source of this carbohydrate.

As certain steroid hormones give rise to glucuronides in the body,it is possible that glucuronidase may regulate the action of these hormones if one regards the formation of a glucuronide as a method of reducing the toxicity or activity of the steroid molecule or if glucuronide conjugation forms part of the transport or excretion mechanism. Changes in glucuronidase activity produced by the steroid hormones and more especially the newly discovered changes in liver enzyme,which increases the scopeof these compounds,suggests a close relation existing between the enzyme and the steroids.

SUMMARY.

1. Phenol-glucuronide is a convenient substrate for the assay of β-glucuronidase, using the Folin and Ciocalteu (1927) reagent for the estimation of free phenol.

2. β-Glucuronidase from mouse liver, kidney and spleen contains two fractions which may be separated by ammonium sulphate precipitation. Uterus contains only one fraction. For total enzyme and fraction B in liver, kidney and spleen a citrate buffer at pH 5.2 was used in the estimation and for uterine enzyme and fraction A in liver, kidney and spleen a buffer at pH 4.5. A substrate concentration of 0.015M was used in all cases.

3. The mean recovery of added phenol in the assay procedure was 100%, and the standard deviation of a single observation from the mean was 0.6%.

4. The effect of menthol in increasing the glucuronidase activity in liver and kidney is secondary to repair following damage provoked by that agent rather than due to its glucuronidogenic properties. This is confirmed by the changes in enzyme activity observed after administration of many nonglucuronidogenic compounds and by the manner in which the enzyme activity reflected the sex-linked nature of chloroform poisoning in kidney.

5. The two enzyme fractions in liver and kidney respond indifferently to agents causing changes in

the enzyme activity.

6. In uterus, as in other organs, changes in glucuronidase activity reflect changes in growth. The action of oestrone on the enzyme is antagonised by testosterone and progesterone.

7. Oestrone causes a marked increase in glucuronidase activity in liver accompanied by an increase in cell division. Both effects may be antagonised by testosterone and progesterone. Oestradiol, oestriol and oestriol-glucuronide, in doses comparable to that of oestrone, were without effect on liver enzyme and their action in stimulating mitosis is slight.

8. During liver regeneration, as after partial hepatectomy, uterine weight and glucuronidase activity increases in ovariectomised mice in the absence of administered oestrogen. Testosterone and progesterone antagonise this effect.

9. Colchicine, substituted stilbenes and anilines and the antifolic acid factor, known to inhibit cell proliferation, are without effect on the in vitro hydrolysis of phenol-glucuronide by glucuronidase. Saccharate, an efficient inhibitor of glucuronidase in vitro, is without effect on mitosis.

10. Sorbic acid is a most efficient inhibitor of cell proliferation.

11. A depression in the high glucuronidase level in an organ in which mitosis had been stimulated is only obtained after an inhibition of cell division.

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β-Glucuronidase and Tissue Damage

AFTER repeated feeding of menthol to mice, Fishman[1] obtained results which on statistical examination showed an increase in β-glucuronidase in liver, spleen and kidney as compared with organs from untreated animals. Similar results were obtained in dogs fed with borneol. Since the compounds examined are known to be excreted as the glucuronides in certain species, Fishman considered β-glucuronidase to be responsible for glucuronide synthesis *in vivo*, and that he was measuring adaptation on the part of the enzyme in response to the presence of excess substrate. The work of other authors is not, however, compatible with the view that the function of β-glucuronidase in the body is other than hydrolytic[2,3,4].

Using biosynthetic phenol β-*d*-glucuronide[5] for the assay of glucuronidase[6], an attempt was made to confirm Fishman's findings with menthol. Twenty-four hours after intraperitoneal injection of *l*-menthol in vegetable oil into mice there was a marked rise in glucuronidase in liver, but not in kidney. It was difficult to decide whether the spleen enzyme was affected because of the great variation in normal values observed with this organ. Average normal values were the same in the case of each organ for mice of both sexes and drawn from three different colonies. The rise in liver glucuronidase was less marked after repeated feeding of menthol than after injection of much smaller doses.

It was observed that menthol caused gross damage to the liver, but not to the kidneys, and it was considered that this property, rather than the one of giving rise to a glucuronide, might explain the effect on liver glucuronidase. A variety of other substances, known to cause damage to the liver or kidneys, but with one exception unlikely to form glucuronides, were therefore examined for their effects on glucuronidase. Compounds which are relatively non-toxic, but which are known or considered to form glucuronides in the body, were also examined. The substances were injected subcutaneously or intraperitoneally in oil or aqueous solution into groups of 3–9 mice, and the animals were killed after 1–3 days. With several of the substances, the organs were examined histologically

Substance	Dose gm./kgm.	G.U.*/gm. moist tissue Spleen	Liver	Kidney	Histological findings
Normal mice	—	580	280	378	—
Liver poisons					
l-Menthol	0·33	777	933	285	Moderate fatty degeneration and necrosis of liver, with evidence of repair, all most marked with carbon tetrachloride. Little deviation from normal in kidney and spleen.
l-Menthol *β-d*-glucuronide	2·3	995	1104	295	
Carbon tetrachloride	5·33	840	1138	139	
Chloroform	2·0	1079	939	194	Not done.
Kidney poison					
Mercuric nitrate	0·02 to 0·04	718	469	808	Kidney was profoundly damaged, with marked necrosis in the medullary region and hyaline casts. Liver and spleen showed little change from normal.
Liver and kidney poisons					
Yellow phosphorus	0·0075	506	91	338	Marked fatty degeneration of liver with no signs of repair. Small areas of necrosis in kidney, most marked in cortex. Spleen as normal.
Phenyl arsenoxide†	0·001 to 0·002	807	257	471	Extensive fatty changes in liver with severe necrosis and no evidence of repair. Kidney as with phosphorus, but changes more obvious. Spleen as normal.
'Non-toxic' compounds					
Sulphathiazole	43	561	460	368	Not done
Pregnanediol‡	0·33	1001	287	321	
Pregnanediol‡ *β-d*-glucuronide	0·80	627	241	264	

* One G.U. (glucuronidase unit) liberates 1 μgm. phenol from 0·015*M* phenol glucuronide at 38° and *p*H 5·2.
† This compound could conceivably give rise to a glucuronide.
‡ Injected as a suspension in oil.

by Mr. J. G. Campbell of the Royal (Dick) Veterinary College, Edinburgh. As shown by the averaged results in the table above, a significant rise in the β-glucuronidase level only occurred when an organ showed obvious, but not irreparable, damage.

Phenylarsenoxide had no effect on the glucuronidase level in liver or kidney, while phosphorus actually depressed it in the case of liver. Both substances, however, caused extensive damage. These results favour the view that a rise in glucuronidase is associated with tissue regeneration following moderate damage rather than with the damage itself. A series of experiments is in progress to settle this point. The effects observed with menthol glucuronide were presumably due to liberation of menthol by glucuronidase initially present in the body, and are difficult to reconcile with Fishman's views. The rise in liver glucuronidase caused by carbon tetrachloride may to some extent explain its enhancing action on the effect of œstrogens[7], since glucuronide formation plays some part in the metabolism of the latter compounds. It is interesting to note that both carbon tetrachloride[8] and chloroform[9] are known to produce hepatomata after repeated administration to mice. The possibility of menthol behaving similarly is under investigation. In a recent communication to *Science*, Fishman[10] gives figures showing that in some cases of human carcinoma the tumour contained much more glucuronidase than the corresponding normal tissue. He suggests that this may be due to exeessive local accumulation of œstrogens. In view of the bearing of our results on this finding, it was considered that an interim account should be published without further delay.

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β-Glucuronidase and Tissue Proliferation

IN a previous communication[1] it was shown that β-glucuronidase in liver or kidney rises following administration of toxic agents to mice, depending upon the organ or organs affected. It was suggested that a rise in this enzyme is associated with tissue repair rather than the damage itself. This view is confirmed by results for kidney following administration of mercuric nitrate or chloroform to male mice. In each case, the rise in the enzyme did not occur in the early stages of poisoning, but was seen when repair was well advanced.

That there is a relation between tissue proliferation and β-glucuronidase is further borne out by the fact that high enzyme contents were found in the livers of adult mice following sub-total hepatectomy, and in the liver, spleen and kidneys of baby mice. These results, summarized in the table overleaf, may provide a simple explanation of the finding by Fishman and Fishman[2] that administration of œstrogens to ovariectomized mice causes a rise in uterine glucuronidase, and of the high figures for tumour glucuronidase recently described by Fishman[3].

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Age	Treatment	Interval following treatment, days	G.U.*/gm. moist tissue			Histological findings
			Spleen	Liver	Kidney	
Adult	None	—	580	280	380	—
,,	2 gm. chloroform/kgm.	1	1079	939	194	Damage and repair in liver; necrosis without evidence of repair in kidney.
		8	746	711	628	Residual damage and advanced repair in liver and kidney.
,,	20 mgm. mercuric nitrate/kgm.	1	—	436	208	Severe damage without evidence of repair in kidney; liver almost normal.
		3	718	469	808	Repair almost complete in kidney; some cell division in liver.
,,	Sub-total hepatectomy	3	—	1052	—	Very active cell division in liver.
		5	—	882	—	
		8	—	1165	—	
1 day	None	–	5100	1370	881	—
5 days	,,	–	3245	1363	793	—
13 ,,	,,	–	1521	2218	606	—
15 ,,	,,	–	5169	1239	727	—

* One G.U. (glucuronidase unit) liberates 1 μgm. phenol from 0·015 M phenol glucuronide at 38° and pH 5·2 in 1 hr.

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Sex and Organ Specificity in the Response of β-Glucuronidase to Extrinsic Agents

IT was considered that the correlation shown to exist between the β-glucuronidase activity of a tissue and the amount of cell proliferation in progress[1] could explain the rise in uterine glucuronidase found by Fishman and Fishman[2] to follow œstrogen administration to ovariectomized mice. In a preliminary kinetic study of the enzyme in mouse uterus, the *p*H-activity curve for the hydrolysis of phenol β-*d*-glucuronide was found to be almost symmetrical about *p*H 4·5. This is in contrast to the curves

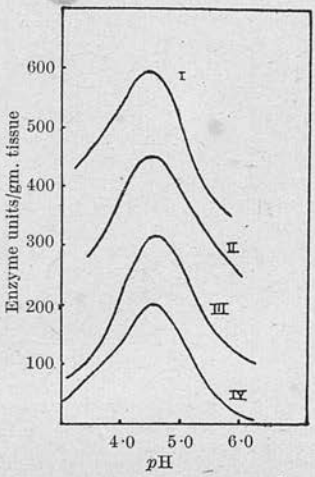

Fig. 1. *p*H-Activity curves for uterine glucuronidase
I, 10-day old mice; II, ovariectomized adults, 3 days after subcutaneous injection of 50 μgm. œstrone; III, normal adult; IV, ovariectomized adults

previously found for liver and spleen glucuronidase in the normal mouse[3], with peaks at *p*H 5·2 as well as at 4·5. Mills[4] has shown that beef spleen glucuronidase can be separated into two fractions, *A* and *B*, with slightly different *p*H optima for the hydrolysis of menthol glucuronide. Since it seemed probable that mouse uterus lacked a glucuronidase fraction present in liver and spleen, it was considered necessary to investigate the possibility that differences between *A* and *B* in distribution and response to extrinsic agents might in some way explain the selective actions of such substances as carbon tetrachloride

and œstrone on liver and uterine tissue respectively.

As can be seen from Figs. 1 and 2, the *p*H activity curves for liver and uterine glucuronidase were unchanged in shape during active growth. The high figure for uterine glucuronidase in infant mice supports the view that in this organ, as in others, the

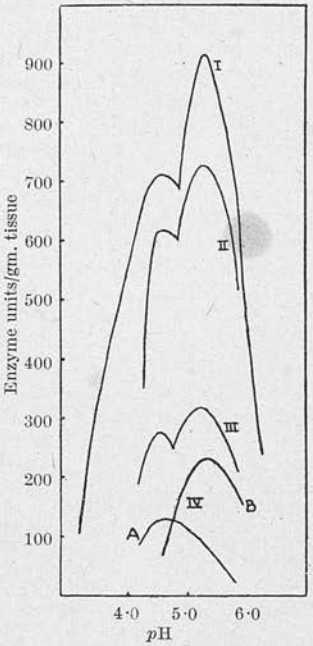

Fig. 2. *p*H-Activity curves for liver glucuronidase.

I, 6-day old mice ; II, adult, 1 day after subcutaneous injection of 5 gm. chloroform/kgm. ; III, normal adult ; IV, the same preparation as III after separation of *A* and *B*

activity of the enzyme is a measure of cell division. The effect of carbon tetrachloride on liver does not suggest any selection between the glucuronidase fractions. Carbon tetrachloride is known to have no effect on kidney glucuronidase[5], in spite of the fact that the *p*H activity curve has been found to have the same general shape as that shown for liver.

In further experiments, fraction *A* was precipitated by making the preparation 38·5 per cent saturated with ammonium sulphate[4] at *p*H 5·2, and the two fractions were then determined separately. All uterine enzyme was found in fraction *A*. The separation achieved is illustrated for a liver preparation in Fig. 2. In the response of liver glucuronidase to chloroform, and kidney to mercuric nitrate, *A* and *B*

were equally affected. Eschenbrenner and Miller[6] found the production of renal necrosis in mice by chloroform, seen only in males, to be prevented by castration, and noted a correlation between kidney morphology and its susceptibility to chloroform. Morphological differences were confined mainly to Bowman's capsule; but this was not involved in the necrosis. The response of kidney glucuronidase to chloroform observed in the male mouse[5] was abolished by castration. While figures for untreated females, males and castrated males did not suggest any differences in *A* or *B* corresponding to the variations in the structure of the kidney, the rise in kidney glucuronidase after injection of normal males with chloroform was confined to fraction *B*. This could be explained on the assumption that the two glucuronidase fractions are unevenly distributed throughout the kidney. From the other results quoted above, it appears that the response by glucuronidase to any agent is independent of the 'fraction' present in the cell affected. For the comparative study of the effects of extrinsic agents on organs such as liver and kidney, it would appear adequate to determine the total enzyme activity at *p*H 5·2. The importance of determining the *p*H-activity curve for each new organ examined is, however, emphasized by the findings for the uterus.

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The Use of Phenol Glucuronide in the Assay of β-Glucuronidase

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(*Received* 11 *June* 1947)

Our present knowledge of the distribution in the body and the physical properties of β-glucuronidase is derived almost entirely from experiments, in which free glucuronic acid was determined by reduction of ferricyanide after enzymic hydrolysis of a conjugated glucuronide. Whilst it is possible to determine very small amounts of glucuronic acid by means of its reducing power (Levvy, 1946), the lack of specificity in the reaction is a serious disadvantage in the routine assay of glucuronidase. Unless the enzyme preparation has been considerably purified, it may contain large amounts of materials other than glucuronic acid which reduce ferricyanide (Levvy, 1948). Other impurities may interfere during the actual titration, if this is cerimetric, obscuring the end point and making it unstable. In studying the enzymic hydrolysis of oestriol glucuronide, Fishman (1939) determined free oestriol by the Kober colour reaction. Its scarcity, however, renders this substrate unsuitable for routine assays. Talalay, Fishman & Huggins (1946) have recently described a method whereby the hydrolysis of phenolphthalein glucuronide can be followed colorimetrically. This method appears to avoid the difficulties mentioned above in the determination of glucuronidase activities. It was found possible in this laboratory to apply the method of King & Armstrong (1934) for phosphatase determination to the assay of glucuronidase, using biosynthetic phenol glucuronide (Williams, private communication) as substrate. (We are greatly indebted to Dr Williams for details of the technique for obtaining this compound from rabbit urine.) Results obtained in studying the kinetics of hydrolysis of phenol glucuronide with enzyme obtained from two different organs, and a description of the procedure for the routine assay of glucuronidase in mouse tissues, are given below.

EXPERIMENTAL

Preparation of phenol β-D-glucuronide. The glucuronide was obtained from the urine of rabbits fed with phenol by the general procedure described by Williams (1943) for the isolation of aminophenol glucuronides. Before use, it was dried over P_2O_5 *in vacuo* for 10 hr. at 80°. Analysis showed it to be free from solvent of crystallization (m.p. 162°, corr.).

The colour reaction for free phenol. Phenol was determined with the reagent of Folin & Ciocalteu (1927). King & Armstrong (1934) give full details of the technique for this estimation. The colour intensity was measured with a Spekker photoelectric absorptiometer, using Ilford no. 602 blue filter. Free glucuronic acid did not interfere in the reaction. Phenol glucuronide in high concentration gave a faint colour, probably due to traces of free phenol (less than 0·2% of the total weight), since it varied from one sample to another. Contrary to the findings of Folley & Kay (1935), the reaction was not particularly sensitive to the Na_2CO_3 concentration and was complete in less than 20 min. at 37°. In constructing the calibration curve (Fig. 1),

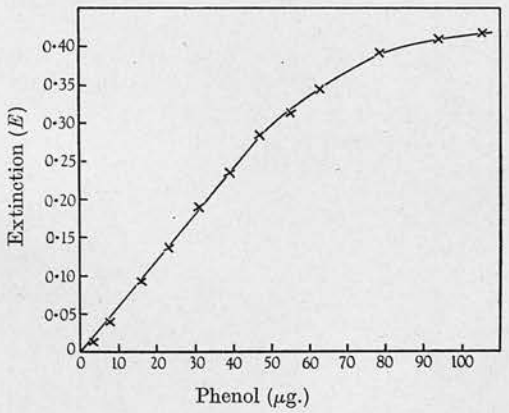

Fig. 1. Calibration curve for estimation of phenol.

0·6 ml. standard phenol solution (Hawk & Bergeim, 1931) was added to 0·2 ml. 0·1 M-citrate buffer (pH 5·2), followed by 2 ml. of a 1 : 5 dilution of the Folin-Ciocalteu reagent. After mixing, 2 ml. of the liquid were taken for colour development as described below for enzyme extracts. Citrate, acetate and formate buffers had no effect on the colour reaction.

Preparation of the enzyme. Preparations from mouse liver and spleen were studied separately in these experiments. The organs were broken up in glass homogenizers (Umbreit, Burris & Stauffer, 1945). Unless stated to the contrary, the enzyme was partially purified following the method described by Talalay *et al.* (1946). The homogenate was brought to pH 5·0 or 5·2 with acetic or citric acid. Protein which separated after 30 min. at 37° was removed by centrifuging, and the enzyme in the supernatant liquid precipitated by adding an equal volume of saturated

$(NH_4)_2SO_4$ solution (SAS). The precipitate was dissolved in a convenient volume of water. No change in the amount of enzyme present was noted after purification in this way.

The effect of pH. The pH for optimum hydrolysis was determined over the range 3–6·5 at a substrate concentration of 0·01 M in citrate buffer. To 0·2 ml. of 0·1 M-buffer and 0·2 ml. of 0·04 M-substrate warmed to 37° was added 0·4 ml. enzyme, and the mixture was incubated at 37° for 1 hr. The initial effect of substrate and enzyme on the pH of the buffer was determined electrometrically. No further change in the pH occurred during the incubation.

With crude homogenates of liver and spleen, considerable amounts of substances giving the colour reaction were liberated in absence of substrate. It would appear that an enzymic process was responsible for the production of these compounds since the amount formed varied with the pH, with an optimum at 3·6–4·6. Partial purification of the homogenates as described above made the blank much smaller and very nearly independent of the pH. With purified enzyme from liver and spleen, the curves for variation in hydrolysis with pH assumed the shapes shown in Fig. 2. Homogenates from five mice were pooled for each organ before purifying, and all points on the curve were determined simultaneously. Corrections were applied for the enzyme blank at the appropriate pH (corresponding to about 200 μg. phenol/g. for spleen and about 100 μg./g. for liver) before plotting the points. From Fig. 2 it can be seen that for enzyme from both sources the rate of hydrolysis rose to a maximum at pH 5·2 as the pH was lowered. After a drop at pH 4·75, the velocity again rose slightly at pH 4·5 and then fell off rapidly. The subsidiary peak at pH 4·5 was seen in other experiments when the pH increment was made small enough. A possible explanation of the shape of the curves in Fig. 2 is given by the work of Mills (1947), who found that β-glucuronidase from beef spleen could be separated into two fractions with slightly different pH optima for the hydrolysis of menthol glucuronide. In acetate buffer, some results were obtained which suggested the pH optimum to be lower than in citrate, but this point was not further investigated. It was decided that for the routine assay of the enzyme the hydrolysis of phenol glucuronide should be done at pH 5·2 in citrate buffer.

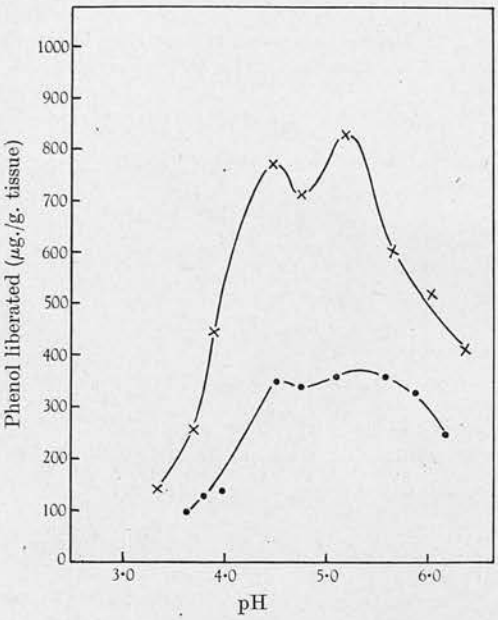

Fig. 2. The variation in hydrolysis rate of phenol β-D-glucuronide with pH. Results expressed in terms of phenol liberated/g. moist tissue. ×—× spleen enzyme; •—• liver enzyme.

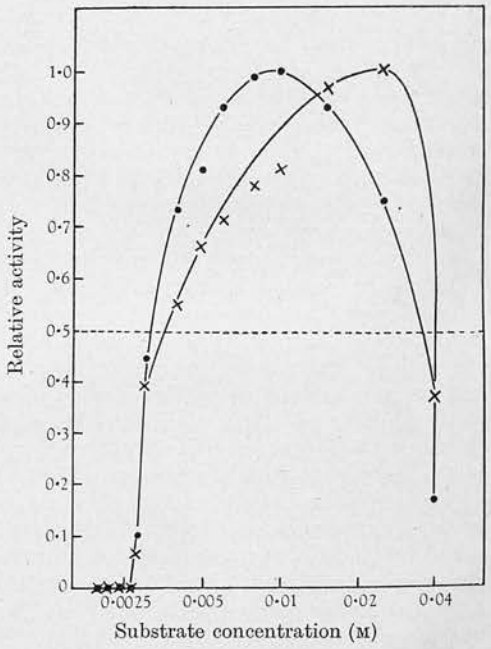

Fig. 3. The variation in initial hydrolysis rate with substrate concentration. Results expressed as fraction of maximum observed activity. ×—× spleen enzyme; •—• liver enzyme.

The effect of substrate concentration. The effect of varying the substrate concentration at constant pH is shown in Fig. 3. The citrate buffer and substrate solutions were adjusted to pH 5·2. Addition of enzyme caused no appreciable change in pH. Three experiments were done with enzyme from each source and the averaged results are shown in the figure. It will be noted that inhibition by excess

substrate was marked in both cases. The substrate optimum for spleen (0·025 M) was greater than for liver (0·01 M). The results did not lend themselves to the accurate calculation of the Michaelis-Menten constant, K_m, by the method of Lineweaver & Burk (1934), but the value obtained graphically from Fig. 3 by finding the substrate concentration at

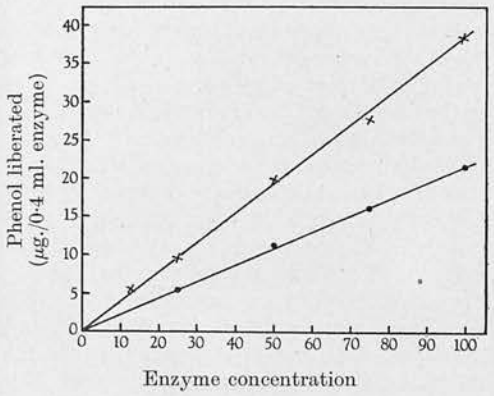

Fig. 4. The relation between enzyme concentration and phenol liberated under standard conditions. Enzyme concentration in arbitrary units. ×—× spleen enzyme; •—• liver enzyme.

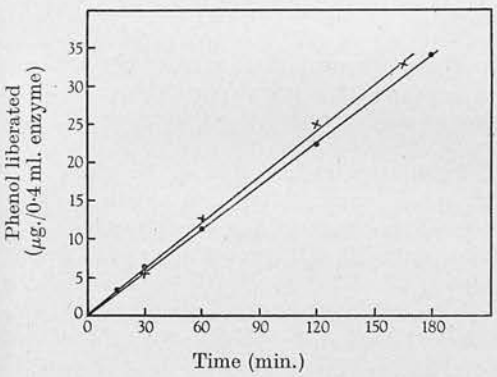

Fig. 5. Phenol liberated after varying incubation periods. ×—× spleen enzyme; •—• liver enzyme.

which half the observed maximum velocity of hydrolysis was developed was approximately the same (0·0035 M) for enzyme from either source. The difference in shape between the curves for liver and spleen may be due to the presence of interfering substances in the tissue extracts rather than a qualitative difference in the enzyme system. A substrate concentration of 0·015 M was adopted for further work with enzyme from both sources.

The effect of varying the enzyme concentration and the duration of hydrolysis. As shown in Fig. 4, the amount of phenol liberated in 1 hr. at 37° and pH 5·2 (citrate buffer) from 0·015 M-phenol glucuronide was directly proportional to the enzyme concentration. Experiments carried out under the same conditions, but in which the incubation period was varied and the enzyme concentration kept constant, showed the reaction velocity to be unchanged after 3 hr. (Fig. 5).

The assay of β-glucuronidase

Reagents

Citrate buffer. (*a*) 0·3 M, pH 5·2 (glass electrode); (*b*) 0·1 M, prepared from 0·3 M.

Substrate solution. 0·06 M-Phenol glucuronide adjusted to pH 5·2; stored in the refrigerator.

Folin-Ciocalteu reagent. (*a*) Stock solution (British Drug Houses Ltd.); (*b*) Stock solution diluted 1 to 5 and prepared daily.

N-*Sodium carbonate solution.*

Saturated ammonium sulphate solution. Adjusted to pH 5·2.

Procedure

Spleen. The dissected organ was weighed in a chilled homogenizer tube and homogenized with 3 ml. water. The cell-free homogenate was transferred to a graduated centrifuge tube, using 1 ml. wash water. After addition of 0·5 ml. 0·3 M-buffer, the tube was maintained at 37° for 30 min. Coagulated protein was separated by centrifuging for 15 min. The volumes of precipitate and supernatant liquid were noted and the latter was transferred to another graduated centrifuge tube. An equal volume of SAS was added and the tube centrifuged for 30 min. The liquid was discarded, and the residue dissolved in water and made up to a volume of 2 ml. The concentration of enzyme was then such that, in the case of normal animals, 0·4 ml. of the solution gave readings of 20–40 μg. phenol after corrections had been applied for enzyme and substrate blanks. This volume of the enzyme solution was added to 0·2 ml. of 0·1 M-buffer and 0·2 ml. substrate in a stoppered 10 ml. centrifuge tube. Prior to addition of the enzyme, the substrate and buffer were warmed to 37°. After incubation of the hydrolysis mixture for 1 hr. at 37°, 2 ml. of the diluted Folin-Ciocalteu reagent were measured into the tube. Protein was removed by centrifuging for 3 min. and 2 ml. of the supernatant transferred to a 10 ml. stoppered centrifuge tube containing 4 ml. Na_2CO_3 solution. The contents of the tube were mixed and the colour developed by incubating for 20 min. at 37°. Assays were carried out in duplicate.

Liver. The procedure was exactly as described above except that at certain stages the volumes used differed. Homogenization was done with 5 ml. water and 3 ml. of water were added in transferring the homogenate to the first graduated tube. In adjusting the pH, 1 ml. 0·3 M-buffer was used. The solution of the enzyme after SAS precipitation was

made up to 4 ml., this volume giving readings of 20–40 μg. phenol in the assay after correction for blanks with livers from normal animals.

Controls. Every assay was controlled by incubating mixtures of enzyme and buffer containing 0·2 ml. water in place of substrate. The purified enzyme shows a small rise in the blank during incubation. Controls for phenol in the substrate were also done at frequent intervals, 0·2 ml. of the solution being incubated with 0·2 ml. buffer and 0·4 ml. water. Since there appeared to be a very small but perceptible increase in the reading when the substrate was incubated at pH 5·2, this procedure was considered preferable to that in which substrate is added to the enzyme after incubation (cf. Talalay *et al.* 1946).

RESULTS

The recoveries obtained in a series of experiments in which 0·2 ml. standard phenol solution was added to 0·2 ml. 0·1 M-buffer and 0·4 ml. enzyme (spleen and liver mixed) are shown in Table 1. In some experiments the mixture was incubated whilst in others the Folin-Ciocalteu reagent was added at once. Each figure shown in the table is the result of a determination carried out in duplicate and the results have been corrected for the enzyme blank (5·2 μg. unincubated, 7·7 μg. incubated).

Table 1. *Recovery of phenol added to buffered enzyme*

Phenol added	Phenol recovered before incubation		Phenol recovered after incubation	
(μg.)	(μg.)	(%)	(μg.)	(%)
3·9	3·7	95	4·1	105
7·9	8·0	101	8·0	101
15·7	15·6	99	15·8	101
31·4	31·1	99	31·2	99
62·9	63·1	100	63·0	100
105·0	105·0	100	104·9	100

In a series of 30 determinations in which 31·4 μg. phenol were added to enzyme and buffer as above, and determined at once, the mean recovery was 31·5 μg. (100·3 %), and the standard deviation of a single determination from the mean was 0·19 μg. (0·6 %).

DISCUSSION

From the results given above it can be seen that phenol glucuronide is a convenient substrate for the assay of β-glucuronidase, using the reagent of Folin & Ciocalteu for estimation of phenol liberated by the enzyme. As compared with phenolphthalein glucuronide (Talalay *et al.* 1946), a much higher concentration of phenol glucuronide is required for optimum hydrolysis, and inhibition of the enzyme by excess substrate is more pronounced.

It has been generally assumed without any experimental justification that the conditions for optimum hydrolysis by β-glucuronidase are always the same no matter the source of the enzyme. The data obtained in this work for mouse liver and spleen glucuronidase support the assumption. Masamune (1934), in his pioneer work on glucuronidase, found hydrolysis of phenol glucuronide by beef kidney extracts, determined by estimation of glucuronic acid, to be at a maximum at pH 5·3–5·6 in citrate buffer. This is in good agreement with our own figure of 5·2 for mouse liver and spleen extracts.

SUMMARY

1. Phenol glucuronide is a convenient substrate for the assay of β-glucuronidase, using the Folin & Ciocalteu (1927) reagent for the estimation of free phenol.

2. In the procedure adopted for assay of glucuronidase in mouse liver and spleen, hydrolysis of phenol glucuronide was carried out in citrate buffer at pH 5·2 and a substrate concentration of 0·015 M.

3. The mean recovery of added phenol in the assay procedure was 100 %, and the standard deviation of a single observation from the mean was 0·6 %.

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β-Glucuronidase and Cell Proliferation*

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After repeated feeding of menthol to mice, Fishman (1940) 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 by menthol and borneol. In Fishman's own interpretation of these important experiments, β-glucuronidase is assumed to be responsible for glucuronide synthesis in the body. A synthetic role for the enzyme has, however, still to be demonstrated, its physical properties and distribution in the body having been studied solely by means of its hydrolytic action on conjugated glucuronides. Since menthol and borneol have been proved to be excreted as the glucuronides in, e.g. the dog, and may conceivably behave in the same way in the mouse, Fishman suggested that in his experiments he was measuring adaptation by glucuronidase in response to the presence of excess substrate for its hypothetical synthetic action. Later this theory was extended to explain the elevation in uterine glucuronidase observed after administration of oestrogens to ovariectomized mice (Fishman & Fishman, 1944; Fishman, 1947). 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 was, however, observed in its hydrolytic action *in vitro*, menthol glucuronide being used throughout in the assay of uterine glucuronidase under conditions found to be optimal for hydrolysis by spleen preparations.

Fishman determined the activity of his enzyme extracts by measuring, by means of its reducing power, glucuronic acid liberated from menthol glucuronide (Fishman, 1939). Sources of error in this procedure, arising largely from its lack of specificity, have been pointed out by other authors (Graham, 1946; Levvy, 1946, 1948), and have led to the development of more satisfactory methods of assay (Talalay, Fishman & Huggins, 1946; Kerr, Graham & Levvy, 1948).

Using phenol glucuronide as substrate in the assay of glucuronidase (Kerr *et al.* 1948), an attempt was made to confirm Fishman's findings (1940) with menthol. Within 24 hr. of a single intraperitoneal injection of L-menthol into mice, there was a marked rise in glucuronidase activity in liver, but not in spleen and kidney. Liver damage was observed and confirmed histologically, and it was subsequently shown that a rise in β-glucuronidase in liver or kidney, depending upon the organ or organs attacked, followed administration of a variety of toxic agents to mice. A more extensive examination of the action of menthol revealed, in addition to the effect on liver, delayed damage to kidney, followed by an increase in glucuronidase activity in this organ also. An increase in the glucuronidase activity of an organ was found, in general, to be associated with active cell proliferation provoked by injury, rather than with the injury itself, and high values were seen in the livers of adult mice after sub-total hepatectomy, and in the liver, spleen and kidneys of infant mice.

* Preliminary accounts of parts of this work have been published elsewhere (Kerr & Levvy, 1947; Kerr, Levvy & Campbell, 1947), and the principal findings were described in a paper read to the Biochemical Society on 27 September 1947 (Levvy, Kerr & Campbell, 1948).

EXPERIMENTAL AND RESULTS

Enzyme assay. In the assay of kidney glucuronidase it was assumed that the conditions for optimum hydrolysis of phenol β-D-glucuronide would be the same as those previously found to hold for spleen and liver preparations (Kerr *et al.* 1948). All preparations of the enzyme were diluted to final volumes giving readings of 20–40 μg. phenol in the assay, after correction for blanks. The results are shown in the tables and figures in terms of glucuronidase units (G.U.)/g. moist tissue, where 1 G.U. liberates 1 μg. phenol in 1 hr. from 0·015M-phenol glucuronide at 38° and pH 5·2. The standard error is given wherever possible. Although frequently based upon too small a group of animals to have any statistical value, it shows the variation in the individual figures in a convenient form.

Histology. Portions of organs from animals used for enzyme assay, or whole organs from other animals treated similarly, were fixed immediately in Susa and taken in the usual way through the ethanols to a mixture of chloroform and cedarwood oil, and finally cleared in pure cedarwood oil. After embedding in paraffin wax, sections were cut at 8 μ. and stained with Mayer's haematoxylin and eosin. The distribution of fat was studied in frozen sections, prepared from tissues rapidly fixed by heat in formol saline, and stained with haematoxylin and Sudan III.

Damage, repair and cell division are shown in the tables by an arbitrary system of + signs. In the case of damage, + indicates that while definite it was neither severe nor extensive, and + + + that it was at its greatest for the toxic agent in question. The course of repair is measured likewise, + + + indicating that replacement of damaged tissue by normal cells is practically complete. Under cell division, an estimate is given of the number of mitotic and amitotic figures and hyperchromatic nuclei *in excess of normal.* No histological findings are given for spleen since deviations from normal could never be distinguished in this organ.

Normal mice and vehicle controls. Average values for β-glucuronidase in each organ were the same for normal adult mice (30–40 g.) of both sexes and drawn from three different colonies, and all the results are grouped together in the tables. Spleen showed greater variation in its normal glucuronidase activity than did liver or kidney. Intraperitoneal injection of relatively large volumes of 0·9 % sodium chloride solution, olive oil or nut oil (the vehicles used for administration of toxic agents) had no effect on glucuronidase in any of the three organs examined after an interval of 1–2 days. These results are not shown. The relatively small number of experiments in which nut oil was used as a vehicle, olive oil being unobtainable, are included in the tables with those done with the latter as medium.

Effects produced by a single injection of L-*menthol.* Intraperitoneal injection of L-menthol (Table 1) caused a rapid rise in liver glucuronidase activity, reaching a maximum after 24 hr. and persisting for 7 days. Greatest liver damage was observed after 24 hr., but repair processes were not perceptible at this time. After 14 days, repair was almost complete and the enzyme level had returned to its original value, although cell division still seemed to be slightly in excess of normal. In the first 24–48 hr., kidney was normal in structure and in its enzyme activity, but after 3 days damage was evident and the figure for glucuronidase had risen after 7 days. At the end of 14 days this organ was normal in all respects. No effect of menthol on spleen glucuronidase was observed at any stage. Sex did not influence the results obtained with liver and kidney.

Mills (1947) found beef spleen glucuronidase to consist of two fractions with slightly different pH optima for the hydrolysis of menthol glucuronide, and the pH activity curves for hydrolysis of phenol glucuronide by enzyme from mouse spleen and liver (Kerr *et al.* 1948) had subsidiary peaks at pH 4·5. An experiment was done in which spleen, liver and kidney glucuronidase activities, 24 hr. after injection of menthol, were compared with normal at pH 4·5 instead of 5·2. The change in pH had no appreciable effect on the results compared with those shown in Table 1.

No details of the toxic action of menthol could be found in the literature. A brief description of the changes seen in liver and kidney may be of interest. In the liver, the first deviation from normal was cloudy swelling, followed by fatty change and necrosis surrounding the central vein and extending about a third of the way into the lobule. The nuclei showed hypertrophy and hyperchromatism, many

Table 1. *Changes in β-glucuronidase and histological findings after injection of mice with* L-*menthol*

(333 mg. Menthol/kg. injected intraperitoneally in olive oil. Average enzyme activity and standard error expressed as G.U./g. moist tissue (see text). Number of animals in group shown by figures in brackets.)

Interval (days)	Spleen enzyme	Liver				Kidney			
		Enzyme	Damage	Cell division	Repair	Enzyme	Damage	Cell division	Repair
Untreated	636±70 (23)	273±13 (23)	—	—	—	363±24 (11)	—	—	—
0·125–0·5	720±63 (9)	467±24 (9)	—	—	—	381±40 (9)	—	—	—
1	690±41 (3)	823±135 (3)	+ + +	0	0	285±46 (3)	0	0	0
2	738±86 (3)	884±74 (6)	—	—	—	344±71 (3)	—	—	—
3	903±208 (3)	953±39 (3)	+ +	+ + +	+	—	+ +	0	0
7	646±86 (6)	775±46 (7)	+	+ +	+ +	603±52 (7)	0	+	+ + +
14	600±14 (3)	318±17 (3)	+	+	+ +	337±23 (3)	0	0	+ + +

binucleate cells appeared (amitotic division), and at a later stage mitotic division became evident. The Küpffer endothelial cells were swollen. In the case of kidney, the damage was not severe, being confined to the distal portions of the convoluted tubules and to some glomeruli, the endothelium of which was swollen and in places necrotic. Intraperitoneal injection of mice with large doses of menthol (about 0·7 g./kg.) caused prolonged depression of the respiration and unconsciousness. No attempt was made to determine the lethal dose.

Repeated administration of L-*menthol and* L-*menthol* β-D-*glucuronide*. Results for β-glucuronidase in spleen, liver and kidney, after intraperitoneal injection of mice with L-menthol or its glucuronide twice or thrice daily for varying periods (Table 2), were similar to those obtained after a single injection of L-menthol. The glucuronide was injected as a neutral solution in 0·9 % sodium chloride solution and menthol itself as a solution in olive oil. (For the preparation of neutral solutions of acid compounds in 0·9 % sodium chloride solution, see Chance, Crawford & Levvy, 1945.) Repeated oral administration (Odell, Skill & Marrian, 1937) of menthol produced an increase in liver glucuronidase activity of the same order as the injections, except in mice receiving a total dose of 9·3 g./kg. in which the rise was barely perceptible. The latter was, however, as great as that obtained by Fishman (1940) and proved to be statistically significant ($P = 0·01$). This experiment was carried out exactly as described by Fishman except that three of the mice were given a solution of menthol in olive oil instead of an emulsion in soap solution. The change in vehicle had no effect on the response of the enzyme, and only solutions in oil were used in the other feeding experiments (total dose 1·2 and 2·0 g./kg.). No histology was done in the experiments listed in Table 2, except in the case of menthol glucuronide, which produced changes similar to those seen after a single injection of menthol.

Changes produced by single injections of a variety of substances. The changes in β-glucuronidase activity and histological findings in liver and kidney after injection of various substances, some of them known liver or kidney poisons, are summarized in Table 3. Spleen was also examined in these experiments. Since any changes in glucuronidase in this organ were relatively small, with wide variation in individual figures, the results are not shown.

Subcutaneous injection of carbon tetrachloride in olive oil caused severe fatty degeneration and early necrosis in liver within 24 hr. After 3 days, damage was extensive, but repair processes had commenced, and after 7 days repair was far advanced. A marked increase in liver glucuronidase activity occurred within 24 hr., and this was maintained for 7 days. There were no marked pathological changes in kidney at any stage, nor was there any rise in glucuronidase in this organ. Intraperitoneal injection of carbon tetrachloride (0·5–2 g./kg.) produced a change in liver glucuronidase similar to that already described for subcutaneous injection.

Mercuric nitrate given subcutaneously in 0·9 % sodium chloride solution had no very marked effect on liver, but produced severe cortical necrosis with hyaline casts in kidney within 24 hr. Kidney glucuronidase activity showed no rise at this stage, but after 3 days, by which time repair was practically complete, it was more than twice its normal value.

The changes in liver after subcutaneous injection of chloroform in olive oil resembled those produced by carbon tetrachloride. Kidney, however, showed an interesting sex specificity in the response of the enzyme to chloroform. In agreement with the observation of Eschenbrenner (1944), this compound was found to cause renal necrosis in male, but not in female mice. The rise in kidney glucuronidase activity, which was confined to male mice, was not seen in the early stages of the damage, but was evident after 8 days, by which time repair was extensive. An increase in liver glucuronidase

Table 2. *Changes in β-glucuronidase after repeated administration of* L-*menthol and* L-*menthol* β-D-*glucuronide*

(Results expressed as in Table 1)

Agent and mode of administration	Total dose (g./kg.)	Interval after 1st administration (days)	Average enzyme activity and S.E. (G.U./g. moist tissue) Spleen	Liver	Kidney
Untreated	—	—	636±70 (23)	273±13 (23)	363±24 (11)
L-Menthol, orally	1·2	3	843±185 (3)	741±146 (3)	—
	2·0	1	—	895±77 (6)	—
	9·3	5	499±48 (6)	369±40 (6)	260±2 (2)
L-Menthol, intraperitoneally	0·8	2	254±38 (3)	1149±136 (3)	—
	1·2	3	599±42 (6)	869±58 (6)	—
L-Menthol β-D-glucuronide, intraperitoneally	2·3	1·5	995±95 (3)	1104±222 (3)	295±13 (3)

Table 3. *Effects of various agents on liver and kidney*

(Results expressed as in Table 1)

Agent and dose	Interval (days)	Sex	Liver				Kidney			
			Enzyme	Damage	Cell division	Repair	Enzyme	Damage	Cell division	Repair
None	—	♀, ♂	273±13 (23)	—	—	—	363±24 (11)	—	—	—
Carbon tetrachloride (5·3 g./kg.)	1	♂	1138±148 (3)	+ + +	0	0	139±9 (3)	+ (?)	0	0
	7	♂	927±48 (4)	+	+ + +	+ +	323±45 (4)	0	0	0
$Hg(NO_3)_2$ (20 mg./kg.)	1	♂	436±28 (2)	+	0	0	208±42 (3)	+ + +	0	0
	3	♂	469±29 (5)	0	+	0	808±61 (5)	0	+	+ + +
Chloroform (2 g./kg.)	1	♂	939±90 (7)	+ + +	+ +	0	194±16 (6)	+ + +	0	0
	8	♂	711±34 (3)	+	+ +	+ +	628±119 (3)	+	+ +	+ +
	1	♀	583±27* (3)	—	—	—	251±18 (3)	0	0	0
	8	♀	608±44 (3)	+	+ +	+ +	274±15 (3)	0	0	0
Yellow phosphorus (7·5 mg./kg.)	2	♂	91±23 (3)	+ + +	0	0	338±35 (3)	+ (?)	0	0
	5	♂	744±37 (3)	+ +	+ +	+	462±89 (3)	0	0	0
	10	♂	429±80 (3)	0	0	+ + +	309±32 (3)	0	0	0
Sulphathiazole (43 g./kg.)	3	♂	460±29 (3)	+	0	0	368±13 (3)	0	0	0
Pregnanediol (333 mg./kg.)	1·7	♀	287±65 (3)	0	0	0	321±64 (3)	0	0	0
†Pregnanediol β-D-glucuronide (800 mg./kg.)	1·7	♀	241±36 (3)	—	—	—	264±19 (3)	—	—	—
Ether (40 min. deep anaesthesia)	1·75	♀, ♂	265±30 (4)	0	0	0	309±63 (3)	0	0	0
Sodium sulphapyridine monohydrate (18–36 g./kg.)	2	♀, ♂	327±17 (7)	+ +	+	+	362±11 (7)	+ (?)	0	0

* Results for glucuronidase in liver obtained after intraperitoneal injection of 0·5 g. chloroform/kg.

† Pregnane-3(α):20(α)-diol glucuronidic acid free from pregnane-3(α)-ol-20-one glucuronidic acid (Sutherland & Marrian, 1947).

activity was observed after injection of as little as 0·2 g. chloroform/kg. subcutaneously.

Yellow phosphorus, injected subcutaneously in olive oil, had no marked effect on kidney, but produced profound and extensive changes in liver (congestion, fatty degeneration and necrosis). From the results of experiments dealt with above it will have been noted that there may be no rise in glucuronidase activity in an organ when damage is at its height. In the case of phosphorus, there was an unmistakable initial drop in the activity of the enzyme, to one-third of its normal value. When, at the end of 5 days, repair was well under way, the enzyme level showed the usual increase, only to fall again when repair was complete.

Of the remaining substances listed in Table 3, ether and pregnanediol produced no pathological changes and had no effects on glucuronidase in either liver or kidney. Pregnanediol glucuronide resembled the parent compound in its effects on the enzyme, but was not examined for histological effects. Ether was given by inhalation, and pregnanediol and its glucuronide were injected intraperitoneally as suspensions in olive oil. Sulphathiazole caused cloudy swelling in liver after subcutaneous injection of a very large dose as a neutral solution in 0·9% sodium chloride solution. There was a small, but significant ($P = 0·05$–$0·02$) rise in liver glucuronidase activity. This compound had no effect of any kind on kidney. Sodium sulphapyridine, given in the same way as sulphathiazole, caused fatty degeneration and necrosis in liver, accompanied by some cell proliferation, but without appreciable change in the enzyme level. In some animals there was slight damage to the kidneys, again without any rise in glucuronidase activity.

Uranyl acetate. Results for glucuronidase in liver and kidney after subcutaneous injection of mice with varying doses of uranyl acetate in 0·9% sodium chloride solution (Fig. 1) illustrate the point that increasing the dose of a toxic agent may retard the rise in glucuronidase activity in the early stages of poisoning, and may even cause an initial drop in the enzyme level. Each point in the figure is an average for a group of three male mice, killed 2 days after injection. Severe tubular 'nephrosis' was noted at this stage with all four doses of the toxic agent. Cell proliferation could also be seen after all but the

largest dose, becoming more marked as the dose fell. In the case of liver, the histological findings were more difficult to interpret as the damage, which was mainly subcapsular, was transitory and rapidly succeeded by intensely active cell proliferation. Only the latter response was observed after injection of the smallest dose of uranyl acetate. In general, however, damage was greater and repair processes

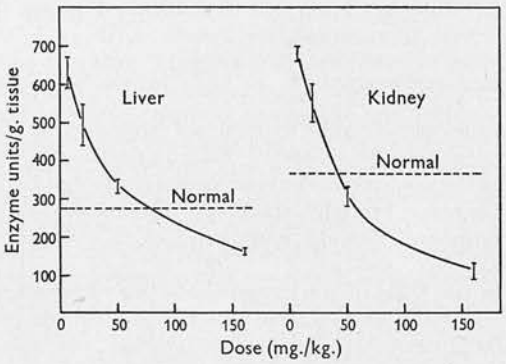

Fig. 1. Liver and kidney glucuronidase activity 2 days after subcutaneous injection of mice with varying doses of uranyl acetate. Mean ± S.E. shown for each point.

slower to appear as the dose was increased. Ten days after injection of the smallest dose of uranyl acetate repair was finished in both liver and kidney, and their enzyme levels had returned to normal. No change in spleen glucuronidase was produced by uranyl acetate.

The possibility that in a severely damaged organ an apparently normal value for glucuronidase may be observed at a certain stage, even though cell proliferation may have commenced, probably explains the fact that only small rises in the liver enzyme were observed after prolonged feeding of menthol (total dose 9·3 g./kg., Table 2) or subcutaneous injection of sodium sulphapyridine (Table 3).

Phenylarsenoxide. Results obtained with this compound (Fig. 2) show the changes in liver and kidney glucuronidase activity at various stages in different degrees of poisoning. Phenylarsenoxide was injected subcutaneously as a neutral solution in 0·9 % sodium chloride solution. Each point in the figure is an average for a group of three male mice, except in the case of the 1 day figures with the larger dose, which are both based on six results. Phenylarsenoxide caused peripheral lobular necrosis and fatty degeneration in liver, and diffuse nephritis in kidney. Damage to both organs was intense 1 day after injection of 1 mg./kg., with no signs of repair. After 3 days, repair processes had become evident, and replacement of damaged tissue was well advanced after 5 days. At the end of 10 days, kidney was entirely normal and liver repair was almost complete. With the smaller dose (0·2 mg./kg.), cell proliferation was marked in both organs after 1 day, and repair was far advanced after 4 days. Phenylarsenoxide had no effect on spleen glucuronidase, and the histological changes produced in liver and kidney were similar for both sexes.

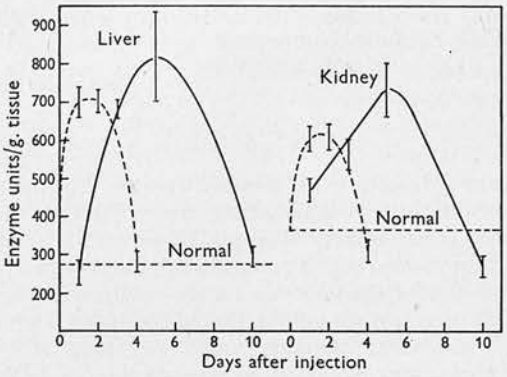

Fig. 2. Liver and kidney glucuronidase activity at varying periods after subcutaneous injection of mice with phenylarsenoxide. ——, 1 mg./kg.; — —, 0·2 mg./kg. Mean ± S.E. shown for each point.

Effects of various substances on the enzyme in vitro. All the substances examined for their effects on β-glucuronidase activity *in vivo*, with the exception of pregnanediol glucuronide, were tested for their effect on the assay *in vitro* in a concentration of 0·1 % (w/v). A solution in the medium used for injection was added to the citrate buffer and shaken vigorously. In no case did the presence of the agent in the incubation mixture affect the activity of β-glucuronidase from normal mice.

Infant mice and partially hepatectomized mice. As shown in Table 4, glucuronidase activity in spleen, liver and kidney was much higher in young mice, ranging in age from 1 to 15 days, than in normal adults. The remaining lobes of liver in adult mice (male and female), 3–8 days after sub-total hepatectomy, were hypertrophied. The glucuronidase level was high and cell proliferation was very active. In preparing the animals, 60 % of the liver was removed by cautery or ligature under ether anaesthesia, the whole operation taking less than 10 min. There was no difference in the final result between the alternative surgical techniques, nor at the various times of examination. One animal (result omitted from Table 4) was comatose and apparently about to expire when killed 3 days after operation. As expected, the remaining fraction of the liver showed no increase in weight, in the glucuronidase activity nor in cell proliferation.

Table 4. *β-Glucuronidase after sub-total hepatectomy and in infant mice*

(Results expressed as in Table 1)

Age (days)	Treatment	Average enzyme activity and S.E. (G.U./g. moist tissue) Spleen	Liver	Kidney
Adult	None	636±70 (23)	273±13 (23)	363±24 (11)
1*	None	5100 (3)	1370 (3)	881 (3)
5*	None	2670 (2)	1294 (2)	702 (2)
5*	None	3820 (2)	1432 (2)	883 (2)
13*	None	1521 (2)	2218 (2)	606 (2)
15	None	5169±2820 (3)	1239±49 (3)	727±108 (3)
Adult	Partial hepatectomy 3–8 days previously	—	1046±88 (10)	—

* Each organ pooled before enzyme assay.

DISCUSSION

Our own results with menthol confirm Fishman's (1940) findings in so far as the enzyme in liver and kidney is concerned. It seems, however, that Fishman suppressed repair processes by overdosage with menthol, and thus obtained a rise in glucuronidase which was only a small fraction of that provoked by the first doses of the compound. No explanation can be offered for our failure to observe the rise in the activity of spleen glucuronidase in menthol-treated mice reported by Fishman, unless the discrepancy has its origin in the wide variation in its glucuronidase level normally shown by spleen. This variation one might expect if glucuronidase activity is a measure of the amount of cell proliferation in progress.

The present work shows that the effects of menthol on β-glucuronidase activity *in vivo* bear no relation to its glucuronidogenic property, but are secondary to its hitherto unsuspected toxic action on liver and kidney. It seems impossible that chloroform, carbon tetrachloride, mercuric nitrate, phosphorus or uranyl acetate should give rise to a glucuronide in the body, and yet all these substances have been found to cause striking changes in glucuronidase activity. Of other substances which caused a rise in glucuronidase, phenylarsenoxide could conceivably form a derivative conjugated with glucuronic acid, and evidence has been obtained that sulphathiazole is partially excreted in rabbits as the glucuronide of a hydroxy derivative (Thorpe & Williams, 1940). In spite of the very large dose injected, sulphathiazole caused only a relatively small rise in glucuronidase activity in mice, and this was confined to liver. No change in glucuronidase was observed after injection of two compounds which are known to be glucuronidogenic, pregnanediol (Venning & Browne, 1936) and sodium sulphapyridine (Scudi, 1944). It should be pointed out that a change in experimental conditions might reveal an effect of sulphapyridine on glucuronidase in liver, since it produced some damage in this organ. The effect of menthol glucuronide on liver glucuronidase was due presumably to menthol liberated by the enzyme initially present. Fishman's theory (see p. 462) provides no explanation for a change in the enzyme brought about by administration of a compound already conjugated with glucuronic acid.

On the basis of the experiments described above it is not possible to decide whether β-glucuronidase is actually concerned in cell proliferation, or whether the increases in activity observed merely reflect an increase in metabolic activity. It is interesting to note, however, that the rise in the enzyme level occasionally slightly preceded the first appearance of cell division which was definitely in excess of normal. Whatever the cause of the parallelism between the glucuronidase activity in an organ and the amount of tissue growth in progress, it provides a straightforward explanation of the changes in the enzyme in liver and kidney which follow administration of menthol and other substances to mice. It seems possible that the same explanation can be applied to the effect of oestrogens on uterine glucuronidase (Fishman & Fishman, 1944; Fishman, 1947), and to a recent observation (Fishman & Anlyan, 1947), which suggests that in some cases of human carcinoma the tumour contained more glucuronidase than the corresponding normal tissue. The possible bearing of our results with carbon tetrachloride on the finding (Pincus & Martin, 1940) that, in liver poisoning produced by this compound, the physiological activity of oestrone is enhanced is of interest.

It is no longer necessary to speculate on the probable role of glucuronidase in the body in order to explain the changes in activity produced by extrinsic agents. The citation by Fishman (1947) of the work of other authors in support of his contention that the enzyme acts synthetically, however, makes it necessary to consider their results from this angle. Florkin, Crismer, Duchateau & Houet (1942) obtained evidence for the condensation of glucuronic acid with borneol in the presence of β-glucuronidase, but the percentage conjugation was very small under extreme conditions, and they concluded: 'Quant à

savoir si cette synthèse enzymatique correspond au mécanisme réalisé *in vivo*, c'est évidemment une autre affaire.' In the work of Lipschitz & Bueding (1939) and Crépy (1946) on the formation of conjugated glucuronides by surviving liver slices, there is no suggestion that the enzyme concerned is β-glucuronidase. De Meio & Arnolt (1944), who studied conjugation of phenol by surviving tissue slices, found that glucuronic acid reversed the inhibition of this process produced by iodoacetate. They also found that feeding phenol and borneol to rats increased phenol conjugation by liver and kidney *in vitro*. Their results are difficult to interpret since it is known that phenol may be conjugated with either sulphuric or glucuronic acid. Even if De Meio & Arnolt are correct in thinking that, contrary to the views of Lipschitz & Bueding (1939), glucuronides are formed by direct condensation of the 'aglucurone' with free glucuronic acid, there is no reason to believe that β-glucuronidase is responsible. With regard to De Meio & Arnolt's second finding, there is, in view of our own work, no need to postulate adaptation by the enzyme or enzymes responsible for conjugation of phenol, since both phenol and borneol may have caused liver and kidney damage in their experiments. Results obtained by Bueding & Ladewig (1939) in studying the effect of chloroform poisoning in guinea pigs on glucuronide synthesis by liver slices are of interest in this connexion. Not only did the liver slices from the poisoned animals show the usual increase in glucuronide synthesis on addition of lactate, but they were apparently more active in forming borneol glucuronide than slices from normal animals. The latter aspect of their results is not touched upon by the authors. While there is thus some evidence to suggest that, following damage to an organ, there may be an increase in its ability to form conjugated glucuronides, it is at present impossible to say whether or not this is due to the rise in β-glucuronidase activity observed when repair is in progress, nor is it certain that the glucuronides are formed directly from free glucuronic acid.

SUMMARY

1. The effect of menthol administration to mice in increasing the β-glucuronidase activity of liver and kidney is due to its toxic action on these organs. The rise in enzyme activity is associated with an increase in cell proliferation following injury. Menthol had no effect on spleen glucuronidase.

2. Among other substances examined, the following caused changes in glucuronidase in liver or kidney in an analogous fashion to menthol: chloroform, carbon tetrachloride, mercuric nitrate, yellow phosphorus, phenylarsenoxide, uranyl acetate, menthol glucuronide and sulphathiazole. The effect of chloroform on kidney glucuronidase was confined to male mice.

3. Livers from adult mice after sub-total hepatectomy, and spleens, livers and kidneys from infant mice showed high glucuronidase activities.

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β-Glucuronidase as an Index of Growth in the Uterus and other Organs. By Lynda M. H. Kerr and G. A. Levvy. (*Department of Biochemistry, University of Edinburgh*)

The β-glucuronidase activity of mouse liver or kidney has been shown to reflect the degree of proliferative activity (Levvy, Kerr & Campbell, 1948), and it was considered that a similar relationship would explain the rise in uterine glucuronidase observed by Fishman & Fishman (1944) after treatment of ovariectomized mice with oestrogens. A comparative study has been 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 the other organs examined, an increase in the activity of this enzyme appeared to be associated with increased growth. A high uterine glucuronidase activity was observed in infant mice as compared with normal adults, and the action of oestrone on the enzyme in ovariectomized mice could be antagonized by testosterone (cf. Fishman, 1947). The value of glucuronidase figures as a biochemical index of growth was illustrated by the discovery of new facts relating to liver and uterus, and it is with these that the present communication is particularly concerned.

After injection of ovariectomized mice with 1·7 mg. oestrone/kg., there was a marked rise in liver glucuronidase. This effect, which was also seen in normal and castrate males, but not in normal females, was antagonized by testosterone, itself without any action on the liver. Histological examination revealed great mitotic activity, with little evidence of damage, in livers showing the response to oestrone. Bullough (1946) found that oestrone stimulated mitotic activity in many organs in the female mouse. His experiments were, however, confined to normal females, and no effect was seen in liver.

One week after injection of ovariectomized mice with $CHCl_3$ or CCl_4, 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 ovariectomized mice were submitted to partial hepatectomy, with similar results. These results can only be explained on the assumption that the body is capable of producing an extra-ovarian growth hormone for uterus. In this case, there is obvious need for care in interpreting certain experiments (for example, see Roberts & Szego, 1947) in which the action of liver damage and regeneration in enhancing the effectiveness of administered oestrogens is claimed to be due to a disturbance of their normal metabolism. In view of the effect of oestrone on liver, a hitherto unsuspected complication must be looked for in the action of this compound on the uterus of the ovariectomized mouse.

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Increased β-Glucuronidase in Response to Tissue Injury. By G. A. Levvy, Lynda M. H. Kerr and J. G. Campbell (introduced by G. F. Marrian). (*Department of Biochemistry, University of Edinburgh and Royal (Dick) Veterinary College, Edinburgh*)

Fishman (1940) found that repeated feeding of menthol to mice caused statistically significant increases in β-glucuronidase in liver, spleen and kidney. He postulated this enzyme to be responsible for glucuronide synthesis *in vivo*, and suggested that he was measuring adaptation by the enzyme in response to the presence of excess substrate. Menthol is known to be excreted as the glucuronide in certain species, but it has not been established that β-glucuronidase acts synthetically.

Using a new method for assay of the enzyme (Kerr, Graham & Levvy, 1947), an attempt was made to confirm Fishman's findings. Twenty-four hours after intraperitoneal injection of *l*-menthol in oil into mice, there was a marked rise in glucuronidase in liver, but not in kidney and spleen. Gross liver damage was noted, and it was considered that this might explain the rise in the enzyme. On histological examination, fatty degeneration, necrosis and evidence of repair were seen in the liver, while the kidneys and spleen showed little deviation from normal.

A variety of substances known to produce liver or kidney damage, but in most cases unlikely to form glucuronides, were injected into mice and liver, kidney and spleen were examined histologically and for their glucuronidase content. Carbon tetrachloride and *l*-menthol-β-D-glucuronide resembled menthol in their effects. Chloroform behaved in the same way as menthol with regard to liver and spleen, but produced severe damage in kidney unaccompanied by any rise in the enzyme. Mercuric nitrate produced profound damage in kidney, followed by rapid repair and a rise in glucuronidase. It had little effect in either respect on liver and spleen. Phenylarsenoxide and yellow phosphorus caused severe liver damage with little evidence of repair. The former had no effect on the glucuronidase level in liver, and the latter depressed it, favouring the view that a rise in the enzyme is associated with repair rather than damage. The results obtained for kidney after injecting chloroform could be explained likewise.

A further series of experiments was carried out with relatively non-toxic compounds known or considered to give rise to glucuronides *in vivo*. Neither pregnanediol, sulphathiazole nor pregnanediol-β-D-glucuronide affected the glucuronidase level in liver, spleen or kidney. No pathological changes in these organs were observed after injection of the first two compounds.

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