3. The catalase concentration in normal rat liver extracts of equal protein content was not significantly changed, either by different protein levels in the diet or by peptone injections. It was raised, however, by serum injections.

4. In confirmation of the work of Greenstein and his group (1941-3) it was found that the growth of a transplanted tumour leads to a fall of liver catalase concentration which, in extreme cases, may reach 5% of normal. There was a very marked individual scatter even in similar stages of tumour growth.

5. The catalase concentration when plotted

against (body weight)/(tumour weight) was found to follow an exponential curve.

6. The protein level of the diet had no significant effect on the course of the process, even in the initial stages. Serum injections had no effect on tumour-bearing rats when started 11 days after transplantation. When started 4 or 5 days after transplantation they were followed by regression of the tumour.

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The β -Glucuronidase Activity of Ox Spleen and the Assay of β -Glucuronidase Preparations

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The existence in animal tissues of an enzyme which hydrolyzes β -glucuronides was first reported by Sera (1915). In 1934, Masamune described a method of preparation of active extracts of this enzyme from ox kidney having pH optima for the hydrolysis of menthylglucuronide and phenylglucuronide in citrate buffers at 5.3 and 5.3-5.6 respectively.

Oshima (1934) reported data for the glucuronidase content of various tissues of the dog and the ox, rich sources being liver, spleen, kidney, ovary, testes and thymus. In 1936 Oshima introduced improvements into the method of Masamune (1934), which were claimed to give a purer preparation. For this preparation the pH optimum for the hydrolysis of menthylglucuronide in acetate buffer was quoted as 5.0-5.2. In the work reported up to that time, little

or no data were presented concerning the degree of purification or the losses at each stage of the preparation of β -glucuronidase.

The first quantitative study of the purification of ox-spleen glucuronidase was reported by Fishman (1939a). By employing ammonium sulphate fractionation after preliminary treatment, Fishman achieved an eightfold concentration of the enzyme and a 140-fold purification. There was, however, an overall loss of 84% by this method. Fishman (1939b) reported that the pH optima of his preparation in an acetate buffer were 5.0 for *l*-menthylglucuronide, 4.4 for borneolglucuronide and 4.3 for oestriolglucuronide.

Florkin, Crismer, Duchateau & Houet (1942) also studied glucuronidase, and demonstrated the reversibility of the hydrolysis of borneolglucuronide *in vitro*.

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A much improved method of preparation of ox-spleen glucuronidase was reported by Graham (1946) in which a 400-fold concentration and a 315-fold purification of the enzyme was achieved. Graham found that a preliminary autolysis of an aqueous extract of acetone-extracted tissue caused an increase in the amount of the enzyme, and that this was accompanied by a liberation of reducing material. These facts were shown to be complicating factors in the assay of crude preparations of the enzyme. It was also demonstrated that pH control was an important factor during the separation of the enzyme from inactive protein by ammonium sulphate fractionation. Graham, however, reported no data on the pH optima or reaction kinetics of his preparation.

Talalay, Fishman & Huggins (1946) reported data concerning the hydrolysis of phenolphthalein glucuronide by fairly crude preparations of glucuronidase from pooled livers, kidneys and spleens of mice. The pH optimum in acetate buffer for this substrate was shown to be 4.5. Data on the kinetics of the reaction were also presented. Talalay *et al.* (1946) and Fishman & Talalay (1947) have used phenolphthalein glucuronide and pH 4.5 in a method of assay of the glucuronidase content of various tissues of the rat.

In the present work further data on the preparation and purification of ox-spleen glucuronidase are presented. The data reveal that two enzymes which hydrolyze *l*-menthylglucuronide are present in ox spleen, and that these two enzymes, which can be separated, have different pH optima. A modified technique for the assay of glucuronidase preparations using *l*-menthylglucuronide is presented along with assay methods employing phenylglucuronide and phenolphthalein glucuronide.

A preliminary report of this work has already appeared (Mills, 1947).

EXPERIMENTAL

Reagents

l-Menthylglucuronide, prepared by the biosynthetic method of Quick (1924).

Phenylglucuronide, prepared by the method of Masamune (1933) as modified by Williams (1945), m.p. 160° (uncorr.).

Phenolphthalein glucuronide, prepared by the method of Talalay *et al.* (1946).

D-Glucurone, prepared by the method of Williams (1940), m.p. 176–177° (uncorr.), $[\alpha]_D^{20} + 20^\circ$.

Trichloroacetic acid (British Drug Houses Ltd.), 20% (w/v) aqueous solution.

Alkaline ferricyanide solution, 5 g. $K_3Fe(CN)_6$ (A.R.), and 10 g. anhydrous Na_2CO_3 in water to 1 l. This is stable for 1 month in a dark bottle; after that time the blank value in the reducing test increases considerably.

Sodium carbonate, 10% (w/v) aqueous solution.

Ceric sulphate, approx. 0.1 N solution, prepared according to Miller & Van Slyke (1936), and diluted 1 in 10 in $N-H_2SO_4$ for daily use.

Sulphuric acid, 18 N (A.R.).

Lissamine green (British Drug Houses Ltd.), 0.05% (w/v) aqueous solution.

Phenol reagent, prepared as described by Folin & Ciocalteu (1927).

All pH measurements were carried out with the glass electrode and Cambridge pH meter.

ASSAY OF GLUCURONIDASE PREPARATIONS

(a) Using *l*-menthylglucuronide

In the methods of Fishman (1939*a*) and Mills (1946) the neutralization of excess trichloroacetic acid, after the removal of precipitated protein from the enzyme digests, was accomplished by *N*-NaOH in the presence of phenolphthalein. This method gave rise to high blank values in the subsequent ferricyanide reduction. The method of Levvy (1946), which employs an alkaline copper tungstate deproteinization, was found to be unsatisfactory if the pH of the enzyme digest was less than 4.6. This criticism applies also to a cadmium hydroxide precipitation, a preliminary exact neutralization of the digest being necessary in both cases to give reliable results.

The most satisfactory procedure so far found involves a trichloroacetic acid deproteinization followed by addition of excess sodium carbonate. It was found that wide variations in the sodium carbonate concentration were allowable in the ferricyanide reduction (cf. Levvy, 1946).

Method. Acetate buffer (0.2 M; 1.0 ml.), 0.5 ml. of 0.02 M *l*-menthylglucuronide solution (pH c. 5) and 0.5 ml. of a suitable dilution of the enzyme solution are incubated in 15 ml. centrifuge tubes for 2 hr. at 38°. The reaction is then stopped and protein removed by the addition of 1 ml. of 20% trichloroacetic acid followed by centrifugation; 2 ml. of the supernatant fluid are transferred to 4 × $\frac{5}{8}$ in. Pyrex tubes and 1 ml. of 10% Na_2CO_3 solution followed by 1 ml. of alkaline ferricyanide solution added. The tubes are closed with glass bulbs, heated in a boiling water bath for 15 min. and then cooled in running water. H_2SO_4 (18 N; 0.25 ml.) is added, followed by 1 drop of 0.05% Lissamine green solution, and the mixture titrated with c. 0.01 N-ceric sulphate solution using a 2 ml. micro-burette. Stirring is performed by a slow stream of N_2 , and a daylight lamp is used for the titration.

The ceric sulphate is standardized against *D*-glucurone and results expressed as glucuronic acid by employing the appropriate correction factor. Controls are carried out using buffer and enzyme solution alone, since it was found that hydrolysis of the substrate in the absence of glucuronidase at pH 3–7 could be neglected.

By using unboiled enzyme extracts in the control tubes, interference caused by the increase in reducing material on incubation of crude extracts is avoided. Graham (1946) first demonstrated this increase in reducing material, and some typical data are presented in Fig. 1. This method does not avoid, however, the increase in enzymic activity which accompanies the increase in reducing material on incubation of such crude extracts. Neither increase occurs in extracts after precipitation with ammonium sulphate.

Fig. 1. Liberation of reducing material (calculated as glucuronic acid) during the incubation of 2 ml. samples of a crude spleen extract at 38°. 1, unboiled extract; 2, boiled extract.

It was also found that the presence of ammonium sulphate at a concentration of 0.05M or less in the enzyme digests did not affect the results of assay.

The glucuronidase reaction was found to follow the equation

$$k = 1/t \log_e a/(a-x)$$

at low substrate concentrations, where t = time in hours, a = original amount of substrate and x = amount of substrate split in time t .

Unit of glucuronidase activity. The unit of glucuronidase activity (menthyl unit) was taken as the activity associated with a value of $k = 0.1$ in the above equation, under the conditions specified.

(b) Using phenylglucuronide

The method is based upon the determination of phosphatase using phenylphosphate (King & Armstrong, 1934; Folley & Kay, 1935). Phenylglucuronide is incubated with the enzyme preparation in a buffer solution, and after a standard time the reaction stopped and the phenol estimated using the reagent of Folin & Ciocalteu (1927).

Method. Acetate buffer (0.2M; 1.0 ml.), 0.5 ml. of 0.02M-phenylglucuronide (Na salt) and 0.5 ml. of enzyme solution are incubated in 15 ml. centrifuge tubes for 1 hr. at 38°; 2 ml. of a 1 in 3 dilution of the Folin-Ciocalteu reagent are added and the protein precipitate removed by centrifugation. 3 ml. of the supernatant fluid are added to 3 ml. of 10% Na_2CO_3 and the mixture incubated at 38° for 40 min. Water (2 ml.) is added and the blue colour estimated in the Spekker photoelectric absorptiometer using Ilford no. 608 red filters. The phenol liberated is estimated by using a calibration curve determined with phenol solutions treated as in an enzyme assay.

Controls were set up in which the buffer and substrate were incubated together for 1 hr. at 38° and then the enzyme, immediately followed by the diluted Folin reagent, added at the end of this time.

Since the liberation of phenol was found to follow a linear course with time and to be directly proportional to the enzyme concentration, the amount of phenol liberated is, therefore, directly relatable to the amount of enzyme present.

1 phenyl unit = 1 μg. phenol liberated/hr. under the above conditions.

(c) Using phenolphthalein glucuronide

The method is essentially that of Talalay *et al.* (1946) with the difference that the enzyme digests consist of 0.5 ml. of 0.2M-acetate buffer, 0.2 ml. of 0.005M-phenolphthalein glucuronide (Na salt) and 0.3 ml. of enzyme solution, and that protein is removed with trichloroacetic acid before bringing the pH to 10.4. The colour is estimated in the Spekker photoelectric absorptiometer using an Ilford 605 yellow-green filter.

1 phenolphthalein unit = 1 μg. phenolphthalein liberated/hr. under the above conditions.

Determination of protein nitrogen

The protein in 1 ml. samples was precipitated with 1 ml. of 20% trichloroacetic acid and the mixture heated to 80° for 10 min. The precipitate was removed by centrifugation and washed on the centrifuge with 5 ml. of 5% (w/v) trichloroacetic acid. If ammonium sulphate was present in the original sample, the washing was repeated a further four times. The protein precipitate was dissolved in 3 ml. of 18N-sulphuric acid and the protein N estimated, after digestion, by the micro-Kjeldahl method of Ma & Zuazaga (1942), employing the Markham (1942) distillation unit.

PREPARATION OF β -GLUCURONIDASE

The finding of Graham (1946) that acetone precipitation of the enzyme from aqueous solution followed by extraction of the precipitate with water, as employed by Fishman (1939a), gave rise to serious

losses was confirmed in the present work. It was also found, in accordance with Graham (1946), that by extracting the tissue pulp with two successive lots of 2 vol. of acetone and subsequent extraction of the solid residue with water, one could avoid these serious losses. Ethanol precipitation, as employed

influenced by both these factors. It is quite obvious therefore that the results of ammonium sulphate fractionation of protein mixtures will be significantly influenced by variations in pH and temperature, factors which are often neglected during salt fractionation of proteins.

Fig. 2. Variable solvent solubility test on spleen extract at stage C of preparation, carried out at pH 5. Ammonium sulphate used as precipitant and glucuronidase assayed at pH 5 using *l*-menthylglucuronide.

by Masamune (1934) and Oshima (1936), also gave rise to as much as a 50% loss of activity.

In the method of Fishman (1939*a*) there was no control of the pH during ammonium sulphate fractionation. Cohn (1925) has shown that the solubility of proteins in salt solutions obeys the equation

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

where S =solubility, I =ionic strength, and β and k are constants. Cohn found that while k is independent of temperature and pH, β is markedly

Graham (1946) took pH into account during ammonium sulphate fractionation, but did not indicate how the limits employed were derived.

In order to obtain precise data in the present work the variable solvent solubility test as defined by Falconer & Taylor (1946) was applied to crude glucuronidase solutions at two different pH values.

Samples (4 ml.) of a glucuronidase solution, adjusted to the requisite pH, were introduced into a series of 15 ml. centrifuge tubes and saturated (NH₄)₂SO₄ at the same pH added to give the required ionic strength. The tubes were

allowed to stand at 0° for 18 hr. and the precipitates, removed by centrifugation at 0° , were dissolved in water and assayed at pH 5 for glucuronidase activity using *l*-menthylglucuronide. Protein N determinations were carried out on the supernatant solutions.

The data obtained at pH 5.0 and 7.0 (Figs. 2 and 3 respectively) reveal that two proteins exist in spleen

conditions of temperature and pH which make $\beta_A - \beta_B$ a maximum are optimum for separation.

These constants must be determined on the pure phases to give absolute values, but the direction of difference can be obtained from the solubility test. If in the present work the fraction precipitated at lower salt concentration gives k_A and the other

Fig. 3. Variable solvent solubility test on spleen extract at stage C of preparation, carried out at pH 7. Details as for Fig. 2.

extracts, both having glucuronidase activity. Falconer & Taylor (1947) indicated methods of separation of two phases (A and B) based upon determination of the constants β and k . They stated that if for two fractions k_A is greater than k_B then a dilution of the original solution aids separation of phase A in a pure form. If k_B is greater than k_A then concentration aids in obtaining A in pure form. Also con-

fraction k_B then it is obvious from the data that k_B is greater than k_A .

A method of purification of glucuronidase was worked out using the data obtained above. Throughout the procedure which follows, the pH was controlled at each stage by means of a glass electrode, and all operations requiring temperature control were carried out in a cold room at 0° .

Stage A. Fresh ox spleens were stripped of fat and finely minced. Redistilled acetone (3 l.) was stirred into 1.5 kg. of this mince and the mixture filtered and sucked dry. The pressed filter cake was stirred with a further 3 l. of acetone and the mixture filtered with suction and pressed well. The filter cake was broken up and dried in air at room temperature for about 30 min. The spleen powder was stirred with 3 l. of water for 1 hr., strained through gauze, clarified in the Sharples super-centrifuge and dialyzed against running tap water for 24 hr. (solution A).

Stage B. Solution A was adjusted to pH 5.0 with 2N-acetic acid and M-acetate buffer (pH 5.0) added (20 ml./l.). A voluminous precipitate formed. The mixture was allowed to stand at 38° for 4 hr. and the precipitate removed in the Sharples super-centrifuge. The filtrate was finally clarified by filtration using Standard Super-Cel (Johns-Manville Co. Ltd.) (solution B).

Stage C. Solid $(\text{NH}_4)_2\text{SO}_4$ was stirred into solution B to 60% saturation and the mixture allowed to stand at 0° for 18 hr. The supernatant liquid was siphoned off and the precipitate removed by filtration using Standard Super-Cel (10 g./l.). The filter cake was sucked dry and then extracted five times with 100 ml. portions of water. The extracts were combined and dialyzed against running tap water for 24 hr. (solution C).

Stage D. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to solution C to 1.2M and the pH adjusted to 5.0 with 2N-acetic acid. The mixture was allowed to stand at 0° for 6 hr., the precipitate removed at the centrifuge and discarded. To the solution $(\text{NH}_4)_2\text{SO}_4$ was added to 1.8M, the pH readjusted to 5.0 and the mixture allowed to stand at 0° for 18 hr. The precipitate was removed at the centrifuge at 0°, dissolved in water and dialyzed against running tap water for 24 hr. (solution D). This stage removes a considerable amount of inactive protein.

Stage E. Solution D (about 500 ml.) was adjusted to pH 5.0 with N-acetic acid, and saturated $(\text{NH}_4)_2\text{SO}_4$, previously adjusted to pH 5.0, was added to 1.26M. The mixture was allowed to stand at 0° for 6 hr. and the precipitate removed at the centrifuge at 0° and discarded. To the supernatant liquid saturated $(\text{NH}_4)_2\text{SO}_4$ at pH 5 was added to 1.54M, the solution allowed to stand at 0° for 18 hr., the precipitate removed at the centrifuge at 0°, dissolved in 25 ml. of water and dialyzed against running water for 24 hr. (fraction I). To the supernatant liquid from the above precipitation, $(\text{NH}_4)_2\text{SO}_4$ at pH 5.0 was added to 1.78M, the

mixture allowed to stand at 0° for 18 hr., the precipitate removed at the centrifuge at 0°, dissolved in 200 ml. water, and dialyzed against running water for 24 hr. (fraction II).

Stage F. Fraction I was reprecipitated with saturated $(\text{NH}_4)_2\text{SO}_4$ at pH 5 between 1.26 and 1.52M at 0°, and the precipitate was washed on a centrifuge at 0° with 1.52M- $(\text{NH}_4)_2\text{SO}_4$ at pH 5, dissolved in the minimum amount of

Fig. 4. pH-activity curve of spleen extract at stage D of preparation. Glucuronidase assays using *l*-menthyl-glucuronide.

water and dialyzed against running water for 24 hr. Fraction II was reprecipitated from the combined supernatant liquids with saturated $(\text{NH}_4)_2\text{SO}_4$ at pH 5 between 1.56 and 1.76M at 0° and the precipitate dissolved in about 200 ml. of water and dialyzed against running water for 24 hr.

Stage G. Stage F was repeated to obtain more pure fractions.

Data for the activity of a typical preparation at each stage of purification are presented in Table 1.

Table 1. Activity of β -glucuronidase preparations at various stages of purification

Stage	Vol. (ml.)	Assays at pH 4.5		Assays at pH 5.0		Ratio units at pH 5.0 pH 4.5
		Units*/ml.	Total units	Units*/ml.	Total units	
A	3600	3.14	11,304	2.99	10,764	0.95
B	3400	4.02	13,668	3.90	13,260	0.97
C	780	17.13	13,361	16.45	12,831	0.96
D	580	16.79	9,738	16.45	9,541	0.98
E: Fraction I	35	133.12	4,659	114.61	4,011	0.86
Fraction II	220	15.91	3,502	17.85	3,927	1.12
F: Fraction I	33	98.00	3,234	82.12	2,710	0.84
Fraction II	230	11.92	2,741	13.95	3,208	1.15
G: Fraction I	24	111.20	2,669	90.04	2,161	0.81
Fraction II	31	72.61	2,251	85.63	2,654	1.18

* Menthyl unit of glucuronidase activity, see p. 127.

Fig. 5. pH-activity curves of fractions I and II at stage E of preparation. Glucuronidase assay using *l*-menthylglucuronide. I, fraction precipitated between 1.26 and 1.54 M $(\text{NH}_4)_2\text{SO}_4$; II, fraction precipitated between 1.54 and 1.78 M $(\text{NH}_4)_2\text{SO}_4$.

Fig. 6. pH-activity curves of fractions I and II at stage G of preparation. Glucuronidase assays using *l*-menthylglucuronide. Details as for Fig. 5.

pH-activity curves using *l*-menthylglucuronide were determined for the preparations at stages D, E and G, and typical data are presented in Figs. 4, 5 and 6 respectively. The pH-activity curve at stage D in some preparations showed evidence of two peaks; in others, the curve shown in Fig. 4 was obtained. pH-activity curves for the pure fractions were also determined using phenyl and phenolphthalein glucuronides, and the data for all three substrates are shown in Table 2.

Table 2. pH optima of ox-spleen β -glucuronidases

Substrate	β -Glucuronidase	
	I	II
<i>l</i> -Menthylglucuronide	4.5	5.0
Phenylglucuronide	4.5	5.2
Phenolphthalein glucuronide	4.5	5.2

DISCUSSION

It would appear from the work of Masamune (1934), Oshima (1936), Fishman (1939 *a, b*) and Talalay *et al.* (1946) that these authors considered they were dealing with a single enzyme. This conclusion may reasonably be drawn from the data they presented on the kinetics of the hydrolytic reaction. While presenting no data upon optimum pH or reaction kinetics, Graham (1946) gave no evidence which could indicate that there was more than one enzyme present in his extracts.

By the application of the variable solvent solubility test it has been possible to show the presence in spleen extracts of two proteins having β -glucuronidase activity. The fact that the two enzymes have different pH optima makes extremely difficult the interpretation of the specific property solubility test of Falconer & Taylor (1946). At pH 5 the two proteins having glucuronidase activity are precipitated by ammonium sulphate within the limits 31.5–38.5% and 38.5–44% saturation, these two fractions having pH optima for the hydrolysis of *l*-menthylglucuronide of 4.5 and 5.0 respectively. Fishman (1939 *a*) used the limits 37–50% saturation and Graham (1946) 36–46% saturation with ammonium sulphate in the preparation of their enzyme extracts. It is obvious, therefore, that these workers eliminated the major portion of the enzyme having optimum pH 4.5 for the hydrolysis of *l*-menthylglucuronide. It is probable, therefore, that the data of Fishman (1939 *b*) concerning pH optima and Michaelis constants may be substantially correct for one of the enzymes.

In later work Talalay *et al.* (1946) used a preparation from a mixture of livers, spleens and kidneys of mice and made no attempt at fractionation. The data presented concerning the optimum pH for the hydrolysis of phenolphthalein glucuronide (4.5) and

reaction kinetics will need revision, since no account is taken of the dual nature of the spleen enzyme and any possible differences of behaviour of the liver and kidney enzymes. Further work on the reaction kinetics of these two enzymes and also upon liver and kidney glucuronidases is in progress.

SUMMARY

1. A reinvestigation of the methods for the purification of ox-spleen β -glucuronidase using the variable solvent solubility test has revealed the

presence of two fractions having different pH optima. The two enzymes have been separated and purified by fractional precipitation with ammonium sulphate and shown to have the following pH optima: with *l*-menthylglucuronide 4.5 and 5.0; phenylglucuronide 4.5 and 5.2; phenolphthalein glucuronide 4.5 and 5.2.

2. Methods of β -glucuronidase assay using *l*-menthylglucuronide, phenylglucuronide and phenolphthalein glucuronide are presented.

3. The relation of these results to those of previous workers is discussed.

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The Colorimetric Estimation of Stilboestrol, Hexoestrol and their Glucuronides in Urine

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Any attempt to estimate the synthetic oestrogens stilboestrol (4:4'-dihydroxy- $\alpha\beta$ -diethylstilbene) and hexoestrol or their detoxication products in urine is complicated by the presence of other phenolic substances which may give rise to interfering colours in procedures depending on colorimetry, or lead to high blank values from control urines. The dual problem presented in such work is to retain a sufficient quantitative recovery of the oestrogen while reducing blank values to a minimum. Sahasrabudhe & Wilder Smith (1947) have largely overcome this

* Part of the work reported in this paper was carried out at the National Institute for Research in Dairying, Shinfield, Reading.

problem in the case of dienoestrol by taking advantage of its diene structure and its reactivity with maleic anhydride. For the two more stable oestrogens, stilboestrol and hexoestrol, it appears that no satisfactory method of estimation in the presence of urine has yet been proposed, though attempts have been made to apply the Dingemans (1940) method to this problem (Dingemans & Tyslowitz, 1941; Bass & Salter, 1943). The present communication describes methods of preliminary extraction which enable estimations of these two clinically important oestrogens and of their glucuronides to be made in urine by the nitration method proposed by Malpress (1945).