# The Preparation and Properties of β-Glucuronidase

## 6. ACTIVITY IN RAT-LIVER PREPARATIONS

By P. G. WALKER and G. A. LEVVY
*Rowett Research Institute, Bucksburn, Aberdeenshire*

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Previous papers in this series have dealt with the fractionation and activity of β-glucuronidase in mouse-liver suspensions ('homogenates'). Identical fractionation of the enzyme was achieved by centrifuging at high speeds or by buffering to slightly acid pH. The sedimentable fraction was associated with subcellular particles of all sizes, and these were agglutinated in acid buffers. In water suspensions (Kerr & Levvy, 1951; Walker & Levvy, 1951), the sedimentable fraction was fully active at the usual tissue concentrations employed for β-glucuronidase assay with phenolphthalein glucuronide, and accounted for slightly less than half the total enzyme activity in preparations of normal adult liver.

In suspensions of mouse liver prepared in isotonic media (Walker, 1952), nearly all the enzyme was in the sedimentable fraction. These preparations, however, did not display full glucuronidase activity until some measure of disruption of the subcellular particles had occurred, with release of part of their enzyme content to the solution. This process of activation was irreversible, and was considered to be due to readier access of the substrate to the enzyme remaining in the particles. Soluble enzyme in mouse-liver suspensions was fully active under all conditions of assay. In this connexion, soluble enzyme was defined as that fraction which was not sedimented after 15 min. at 25000 *g* nor precipitated by acetate buffer, and which gave optically clear preparations under the phase-contrast microscope (Walker & Levvy, 1951).

A similar approach has been made to the study of β-glucuronidase in rat liver, in which part of the enzyme is known to be precipitable at acid pH (Becker & Friedenwald, 1949). A complication was encountered at an early stage due to reversible inhibition of soluble and insoluble enzyme by material present in the tissue.

## EXPERIMENTAL

*Preparation and fractionation of the enzyme.* In most of the experiments, hooded Lister strain rats were used. For purposes of comparison, a few later experiments were done with albino Wistar strain rats. The liver was ground for 1 min. at room temperature in the glass homogenizer with water or 0·25 M-sucrose solution, diluted with the homogenizing fluid to give a tissue concentration of 0·5–1 % (w/v), and sampled for the measurement of enzyme activity. As described for mouse liver (Walker & Levvy, 1951), other samples were fractionated at room temperature, either by adding acetate or citrate buffer (pH 5·2, final concentration 0·1 N) and centrifuging at low speed (1500 *g* for 15 min.), or by centrifuging the unbuffered preparation at high speed (25000 *g* for 15 min.). With buffered preparations, both supernatant and sediment were taken for assay, the latter being washed and resuspended in the buffered homogenizing fluid with the aid of the glass homogenizer. After centrifuging unbuffered preparations at high speed, only the supernatant could be sampled for assay since the sediment was not well enough packed to permit complete separation of the two fractions.

*Enzyme assay.* The enzyme preparations, fractionated and unfractionated, were diluted if necessary to a final volume of 200 ml./g. moist liver, and 0·5 ml. was transferred to a tube containing 0·5 ml. 0·01 M-phenolphthalein glucuronide solution (Talalay, Fishman & Huggins, 1946) and 3 ml. 0·1 N-acetate or citrate buffer, as a rule pH 5·2. Where buffer had been used in the fractionation, the same buffer was used in the assay. The remainder of the procedure followed that described by Kerr & Levvy (1951). Results are expressed as glucuronidase units (G.U.), where 1 G.U. liberates 1 μg. phenolphthalein in 1 hr. at 37°.

Under the above conditions of assay, the enzyme in preparations of rat liver did not always display full potential activity (see below). The latter could be most conveniently measured by adding the surface-active agent, Triton X-100 (Rohm and Haas Co.), as a solution in the buffer, to the incubation mixture to give a final concentration of 0·075 % (w/v).

*Endogenous inhibitor.* Preparations of the inhibitor for rat-liver glucuronidase present in this and other tissues (see below) were obtained free from enzyme activity by immersing a water suspension of the tissue in a boiling-water bath for 2 min. The cooled preparation was made 0·1 N with respect to acetate or citrate buffer, pH 5·2, and unless stated to the contrary was diluted to give a final tissue concentration of 0·5 % (w/v). To measure inhibitory power, 0·5 ml. of the preparation was added in place of buffer to the incubation mixture during the assay of preparations of soluble, inhibitor-free enzyme. The order of addition of enzyme, inhibitor and substrate did not influence the measure of inhibition. Very often, inhibitor and enzyme were prepared from separate samples of the same liver preparation.

## RESULTS

### *The action of an endogenous inhibitor in water suspensions of rat liver*

The following series of experiments, based on those previously done with mouse liver (Kerr & Levvy, 1951; Walker & Levvy, 1951), led to the belief that glucuronidase in water suspensions of rat liver is subject to the action of an endogenous inhibitor.

Kerr & Levvy (1951) found that the use of too high a tissue concentration in the assay of water suspensions of mouse liver led to anomalous results for glucuronidase activity. A similar phenomenon has been noted in dog and rat tissues by Fishman (1952). This effect was not seen in mouse-liver preparations (Kerr & Levvy, 1951) at the usual tissue concentrations (about 2·5 %, w/v) taken for assay by the phenolphthalein glucuronide method, nor at any tissue concentration once all the enzyme in the preparation had been converted into a soluble form. The effect of varying the tissue concentration on the glucuronidase activity of acetate-buffered water suspensions of rat liver is shown in Table 1. Addition of Triton X-100 was adopted as a convenient way of making all the enzyme soluble, as found for mouse liver by Walker & Levvy (1951). A 0·5 % suspension of rat liver had the same order of glucuronidase activity as a 2·5 % suspension of mouse liver, but even at this or greater dilution rat-liver preparations in absence of Triton X-100 still varied in enzyme activity according to the tissue concentration employed, and never attained the constant values observed in presence of the surface-active agent. The percentage differences were greater with infant than with adult liver. A tissue concentration of 0·06 % (w/v) was the lowest giving measurable release of phenolphthalein under the assay conditions. Triton X-100 was equally effective in concentrations in the assay tubes ranging from 0·015 to 0·375 % (w/v).

Table 1. *Effect of tissue concentration on the glucuronidase activity of water suspensions of rat liver (hooded Lister strain)*

(Serially diluted suspensions assayed in acetate buffer, pH 5·2, in presence and absence of 0·075 % Triton X-100. Results expressed as G.U./mg. moist liver. For definition of G.U. in tables see text.)

| Tissue concentration* (g. liver/100 ml. suspension) | G.U./mg. liver: Normal adult liver, With Triton | Normal adult liver, Without Triton | Infant (4-day-old) liver, With Triton | Infant (4-day-old) liver, Without Triton |
|---|---|---|---|---|
| 1·0 | 27·6 | 16·1 | 15·8 | 4·8 |
| 0·5 | 26·8 | 15·6 | 14·8 | 4·8 |
| 0·25 | 26·4 | 18·4 | 14·8 | 5·4 |
| 0·13 | 26·4 | 21·0 | 15·0 | 7·6 |
| 0·06 | 27·0 | 22·5 | 14·2 | 8·5 |

* These concentrations reduced to one-eighth in the assay tubes.

As previously found for mouse liver (Kerr & Levvy, 1951), the glucuronidase activity in water suspensions of rat liver could be fractionated on the low-speed centrifuge after buffering to pH 5·2 with acetate or citrate. Fig. 1 shows results thus obtained, the activities before and after fractionation being measured in presence and absence of Triton X-100. A single adult- or infant-liver preparation was used for all measurements, and fractionation was done immediately or after 2 hr. incubation in the buffer. Incubation in acetate or citrate is a preliminary to the preparation of tissue extracts for some methods of glucuronidase assay.

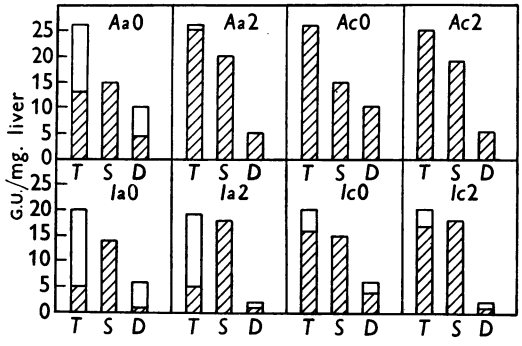


Fig. 1. The glucuronidase activity in presence and absence of 0·075 % Triton X-100 of water suspensions of rat liver (hooded Lister strain), buffered with acetate or citrate to pH 5·2 and fractionated before and after incubation at 37° for 2 hr. All preparations were diluted to 200 ml./g. liver before assay. Results are expressed as G.U./mg. liver. For definition of G.U., see text. The total height shows the activity in presence of Triton X-100, and the height of the hatched area the activity in its absence. *T*, unfractionated suspension; *S*, soluble fraction; *D*, sedimentable fraction; *A*, adult liver; *I*, infant (4-day-old) liver; *a*, buffered with acetate; *c*, buffered with citrate; *O*, fresh suspension; 2, incubated suspension.

Assays conducted in presence of Triton X-100 revealed a simple transfer of enzyme activity from the sedimentable to the soluble fraction during the incubation of the preparation in either buffer. There was no change in the activity of the unfractionated preparation which was always equal to the sum of the activities in the two fractions. Citrate and acetate gave similar fractionation of activity. (Control experiments showed that addition of Triton X-100 to the fresh preparation before fractionation made all the enzyme soluble.)

Assays in absence of Triton X-100 were more difficult to interpret. Considering acetate-buffered preparations first, the soluble fraction invariably displayed full enzyme activity, but the activity of

the sedimentable fraction separated prior to incubation was greatly depressed in absence of the surface-active agent. This was also true of the unfractionated suspension, so much so that it was exceeded in activity by the soluble fraction alone. After incubating the suspension for 2 hr. in acetate buffer, a difference became apparent between adult- and infant-liver preparations. The adult-liver preparation approached full activity, as did its sedimentable enzyme, whilst the infant-liver preparation retained its original depressed value, in spite of the fact that nearly all the enzyme had gone into solution. The disparity between fractionated and unfractionated preparations of infant liver assayed in absence of Triton X-100 thus became greater after incubation in acetate buffer. Although the inhibitor was lacking or without effect in separated soluble enzyme fractions, it must have acted on soluble enzyme in the unfractionated suspension. It is therefore evident that the effect of Triton X-100 in overcoming the endogenous inhibitor did not depend entirely on its action in converting all the enzyme in suspensions into a soluble form.

When citrate buffer was used instead of acetate, the effect of the inhibitor became smaller in infant-liver preparations and disappeared entirely in adult preparations. This was apparently due to citrate causing reversal of the inhibition since the use of other buffers (propionate, lactate, phthalate) or dilute hydrochloric acid to bring the pH to 5·2 gave results similar to those obtained with acetate.

Other series of experiments confirmed and extended the results shown in Fig. 1. The effect of fractionation on acetate-soluble enzyme was reversible: adding the sediment led to a net loss in activity. The inhibitor in infant (4-day-old) liver suspensions was stable to 6 hr. incubation in acetate buffer. Incubation for 2 hr. in acetate buffer reduced the effect of the inhibitor in liver from 30-day-old animals by about 50 % and, as we have already seen (Fig. 1), almost completely destroyed that in adult liver. ('Adult' rats were at least 3 months old.)

Immediate fractionation of acetate-buffered suspensions followed by incubation of the separate fractions for 2 hr. showed changes in the inhibitor, uncomplicated by changes in enzyme distribution. The activity of the inhibitor-free soluble fraction was unchanged by incubation, as was the total activity, measured in presence of Triton X-100, of the sedimented fraction. Incubation in acetate buffer destroyed the inhibitor in the sediment from adult liver, but left that from infant liver unaffected.

As proved later (Table 8), high-speed centrifuging (25000 *g* for 15 min.) of unbuffered water suspensions of rat liver led to fractionation of the total enzyme activity identical to that produced by acetate or citrate buffer, pH 5·2. The soluble fraction obtained on the high-speed centrifuge, however, contained some of the inhibitor, as shown in Table 2. Before incubation in acetate buffer, Triton X-100 caused a rise in activity in the soluble as well as the sedimentable fraction. Incubation in the buffer destroyed the inhibitor in both adult-liver fractions, but not in the infant-liver preparations.

Recentrifuging the high-speed supernatant at low speed after buffering with acetate freed the soluble enzyme from inhibitor which now sedimented. (This sediment always contained a trace of adsorbed enzyme which could not be removed by washing with the buffer.) The insolubility of the inhibitor in the buffer explains the fact that fractionation of buffered suspensions always yielded the soluble enzyme in an inhibitor-free state (cf. Fig. 1).

Table 2. *The glucuronidase activity in presence and absence of* 0·075 % *Triton X*-100 *of the soluble and sedimentable fractions separated by centrifuging unbuffered suspensions of rat liver at* 25000 *g for* 15 *min.*

(Hooded Lister strain rats were used. The fractions were buffered with acetate (pH 5·2, final concentration 0·1 N) and diluted to 200 ml./g. liver. Samples were taken for assay before and after 2 hr. incubation. One sample of the buffered high-speed supernatant, unincubated, was centrifuged at 1500 *g* for 15 min. and the sediment was discarded before assay. Results are expressed as G.U./mg. liver.)

| | G.U./mg. liver | | | | |
|---|---|---|---|---|---|
| | High-speed supernatant | | | Sediment* | |
| | With Triton | Without Triton | Acetate-soluble enzyme (without Triton) | With Triton | Without Triton |
| Adult male | | | | | |
| Before incubation | 17·2 | 11·8 | 16·4 | 9·2 | 6·4 |
| After incubation | 17·4 | 17·4 | — | 9·2 | 8·8 |
| Infant (5 days old) | | | | | |
| Before incubation | 10·8 | 5·6 | 10·3 | 4·0 | 1·6 |
| After incubation | 10·8 | 6·4 | — | 3·8 | 1·4 |

* Total activities are low due to losses in the separation and washing of this fraction.

Table 3. *The effect of Triton X-100 (0·075 %, w/v) on the glucuronidase activity of water suspensions of various rat and mouse tissues*

(Rat preparations (hooded Lister strain) were diluted to 200 ml./g. moist tissue, and mouse preparations to 40 ml./g. Assays were done in acetate buffer, pH 5·2, and results are shown as the mean ± S.E.)

| Tissue | Number of experiments | G.U./mg. moist tissue: With Triton | G.U./mg. moist tissue: Without Triton | Inhibition* (%) |
|---|---|---|---|---|
| Adult rat liver | 7 | 25·94 ± 1·47 | 14·83 ± 1·86 | 43·9 ± 4·65 |
| Infant rat liver | 7 | 19·97 ± 1·57 | 4·40 ± 0·42 | 77·7 ± 2·02 |
| Adult rat kidney | 3 | 5·67 ± 0·09 | 3·93 ± 0·41 | 30·7 ± 5·25 |
| Adult rat spleen | 3 | 20·27 ± 0·87 | 19·07 ± 0·67 | 5·9 ± 0·09 |
| Adult mouse liver | 6 | 3·17 ± 0·15 | 3·10 ± 0·12 | 2·0 ± 0·63 |
| Adult mouse kidney | 5 | 3·20 ± 0·36 | 3·02 ± 0·28 | 5·8 ± 1·18 |

* The significance of the difference was tested by calculating $t$ (= mean/S.E.).

For comparison with rat liver, a study was made of the effects of Triton X-100 on the glucuronidase activities of unfractionated water suspensions of rat kidney and spleen, and mouse liver and kidney (Table 3). Activation was marked in rat kidney and slight, but in every case significant ($P < 0·05$), in rat spleen and mouse liver and kidney. An occasional small increase in the activity of water suspensions of mouse liver on addition of Triton X-100 has previously been noted (Walker & Levvy, 1951).

*Properties of the endogenous inhibitor.* The following properties of the endogenous inhibitor in water suspensions of rat liver were confirmed in experiments with preparations of enzyme-free inhibitor (see Experimental section), tested against inhibitor-free, acetate-soluble enzyme. In the untreated preparation, it was partly insoluble and partly in solution, but it was completely precipitated, without loss in inhibitory power, by buffering to pH 5·2. Its action was completely reversed by Triton X-100 and in adult preparations by citrate also. The inhibitor from infant liver was stable to incubation in acetate buffer, pH 5·2, but the inhibitor from adult liver was destroyed after 2 hr. incubation. When precipitated from preparations of soluble enzyme by addition of acetate buffer, the inhibitor always adsorbed traces of enzyme which could not be removed by washing with the buffer.

Unbuffered suspensions of adult or infant liver could be boiled for at least 10 min. without loss in inhibitory power, and this was made the basis of the preparation of enzyme-free inhibitor. The inhibitor in boiled or unboiled preparations was non-dialysable. Hyaluronidase ('Hyalase', Benger Laboratories Ltd.) added in a concentration of 100 Benger units/ml. to boiled or unboiled suspensions of infant rat liver, buffered with acetate to pH 5·2, caused no fall in inhibitory power after 2 hr. incubation. Similar experiments with adult liver showed no acceleration of the destruction of inhibitor by hyaluronidase.

Glucuronidase in mouse liver (Walker & Levvy, 1951; Walker, 1952) could be brought completely into solution without enzyme inactivation by shaking suspensions with Ballotini, Grade 12 (Chance Bros. Ltd.) in the Mickle tissue disintegrator (Mickle, 1948) for 10–30 min., or by treating the liver with water in the Waring Blendor for 3 min. Increasing the period of grinding the tissue with water in the glass homogenizer from 1 to 5 min. caused little change in the distribution and activity of the enzyme. Grinding the liver in acetone (glass homogenizer) followed by resuspension of the dry powder in water gave most of the enzyme in a soluble form, with only small losses in overall activity.

Table 4 shows the results obtained in similar experiments with rat liver. Here the possible effects of the treatment on the inhibitor had to be considered. With respect to the enzyme, the action of acetone in rat liver was the same as in mouse liver, but it had the additional effect of destroying the inhibitor in rat liver. The inhibitor was not found in the residue from the evaporated acetone extract. Unlike the enzyme in mouse liver, glucuronidase in rat liver showed marked inactivation after short periods of treatment in the Waring Blendor. A large part of the remaining enzyme was in a soluble state, and there appeared to have been some destruction of the inhibitor. Increasing the period of treatment with water in the glass homogenizer from 1 to 5 min. had little effect on either the enzyme or the inhibitor in rat liver.

In experiments with separate preparations of rat-liver enzyme and inhibitor (Table 5), the action of the Waring Blendor in destroying both was confirmed. The effects of shaking with Ballotini in the Mickle tissue disintegrator were even more striking.

*Mode of action of the inhibitor.* In the experiments described in this and the next section, preparations of enzyme-free inhibitor and inhibitor-free enzyme only were used, and assays were done in acetate buffer in absence of Triton X-100.

Table 4. *The effects of mechanical disintegration and acetone on the glucuronidase activity of adult rat liver (hooded Lister strain)*

(Samples of a single liver treated as shown. Activities measured in presence and absence of 0·075% Triton X-100 before and after fractionation with acetate buffer, pH 5·2.)

| Treatment | Period (min.) | G.U./mg. liver: Total activity, With Triton | Total activity, Without Triton | Soluble enzyme, With Triton | Soluble enzyme, Without Triton |
|---|---|---|---|---|---|
| Glass homogenizer and water | 1* | 32·8 | 19·6 | 21·3 | 21·6 |
| | 5 | 32·4 | 20·5 | 22·0 | 22·0 |
| Glass homogenizer and acetone† | 1 | 30·2 | 28·8 | 26·7 | 27·0 |
| Waring Blendor and water | 2 | 27·0 | 23·4 | 22·5 | 22·6 |
| | 5 | 22·2 | 20·0 | 19·1 | 19·0 |

* Provides control for other experiments.

† Dry powder resuspended in water.

Fourfold variation in the concentration of the rat-liver enzyme caused no significant change in the percentage inhibition observed on adding rat-liver inhibitor in a concentration of either 0·0125 or 0·0625% (w/v) in the assay tube. Fig. 2 shows the effect of increasing inhibitor concentration at fixed

Table 5. *Inactivation of rat-liver enzyme and destruction of inhibitor by mechanical treatment*

(Hooded Lister strain rats were used. Preparations of boiled, enzyme-free inhibitor and acetate-soluble, inhibitor-free enzyme from adult liver were treated and tested separately. Inhibitor tested with untreated enzyme. Assay in acetate buffer, pH 5·2, in absence of Triton X-100.)

| Treatment | Period (min.) | Residual enzyme activity (G.U./mg. liver) | Action of treated inhibitor (% inhibition) |
|---|---|---|---|
| None | — | 19·2 | 37 |
| Waring Blendor | 2 | 16·8 | 24 |
| | 5 | 13·0 | 13 |
| | 10 | 10·4 | 7 |
| Tissue disintegrator | 2 | 11·6 | 17 |
| | 5 | 2·9 | 6 |
| | 10 | 1·2 | 3 |

enzyme and substrate concentration. Inhibition increased to a maximum, always well below 100%, which was characteristic of the tissue of origin of both inhibitor (Fig. 2*a*) and enzyme (Fig. 2*b*). Two points are particularly noteworthy. A given inhibitor preparation had a greater effect on infant- than on adult-rat-liver enzyme. Adult-rat-liver enzyme was powerfully inhibited by boiled preparations of mouse liver and kidney. We have already seen (Table 3) that the enzyme in suspensions of these two mouse tissues was little affected by endogenous inhibitor. Mouse-liver enzyme was not appreciably affected by inhibitor preparations from the same tissue in concentrations in the assay as high as 5% (w/v), nor by inhibitor prepared from

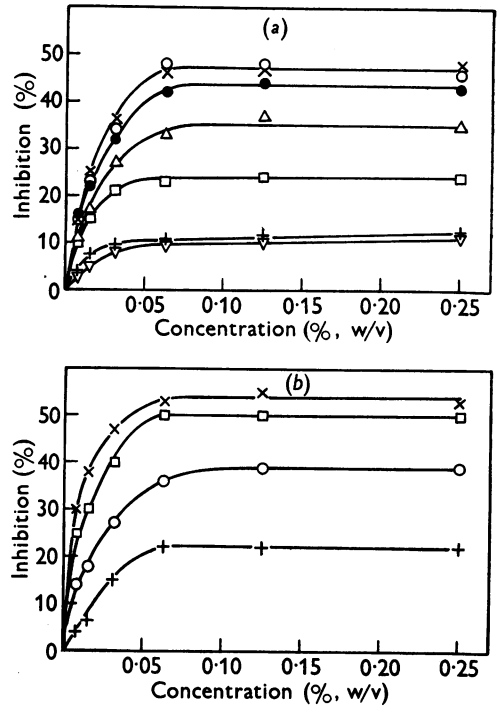


Fig. 2. Effect of varying inhibitor concentration with (*a*) a single preparation of adult-rat-liver enzyme and inhibitor of varied origin and (*b*) a single preparation of adult-rat-liver inhibitor and enzyme of varied origin. Inhibitor concentrations shown in terms of boiled tissue in the assay tube. Hooded Lister strain rats were used. Assay in acetate buffer, pH 5·2. Substrate concentration 0·00125M. ×, infant rat liver; ○, adult rat liver; □, adult rat kidney; +, adult rat spleen; ●, adult mouse kidney; △, adult mouse liver; ▽, adult mouse spleen.

any of the other tissues studied, at a concentration of 0·25%.

That pH was not a factor in the action of the tissue inhibitor was shown by experiments with

enzyme and inhibitor prepared from adult and infant rat liver. Identical figures for percentage inhibition were obtained in assays done in acetate buffer at pH 4·5 and 5·2. Inhibitor sedimented in acetate buffer, as already noted, adsorbed small amounts of soluble enzyme (never more than 10 % of the total activity). In presence of substrate, adsorption increased to as much as 40 % of the total soluble enzyme activity. However, adsorption by rat-liver inhibitor increased just as much with mouse-liver as with rat-liver enzyme, although in the former case there was no inhibition of adsorbed enzyme. While adsorption may well have been important in the mechanism of inhibition, the final effect was thus determined by other factors.

The possibility was considered that in those tissues in which it is affected by endogenous inhibitor, glucuronidase might exist in two forms, only one being open to inhibition. A variety of experiments was done with rat liver to test this possibility. Preparations of soluble enzyme were divided into fractions of approximately equal activity by making them 38 % saturated with ammonium sulphate (cf. Mills, 1948; Kerr, Campbell & Levvy, 1949). Suspensions were divided into soluble and insoluble enzyme fractions and the latter brought into solution by incubation in acetate buffer. In neither case was any evidence obtained for differences in the response of the various fractions to a given inhibitor preparation.

Becker & Friedenwald (1949) found that rat-liver glucuronidase was inhibited up to a limit well below 100 % by heparin and by impure hyaluronic acid. The effects of the two inhibitors were not additive. A study was made of the inhibition of acetate-soluble adult-rat-liver enzyme by powdered heparin (B.P., British Drug Houses Ltd.) and by a highly purified preparation of chondroitin sulphate (presented by Dr R. B. Duff). Fig. 3 shows that inhibition in both cases tended to a maximum which was well below 100 % and was greatly increased by decreasing the pH of assay from 5·2 to 4·5. With neither compound was inhibition reversed by Triton X-100. Further experiments with heparin showed that it inhibited mouse-liver as strongly as rat-liver glucuronidase, and that it was as effective in citrate as in acetate buffer (cf. Fig. 1). Table 6 shows that the actions of heparin and chondroitin sulphate on rat-liver enzyme were additive to that of the endogenous inhibitor, but not to each other. Heparin, the more powerful inhibitor, appeared to displace chondroitin sulphate completely from combination with the enzyme.

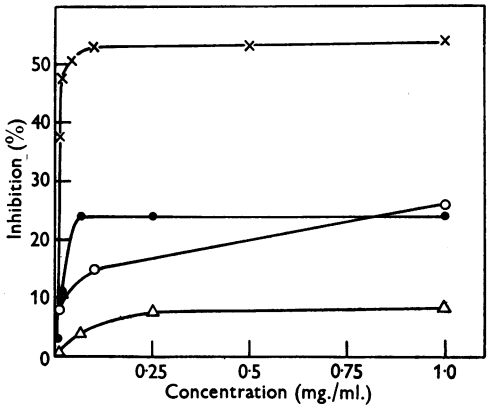


Fig. 3. Inhibition of adult rat-liver enzyme (hooded Lister strain) by varying concentrations of heparin and chondroitin sulphate at pH 4·5 and 5·2 (acetate buffer). Inhibitor concentrations expressed as mg./ml. in assay tubes (1 mg. heparin = 94·95 i.u.). ×, heparin at pH 4·5; ○, heparin at pH 5·2; ●, chondroitin at pH 4·5; △, chondroitin at pH 5·2.

*The effect of varying substrate concentration.* Fig. 4 shows the effect of varying the substrate concentration on the rate of hydrolysis of phenolphthalein glucuronide by acetate-soluble, adult-rat-liver glucuronidase in presence and absence of a fixed concentration of boiled inhibitor from the same liver. Inhibition increased with increasing substrate concentration, suggesting that the inhibitor combined with the enzyme-substrate complex, rather than

Table 6. *Inhibition of adult-rat-liver glucuronidase (hooded Lister strain) by boiled endogenous inhibitor from the same tissue* (0·125 %, *w/v*), *heparin* (0·5 *mg./ml.*) *and chondroitin sulphate* (0·5 *mg./ml.*), *singly and in combination*

(Assay in acetate buffer, pH 4·5. Residual enzyme activity expressed as G.U./ml. acetate-soluble enzyme preparation. In parenthesis opposite each addition, the residual activity is shown in each column as a percentage of that for the control system.)

| | Residual activity (G.U./ml. enzyme) | | | |
|---|---|---|---|---|
| Addition | Enzyme | Enzyme + endogenous inhibitor | Enzyme + heparin | Enzyme + chondroitin sulphate |
| None (control) | 132 | 65 | 60 | 96 |
| Chondroitin sulphate | 96 (73 %) | 49 (75 %) | 60 (100 %) | — |
| Heparin | 60 (45 %) | 29 (45 %) | — | 60 (63 %) |
| Endogenous inhibitor | 65 (49 %) | — | 29 (48 %) | 49 (51 %) |

with the enzyme alone, a conclusion supported by the results of the experiments mentioned above on the adsorption of enzyme by inhibitor. This uncommon type of inhibition has been described by Burk (quoted by Ebersole, Guttentag & Wilson, 1944), who called it 'uncompetitive'. The term 'combined inhibition' seems more apt.

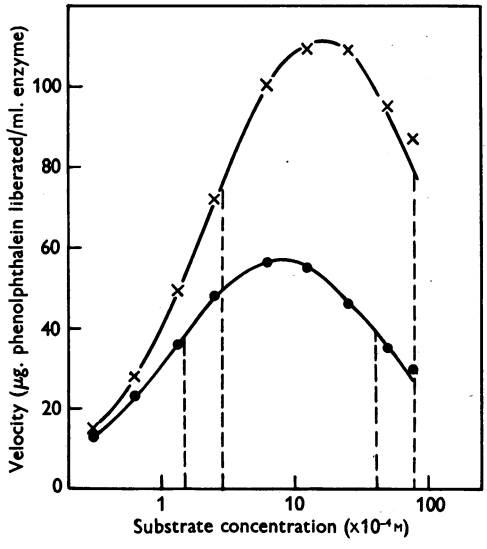


Fig. 4. The effect of varying the substrate concentration ($S$) on the rate of hydrolysis ($v$) of phenolphthalein glucuronide in presence (●) and absence (×) of 0·125% boiled tissue. Acetate-soluble enzyme from adult hooded Lister strain rat liver assayed in acetate buffer, pH 5·2. The points are experimental whilst the lines were calculated by substituting for $K_s$, $K_2$, $n$ and $V_m$ (Table 7) in the equation

$$v=\frac{V_m S}{S+K_s+S^n/K_2}.$$

The broken vertical lines show the values of $K_s$ and $K_2$.

Inhibition by excess substrate, always pronounced with glucuronidase, became apparent at a lower concentration when the inhibitor was present. Each series of points in Fig. 4 was analysed by the method of Lineweaver & Burk (1934, case 3) for $K_s$, the dissociation constant of the active enzyme-substrate complex, $K_2$, the dissociation constant of the inactive complex, $n$, the number of substrate molecules per active enzyme centre in the inactive complex, and $V_m$, the theoretical maximum rate of hydrolysis for the particular preparation. The figures obtained for these constants are shown in Table 7, and the lines in Fig. 4 were drawn (taking $n=2$) to fit the equation

$$v=\frac{V_m S}{S+K_s+S^n/K_2},$$

where $v$ is the reaction velocity at different values of $S$, the substrate concentration. Table 7 also gives values for $\sqrt{(K_s K_2)}$, the substrate concentration for optimum enzyme activity. As well as a fall in $V_m$, the inhibitor caused an apparent increase in the affinity ($=1/K_s$) of the enzyme for the substrate, but left $n$ unchanged.

Table 7. *Kinetic constants for the hydrolysis of phenolphthalein glucuronide by rat-liver glucuronidase in presence and absence of endogenous inhibitor*

(Calculated by the method of Lineweaver & Burk (1934) from the results shown in Fig. 4.)

| | Without inhibitor | With inhibitor |
|---|---|---|
| $V_m$ (μg. phenolphthalein/ml. enzyme) | 157 | 78 |
| $K_s$ ($\times 10^{-4}$M) | 2·9 | 1·5 |
| $n$ (±S.E.) | 1·91±0·08 | 1·94±0·07 |
| $K_2$* ($\times 10^{-4}$M) | 78 | 41 |
| $\sqrt{(K_s K_2)}$† ($\times 10^{-4}$M) | 15 | 8 |

* Calculated by making $n=2$ exactly.
† Substrate concentration for optimal velocity.

These results afford a striking demonstration of the way in which a tissue impurity can alter the kinetics of an enzyme reaction. The findings were confirmed in several other experiments with both adult- and infant-liver preparations. There was no significant difference between enzyme from adult and infant liver in values for $K_s$ in absence of inhibitor. With a given enzyme preparation, the values of $K_s$ and other constants in presence of inhibitor could be altered by altering the concentration of inhibitor added.

Inhibition by heparin of both rat- and mouse-liver glucuronidase was essentially non-competitive at pH 4·5 (acetate buffer). In non-competitive inhibition, $V_m$ is the only constant to alter. The nature of the inhibition by chondroitin sulphate was not studied.

*Fractionation of rat-liver glucuronidase.* In all subsequent experiments, Triton X-100 was added during the enzyme assay, so that the effects of endogenous inhibitor were abolished. Table 8 shows the partition of glucuronidase activity observed after homogenizing samples of a single adult or infant rat liver in water and in 0·25M-sucrose solution. In general, the results were similar to those previously obtained for the fractionation of mouse-liver glucuronidase (Kerr & Levvy, 1951; Walker & Levvy, 1951; Walker, 1952). The distribution of enzyme activity between the soluble and insoluble fractions was the same after both methods of fractionation employed. In the isotonic medium, practically all the enzyme activity was associated with insoluble material. After homogenizing in water, about 60% of the activity in adult liver and about 80% of the activity in infant liver was soluble.

Table 8. *Fractionation of rat-liver glucuronidase on the high-speed centrifuge, and on the low-speed centrifuge after buffering with acetate to* pH 5·2

(Hooded Lister strain rats were used, and the assay was done in acetate buffer, pH 5·2, in presence of Triton X-100.)

| | G.U./mg. liver | | | | |
|---|---|---|---|---|---|
| | | High-speed centrifuge | | Acetate treatment | |
| Homogenizing fluid | Total | Soluble | Insoluble* | Soluble | Insoluble |
| Adult female | | | | | |
| Water | 36·8 | 22·7 | 14·1 | 21·2 | 14·8 |
| 0·25 M-Sucrose | 35·6 | 1·9 | 33·7 | 1·4 | 34·3 |
| Infant (4 days old) | | | | | |
| Water | 17·2 | 13·9 | 3·3 | 13·0 | 4·3 |
| 0·25 M-Sucrose | 17·0 | 1·4 | 15·6 | 1·0 | 16·4 |

* Calculated—see Experimental section.

It would appear that in rat liver, as in mouse liver, practically all the glucuronidase activity is initially associated with subcellular particles, from which it is released in hypotonic media. Suspensions of rat liver in water and in isotonic sucrose solution were examined by phase-contrast microscopy. The preparations were very similar to those of mouse liver (Walker & Levvy, 1951; Walker, 1952). Complete sedimentation of the insoluble enzyme was associated with the removal from the suspension of all but a very few of the smallest visible particles: buffering with acetate led to agglutination of all the visible components. All components of water suspensions, except the fat globules, displayed severe osmotic swelling.

*The variation in rat-liver glucuronidase activity with the state of proliferation of the tissue.* It would appear that the best measure of rat-liver glucuronidase activity is that obtained by the use of the whole suspension in conjunction with Triton X-100. It will have been noted that in previous experiments the activity of hooded Lister strain infant (4-day-old) liver was less than that of adult under these conditions of assay. Fig. 5 shows the change in rat-liver glucuronidase activity throughout the first months after birth for both hooded Lister and albino Wistar animals. With the hooded Lister strain, there was a sixfold rise in liver enzyme activity with age in males, and rather more in females: in females the activity remained constant, but in males it fell after 2 months to rather less than 75% of the female level. Albino Wistar rats differed from the hooded Lister strain in that the liver enzyme activity was much higher immediately after birth and showed a fall in females as well as males after 1 month. Apart from the higher glucuronidase activity in infants, water suspensions of Wistar rat liver behaved identically to those of hooded Lister rat liver of the same age as regards the fractionation of the enzyme and its activation by Triton X-100, and the stability of the inhibitor to incubation in acetate buffer in infants as compared with adults (cf. Fig. 1).

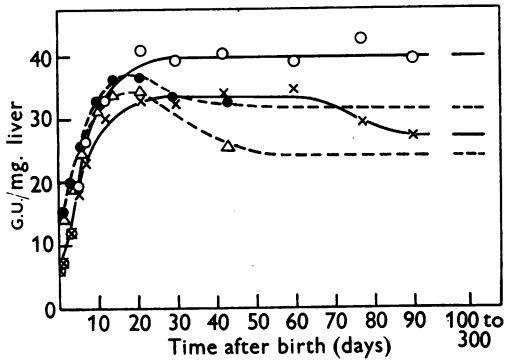


Fig. 5. Variation in hooded Lister and albino Wistar rat-liver glucuronidase activity with age. Assay in acetate buffer, pH 5·2, in presence of Triton X-100. Five Lister or three Wistar rats were used at each point. For definition of G.U., see text. O—O, hooded Lister females; × — ×, hooded Lister males; ●---●, Wistar females; △---△, Wistar males.

Table 9. *Effect of partial hepatectomy on rat-liver glucuronidase activity (hooded Lister strain)*

(Assay in acetate buffer, pH 5·2, in presence of Triton X-100. Values shown as mean ± S.E. followed in parenthesis by the number of experiments.)

| Days after operation | G.U./mg. liver | |
|---|---|---|
| | Males | Females |
| Control | 27·3 ± 1·14 (15) | 39·9 ± 1·45 (11) |
| 1 to 4 | 23·3 ± 0·95 (12) | 33·3 ± 1·51 (9) |
| 6 to 10 | 28·7 ± 0·97 (6) | 40·0 ± 1·63 (6) |

Table 9 shows the effect of partial hepatectomy on liver glucuronidase activity. In both sexes there was a small, but real ($P < 0·05$), fall 1–4 days after operation, followed by a return to normal after 6–10 days.

## DISCUSSION

Although there were minor points of difference, the factors which determined the distribution of glucuronidase in suspensions of rat liver were in general the same as those found for mouse liver (Kerr & Levvy, 1951; Walker & Levvy, 1951; Walker, 1952). After homogenizing the tissue in an isotonic medium, the enzyme was almost entirely associated with the subcellular particles. Homogenizing the tissue in water made a large part of the enzyme activity soluble, and this fraction could be further increased, notably by adding the surface-active agent, Triton X-100, or by incubation in acid buffers. The effects of these measures, and the contrast between the behaviour of β-glucuronidase and of more complex enzyme systems have been discussed in connexion with the enzyme in mouse liver (Walker & Levvy, 1951).

There was an important difference between the enzymes in rat and mouse liver in that the former were powerfully inhibited by an unidentified tissue constituent, apparently similar to the blood plasma anti-glucuronidase briefly described by Fishman, Altman & Springer (1948). The inhibitor in rat liver resembled heparin and hyaluronic acid, also inhibitors for the enzyme (Becker & Friedenwald, 1949), in that it was non-dialysable and did not cause complete inhibition. However, the action of the endogenous inhibitor on glucuronidase was very different from that of heparin. Chondroitin sulphate, which was also found to cause inhibition, behaved like heparin. Triton X-100 overcame the effect of the endogenous inhibitor, but not that of heparin or chondroitin sulphate. Hyaluronic acid was not studied, but the endogenous inhibitor appeared to be distinct from this compound since it was not destroyed by hyaluronidase, and since its action was additive to that of heparin, which is not true of hyaluronic acid (Becker & Friedenwald, 1949). Nevertheless, the properties of the endogenous inhibitor were not inconsistent with the possibility that it was mucopolysaccharide in character.

Two types of tissue preparation have been used for glucuronidase assay. Prior to the introduction of phenolphthalein glucuronide as substrate (Talalay *et al.* 1946), assay procedures made use of buffered extracts of soluble material, separated from cell debris after varying periods of incubation at slightly acid pH. With phenolphthalein glucuronide it became possible to use the crude water suspension, and this course has been widely followed. Thus, Fishman and his collaborators stopped using buffered tissue extracts in all work published subsequent to 1946 (private communication from Dr W. H. Fishman). Sources of error in the use of tissue extracts, arising from incomplete extraction of insoluble enzyme, have been discussed in connexion with mouse liver (Kerr & Levvy, 1951), and, as can be seen from Fig. 1, apply equally to the use of rat-liver extracts. The action of the endogenous inhibitor does not come into the question with buffered tissue extracts, since if not destroyed by incubation it will in any case have been precipitated and removed with cell debris. Crude water suspensions, if not too concentrated, were found satisfactory for the assay of mouse-liver glucuronidase activity (Kerr & Levvy, 1951). This is not true of water suspensions of rat liver due to the presence of the endogenous inhibitor which varies in its effects according to the age of the animal. Addition of Triton X-100 overcame the action of the endogenous inhibitor and led to apparently satisfactory results with water suspensions of rat liver. It should, however, be remembered that tissue preparations may contain other inhibitors, such as heparin and chondroitin sulphate, which are not overcome by Triton X-100.

It is clear that a single method of glucuronidase assay may not yield comparable results with different species and tissues, nor even with a single tissue in different physiological and pathological states. For purposes of comparative tissue assay, however, it may be argued that the final reading should include the effects of such factors as the inhibitor in rat liver. On the other hand, it is by no means certain that this inhibitor has any effect *in vivo*. Moreover, it was shown in the case of mouse liver (Walker, 1952) that the better the subcellular particles in the suspension were preserved, the smaller was their glucuronidase activity. This effect, unlike that of the endogenous inhibitor in rat liver, could be overcome by increasing the substrate concentration. Taking everything into consideration, it would appear that the best one can do is to try to measure the full activity of all the enzyme in the tissue. Whether the physiological function of β-glucuronidase can be deduced from figures thus obtained is another matter.

In rats, the glucuronidase activity of liver from newborn animals and of liver regenerating after partial hepatectomy was found to be less than that of normal adult liver. So far as comparison is possible, the general findings seem to be in agreement with those of Mills, Smith, Stary & Leslie (1950) and Mills (1951). The present results for rat liver are in contrast to those obtained for mouse liver (see for example Walker, 1952), where tissue growth or regeneration was found to be associated with increased glucuronidase activity. Kerr, Campbell & Levvy (1950) found that liver glucuronidase activity was higher in 4-day-old than in adult rats. It can now be seen that this result was obtained because, as shown by the original records, albino Wistar rats were used, and because only the

soluble enzyme fraction in the water suspension was taken for assay. In Wistar rats, the total glucuronidase activity in infants soon reaches and exceeds the ultimate adult value, and in general infant liver has a greater percentage of the enzyme in a soluble form than adult.

## SUMMARY

1. After homogenizing rat liver in isotonic sucrose solution, nearly all the glucuronidase activity was found in the subcellular particles. Homogenizing in water made a large part of the enzyme soluble, and it was brought completely into solution by the surface-active agent, Triton X-100.

2. The activity of soluble and insoluble rat-liver glucuronidase in water suspensions was greatly reduced by a non-dialysable endogenous inhibitor. Inhibition, which was overcome by Triton X-100, was also seen in rat kidney, but was very slight in mouse liver and kidney. Rat-liver enzyme was powerfully inhibited by mouse-liver and kidney preparations.

3. The inhibitor in rat liver was thermostable at neutral pH. It was not completely sedimented on the high-speed centrifuge, but was completely precipitated by buffering to pH 5·2. Incubation in acetate buffer, pH 5·2, destroyed the inhibitor in adult-, but not infant-liver preparations.

4. Inhibition varied directly with substrate and inhibitor concentration, and the kinetics were analysed. The term 'combined inhibition' is suggested for this effect.

5. Inhibition by heparin and chondroitin sulphate was studied. The effects differed in important respects from those of the endogenous inhibitor.

6. The glucuronidase activity of newborn rat liver and of rat liver regenerating after partial hepatectomy was less than that of normal adult rat liver.

7. Sources of error in the assay of β-glucuronidase are discussed, with particular respect to the effects of the endogenous inhibitor in rat liver.

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# The Antibacterial Effects of Substances Structurally Resembling Maleimide

By D. H. MARRIAN, E. FRIEDMANN and JEAN L. WARD
*Department of Radiotherapeutics, University of Cambridge, and the Research Department, Roche Products Ltd., Welwyn Garden City, Herts*

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A series of structurally related compounds, including unsaturated cyclic imides, unsaturated dicarboxylic acids, and derivatives of 1:4-quinones has been investigated by Friedmann, Marrian & Simon-Reuss (1948*a*, *b*, 1949), and their action in inhibiting mitoses in cultures of chick fibroblasts was found to parallel their reactivity towards sulphydryl groups. Since quinones are well known to have some antibacterial effect (Cooper, 1913; Cooper & Nicholas, 1927; Oxford, 1942*a*, *b*; Oxford & Raistrick, 1942; Armstrong, Spinke & Kahnke, 1943; Barber, 1944; Atkins & Ward, 1945; see also Hoffman-Ostenhof, 1947); it seemed of interest to assay the whole group for antibacterial action.