pathology and should prove of value in throwing further light on the pathogenesis of various conditions in which acetonæmia is a prominent feature.

SUMMARY

1. A method has been devised for the estimation as acetone of the individual ketone bodies—acetone, acetoacetic acid, β -hydroxybutyric acid and isopropanol—within the range 0–120 mg. acetone/100 ml.

2. The basis of the method is the diffusion of acetone into an alkaline solution of salicylic aldehyde with the production of an orange-red colour, the intensity of which is measured in a photo-electric colorimeter.

3. The application of the method to biological materials such as blood, milk, urine and rumen liquor is described.

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The Preparation and Properties of β -Glucuronidase

3. FRACTIONATION AND ACTIVITY OF HOMOGENATES IN ISOTONIC MEDIA

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The first paper in this series (Kerr & Levvy, 1951) dealt with the fractionation of glucuronidase activity in water homogenates of mouse liver and other organs which is brought about by buffering to slightly acid pH. Changes in the glucuronidase activity of a tissue with the state of proliferation (Levy, Kerr & Campbell, 1948; Kerr, Campbell & Levvy, 1949, 1950) were seen to be confined to the non-precipitable fraction. In the second paper (Walker & Levvy, 1951), it was shown that an identical fractionation of mouse-liver glucuronidase in untreated water homogenates could be achieved on the high-speed centrifuge, and that the sedimentable fraction was associated with cytoplasmic granules of all sizes. The use of water in preparing tissue homogenates caused pronounced osmotic swelling of the nuclei and other subcellular particles, leading sometimes to disruption. Homogenizing in isotonic media tends to preserve these bodies, and the present paper deals with the partition of glucuronidase activity between sedimentable material and the suspension fluid after homogenizing mouse liver in isotonic sodium chloride or sucrose solution. It was found that liver preparations did not display their full glucuronidase activity until a considerable proportion of the enzyme had been brought into solution.

METHODS

Preparation and fractionation of the enzyme. Unless stated to the contrary, the mouse liver was ground for 1 min. in the glass homogenizer (average speed 5000 rev./min.) with sufficient water, 0.17M-NaCl or 0.25M-sucrose solution to give a final tissue concentration of 10%, and samples were taken for assay. Following the procedure already described (Walker & Levvy, 1951) fractionation was then carried out either by centrifuging at a high speed (25000 g for 15 min.), or by adjusting the pH to 5.2 with acetate or citrate buffer (final concentration 0.1N) and centrifuging at a low speed (1500 g for 15 min.). In the majority of experiments, the figure for sedimented enzyme activity was found by difference from the figures for total and unsedimented enzyme, the assumption being made that the latter was uniformly distributed between supernatant and precipitate. Control experiments to test this assumption, similar to those carried out with water homogenates (Walker & Levvy, 1951), showed that it also held for the homogenates in isotonic media.

Enzyme assay. After homogenizing liver in isotonic media, the enzyme associated with the subcellular particles did not display its full potential activity (see below). Unless otherwise stated, therefore, such homogenates were treated prior to assay by shaking with Ballotini, grade 12 (Chance Bros. Ltd.) in the Mickle tissue disintegrator (Mickle, 1948) for 10 min. When the sedimented material from these homogenates was subjected to direct assay, it was found sufficient to rehomogenize in water.

The enzyme preparations, fractionated or unfractionated, were usually diluted to a final volume of 40 ml./g. moist tissue, and 0.5 ml. was transferred to a tube containing 0.5 ml. 0.01 M-phenolphthalein glucuronide solution (Talalay *et al.* 1946) and 3 ml. 0.1 N-acetate or citrate buffer, pH 5.2. The same buffer was used in the assay as had been used in fractionation. The remainder of the procedure followed that described by Kerr & Levy (1951). Results are expressed as glucuronidase units (G.U.), where 1 G.U. liberates 1 μ g. phenolphthalein in 1 hr. at 37°.

Microscopy. As before (Walker & Levy, 1951), preparations were examined under phase contrast.

RESULTS

Fractionation of homogenates in isotonic media

Table 1 shows typical results obtained for the fractionation of glucuronidase activity in adult and infant mouse liver homogenized in water, 0.17M-sodium chloride and 0.25M-sucrose solution. The

Table 1. *Fractionation of mouse-liver glucuronidase activity with acetate buffer after homogenizing in water or in isotonic solution*

(Insoluble enzyme activity found by direct measurement.)

Homogenizing fluid	G.U.* / g. liver		
	Total	Soluble	Insoluble†
Normal adult:			
Water	2900	1740	1140
0.17M-NaCl	2920	280	2600
0.25M-Sucrose	2850	220	2530
Infant (4 days old):			
Water	4810	3600	1240
0.17M-NaCl	4800	780	4020
0.25M-Sucrose	4720	720	4080

* For definition of G.U. see 'Enzyme assay'.

† Washed once on centrifuge with one-tenth volume of 0.1M-acetate buffer; wash fluid added to soluble fraction.

figures for normal adult were all obtained with samples of the same liver, whilst the infant figures refer to litter mates. Fractionation was carried out with acetate buffer, and in these particular experiments the figures for total, soluble and insoluble enzyme activity were all obtained by direct measurement. The results illustrate the following points. With either adult or infant liver, the total activities for all three types of preparation were in agreement. In each individual case, the figure for the total agreed within experimental error with the sum of the figures for the two fractions. There was, however, a striking difference between water homogenates on the one hand and homogenates in sodium chloride or sucrose solution on the other in the partition of enzyme activity between the two fractions.

In water the greater part of the activity was in a soluble form, but in the two isotonic media the soluble enzyme only accounted for 10–15% of the total activity. As already noted (Kerr & Levy, 1951), the activity of the soluble fraction in a water homogenate of infant liver was higher than that in adult by an amount corresponding to the overall increase in activity. This was certainly not the case for homogenates in isotonic media, although the activity of the soluble fraction showed some increase in infants as compared with adults. Sodium chloride and sucrose did not act in these experiments as precipitating agents for enzyme soluble in water alone, but produced their effect by preserving some form of union between the enzyme and insoluble cell debris during homogenization. This was shown by experiments in which homogenates in water were subsequently made 0.17M with sodium chloride or 0.25M with sucrose. The partition of the enzyme remained characteristic of a water homogenate.

Fig. 1. Supernatant glucuronidase activity after centrifuging unbuffered homogenates of normal adult mouse liver at varying speeds for 15 min. Results expressed as percentages of the initial homogenate activity. O—O, liver homogenized in 0.17M-NaCl; ●—●, same liver homogenized in water and then made 0.17M with NaCl; O—O, liver homogenized in 0.25M-sucrose; ●—●, same liver homogenized in water and then made 0.25M with sucrose. The horizontal lines indicate the values for acetate soluble enzyme in the various preparations.

As with water homogenates (Walker & Levy, 1951), the fractionation of glucuronidase activity produced by centrifuging at a high-speed unbuffered homogenates in isotonic media was identical to that produced by acetate buffer. This is shown in Fig. 1 for adult mouse liver. As a control for the effect of the homogenizing medium on the rate of sedimentation of subcellular particles, the tissue was also homogenized in water alone, and sodium chloride or sucrose was added before centrifuging. In each preparation, sedimentation on the high-speed centrifuge ceased when the figure for acetate

soluble enzyme was reached. At this stage, subsequent addition of acetate buffer to the supernatant caused no further separation of enzyme. As already noted (Walker & Levvy, 1951), sodium chloride caused agglutination and more rapid sedimentation of subcellular particles. Sucrose did not produce this effect. Considering either isotonic medium separately, it can be seen that the completeness of sedimentation of the insoluble enzyme at any given relative centrifugal force (R.C.F.) value was the same as in the appropriate control.

In spite of the higher overall glucuronidase activities of infant liver and liver regenerating after partial hepatectomy (Levy *et al.* 1948), the sedimentation of the insoluble enzyme in homogenates in either isotonic medium followed the same course as that shown in Fig. 1 for the normal adult tissue.

Microscopic observations

Sucrose homogenates. The components of a homogenate of mouse liver in 0.25 M-sucrose resembled closely in appearance those described in similar preparations of rat liver (Hogeboom, Schneider & Pallade, 1948). The nuclei were smooth in outline and structureless, and granules of all sizes were discrete, uniformly dense and spherical. Complete sedimentation of insoluble enzyme was associated with the removal of all but a very few of the smallest visible particles from the suspension. Buffering the homogenate to pH 5.2 with acetate led to agglutination of all the visible components, and consequently much greater ease of sedimentation (*cf.* Walker & Levvy, 1951).

Saline homogenates. Compared with their appearance in sucrose homogenates, the nuclei in homogenates in 0.17 M-sodium chloride were small, dark and granular, with well marked nucleoli. The granules were similar in appearance to those in sucrose homogenates, but were loosely agglutinated.

Water homogenates. As already noted (Walker & Levvy, 1951), the nuclei and granules showed pronounced osmotic swelling. The former were pale with faint nucleoli and were sometimes vacuolated. The latter were discrete, spherical and vesicular, with much less contrast than the granules in sucrose or saline homogenates.

On progressive dilution with water, the nuclei and granules in a saline or sucrose homogenate took on the appearance of those seen in water homogenates, without perceptible fragmentation of the larger granules. On making a water homogenate isotonic with sodium chloride or sucrose, however, the particles did not revert completely to the appearance typical of a homogenate prepared in the isotonic medium. Most of the granules became smaller and darker, but irregular in outline, while the nuclei became slightly crenated.

Factors influencing the partition of enzyme activity in the homogenate

It would appear from the experiments described above that the greater part at least of the soluble glucuronidase activity in a water homogenate of mouse liver is not originally free in the cytoplasm of the intact cell, but is liberated from the insoluble particles during the preparation of the homogenate. The effects were studied of three variables which might conceivably influence the final partition of enzyme activity after grinding the tissue in the glass homogenizer, namely, the temperature of the homogenizer, the period of homogenizing and the tonicity of the medium.

Temperature. The freshly dissected, chilled tissue was homogenized with cold 0.25 M-sucrose solution for 1 min. in a glass homogenizer surrounded with crushed ice. The centrifuge tubes were also surrounded with ice during the subsequent fractionation using the acetate buffer method. Results obtained in this way were identical with those obtained for another sample of the same liver put through the same treatment without temperature control. This was also true for a third sample of liver homogenized at 37° and then fractionated in the usual way.

Period of homogenizing. Portions of adult liver were homogenized for varying periods, without temperature control, and fractionated by buffering with acetate. The results reveal a progressive release of enzyme from the particles to the solution as homogenizing was prolonged (Table 2). The time factor was least marked in water. There was no inactivation of the enzyme in these experiments.

Table 2. *Fractionation of adult mouse-liver glucuronidase activity after grinding in the glass homogenizer for varying periods with water, 0.17 M-NaCl or 0.25 M-sucrose solution*

Period of homogenizing (min.)	Acetate-soluble enzyme (% total activity)		
	Water	0.17 M-NaCl	0.25 M-Sucrose
0.5	64	9	7
1	66	11	8
2	70	15	15
3	73	19	20
5	75	26	25

An attempt was made to reduce the figure for soluble enzyme activity in an isotonic preparation below the lowest given in Table 2. Liver was ground in a chilled mortar with 0.25 M-sucrose solution and strained through bolting silk. The preparation thus obtained was very similar in its microscopic appearance to the usual sucrose homogenate, and had the same percentage soluble enzyme activity as the 0.5 min. homogenate (Table 2).

Tonicity. Fig. 2 shows the partition of enzyme activity after homogenizing adult liver for 1 min. in varying concentrations of sucrose. No change in the value for acetate-soluble enzyme was observed in passing from the isotonic to hypertonic medium, but as the medium was made increasingly hypotonic there was a progressive rise in the activity of this fraction until the figure for water was reached. The overall glucuronidase activity of the preparation did not vary with the concentration of sucrose.

Fig. 2. Acetate-soluble glucuronidase activity in mouse-liver preparations after homogenizing in varying concentrations of sucrose. Results expressed as percentages of the total homogenate activity. ●—●, adult; ○—○, 4-day-old.

Fig. 2 also shows that at all concentrations of sucrose, infant liver had a greater proportion of the enzyme in a soluble form than the adult tissue. The difference became more marked with decreasing concentration of sucrose till in water alone, as already noted, it accounted entirely for the higher overall activity of the infant tissue. Similar curves were obtained for liver homogenized in sodium chloride solutions covering the same range of tonicity.

Measures causing the release of enzyme from insoluble material

Dilution with water. On dilution with water, enzyme was released from the particles in homogenates prepared in isotonic sucrose or sodium chloride solution, to give a distribution identical to that obtained by homogenizing directly in media of the same final tonicity. The change in the distribution of the enzyme appeared to be instantaneous.

Incubation at 37°. It was previously found for water homogenates (Kerr & Levvy, 1951; Walker & Levvy, 1951) that incubation in acetate buffer, pH 5.2, at 37° for 4 hr., converted all the enzyme present into a soluble form, with little change in the microscopic appearance of the insoluble particles. This property of acetate was not shared by citrate,

nor did release of enzyme from the particles occur on incubating unbuffered preparations.

Homogenates in 0.17 M-sodium chloride or 0.25 M-sucrose solution behaved like water homogenates in that incubation for 4 hr. after buffering to pH 5.2 with acetate (final concentration 0.1 N) without change in tonicity resulted in the conversion of enzyme into a soluble form, although conversion was not so complete as in water. When acetate was replaced by citrate buffer (final concentration 0.1 N)

Fig. 3. Changes in the partition of the glucuronidase activity in a homogenate of adult mouse liver in 0.17 M-NaCl solution after incubating at 37° for varying periods. Acetate-soluble enzyme expressed as a percentage of the total homogenate activity. ○—○, unbuffered homogenate; x—x, homogenate made 0.1 N with respect to acetate buffer, pH 5.2.

at pH 5.2, or when the unbuffered preparation was incubated at its natural pH (6.5–6.8), the release of enzyme from the particles was much slower and tended to a maximum at the figure for soluble enzyme in a water homogenate. This is illustrated for a saline homogenate in Fig. 3. In the unbuffered preparations incubation resulted in a change in the microscopic appearance of the particles, resembling that observed when homogenates in isotonic media were diluted with water (see 'Microscopic observations'). No enzyme inactivation was observed in these experiments.

Mechanical disintegration. As previously noted for water homogenates (Walker & Levvy, 1951), shaking with Ballotini in the Mickle tissue disintegrator caused almost complete release of the enzyme from the particles in a homogenate in isotonic sodium chloride or sucrose solution, without appreciable inactivation. The Waring Blender has been found to act similarly in this respect (Table 3), practically all the enzyme in liver or a liver homogenate being converted to a soluble form in 3 min., without any loss in overall activity. At this stage, all the components of the preparation were seen under the microscope to have undergone fragmentation, with complete loss of structure.

It has been customary to use the supernatants from water homogenates for the assay of tissue glucuronidase activity (see Kerr & Levvy, 1951). It can be seen from Table 3 that the use of the Waring Blendor instead of the glass homogenizer can lead to a very different value for the glucuronidase activity of a tissue in such methods of assay (cf. Fishman & Talalay, 1947).

Table 3. *Fractionation of adult mouse-liver glucuronidase activity after treatment for varying periods in the Waring Blendor*

(The starting material consisted of liver which had previously been ground in water, 0.17M-NaCl or 0.25M-sucrose solution in the glass homogenizer for 1 min., or the whole organ to which water was added in the blender.)

Period of treatment (min.)	Acetate-soluble enzyme (% total activity)			
	Water homogenate	Saline homogenate	Sucrose homogenate	Liver and water
0	57*	14	14	—
0.5	71	77	72	—
1	88	89	86	79*
2	95	96	93	90
3	100	100	98	98
5	100	100	100	100

* These figures give a direct comparison of liver treated with water for 1 min. in the glass homogenizer and the Waring Blendor respectively.

Freezing. As in water homogenates (Walker & Levvy, 1951), the particles in sucrose or saline homogenates of liver gave up their glucuronidase activity to the solution on repeated freezing with a mixture of solid carbon dioxide and acetone, and thawing at 37°. The rates of transfer were, however, very different in the two types of isotonic homogenate. In sodium chloride solution, 60% of the enzyme activity became soluble after a single freezing, whereas four freezings were necessary before this stage was reached in sucrose solution.

Treatment with acetone. After homogenizing mouse liver in acetone (Walker & Levvy, 1951) followed by resuspension of the dry powder in water, 0.17M-sodium chloride or 0.25M-sucrose solution, almost all the enzyme activity was soluble.

Treatment with surface-active agents. The actions of Teepol XL (British Drug Houses Ltd), Triton X-100 and Triton A-20 (Rohm and Haas Co.) on homogenates in isotonic media were identical in all respects with their actions on water homogenates (Walker & Levvy, 1951). The first two agents in suitable concentrations caused complete conversion of the enzyme into a soluble form, but Teepol XL differed from Triton X-100 in that high concentrations were inhibitory. Triton A-20 had no effect of any kind.

Distribution of the enzyme according to particle size

Fractional sedimentation of mouse-liver homogenates in 0.25M-sucrose solution was carried out by a procedure which followed that of Schneider & Hogeboom (1950) as closely as our equipment permitted.

Fraction 1. A 10% homogenate (10 ml.) was centrifuged for 10 min. at 700 g and the supernatant was decanted. The residue was resuspended in 8 ml. isotonic sucrose solution and sedimented as before. The debris, consisting of nuclei contaminated with erythrocytes, unbroken liver cells and cytoplasmic granules, was resuspended for assay.

Fraction 2. The combined supernatants from fraction 1 were centrifuged at 5000 g for 10 min., and the supernatant was decanted and again centrifuged at 5000 g for 10 min. Two washings of the combined sediments were carried out by suspending the solid in 5 ml. sucrose solution and centrifuging at 20 000 g for 10 min. The debris, a fairly homogeneous preparation of the larger cytoplasmic granules, was finally resuspended for assay.

Fraction 3. Values for this fraction, consisting of all remaining insoluble material in the preparation, were found by difference, since centrifuging at 25 000 g (the maximum R.C.F. value at our disposal) did not give a sufficiently well packed sediment for clean separation. The supernatant and washings from fraction 2 were combined and aliquots were taken for assay. The figures thus obtained and those for fraction 4 gave by difference the figures for fraction 3.

Fraction 4. The remainder of the preparation was centrifuged at 25 000 g for 20 min. and the supernatant was decanted. After recentrifuging at the same speed, samples of the now optically clear supernatant were withdrawn directly from the centrifuge tube for assay of the 'soluble enzyme' in the homogenate.

Results for representative experiments are shown in Table 4. The main points emerging from this table are as follows. The total enzyme activity of the homogenates was fully accounted for by the activities of the separated fractions, and 80–90% was sedimentable. Of this, four-fifths was in the pure granule fractions, which also showed the highest specific activities. The relatively low activity of the impure nuclear fraction may well have been due to the contaminating granules.

Infant liver and liver regenerating after partial hepatectomy showed a similar distribution of insoluble enzyme activity to that seen in the normal adult tissue. The higher total and specific activities of growing and regenerating liver (cf. Levvy *et al.* 1948) were reflected in every centrifuge fraction.

All fractions of the sedimentable material showed approximately equal (60–70%) release of enzyme

activity to the solution on dilution with water. Under the microscope, there was no perceptible fragmentation of the particles in fraction 2. In infant as compared with adult liver a greater percentage of the enzyme in every fraction (70–80%) was converted to the soluble form.

in a given preparation no matter which method of treatment was adopted, but the results make it clear that it was unnecessary to take more than part of the enzyme into solution before the preparation displayed its full potential activity. The release of enzyme activity from insoluble material was readily

Table 4. *Fractional sedimentation of unbuffered mouse-liver homogenates in 0.25 M-sucrose solution*

Fraction no. and description	N (total)		Enzyme activity		
	mg./g. liver	% total	g.u.* /g. liver	% total	g.u.* /mg. N
Normal adult liver					
Total homogenate	39.0	100	3580	100	92
1. Nuclei, etc.	7.5	19	510	14	68
2. Large granules	6.5	17	1100	31	170
3. Small granules	6.8	17	1430	40	210
4. Soluble enzyme	16.5	42	470	13	29
Sum of fractions	37.3	95	3510	98	—
Infant liver (4-day-old)					
Total homogenate	31.0	100	5100	100	164
1. Nuclei, etc.	6.5	21	750	15	117
2. Large granules	5.1	16	1310	26	256
3. Small granules	5.3	17	2000	39	377
4. Soluble enzyme	14.2	46	950	19	67
Sum of fractions	31.1	100	5010	99	—
Regenerating liver (4 days after partial hepatectomy)					
Total homogenate	36.6	100	4480	100	122
1. Nuclei, etc.	7.2	20	550	12	76
2. Large granules	7.0	19	1360	30	194
3. Small granules	5.9	16	1820	41	309
4. Soluble enzyme	17.0	46	820	18	48
Sum of fractions	37.1	101	4550	101	—

* For definition of g.u. see text.

The activity of the enzyme in homogenates in isotonic media

Kerr & Levvy (1951) found that the use of too high a tissue concentration in the assay of water homogenates of mouse liver led to false low results for the total glucuronidase activity. Full activity was shown at all concentrations once the enzyme had been completely converted into a soluble form by incubation in acetate buffer at 37° for 4 hr.

Table 5 shows that tissue concentration had a similar effect in the assay of homogenates of mouse liver in isotonic sodium chloride or sucrose solution. The activity no longer varied with tissue concentration once the enzyme had been completely converted into a soluble form. Even at low concentrations, however, homogenates in isotonic media, unlike those in water, increased considerably in activity when the enzyme was made soluble.

Table 6 shows parallel figures for the total glucuronidase activity and the percentage soluble enzyme activity in homogenates of adult mouse liver after treatment by various methods which cause the transfer of enzyme activity from insoluble material to the solution. The same final activity was reached

controlled when it was brought about by dilution with water or incubation in acetate buffer, and with these measures full activity was reached when only about half the enzyme had gone into solution, i.e. when the distribution of enzyme activity had become similar to that seen in water homogenates. Whereas these measures released the enzyme by a gradual process affecting all insoluble particles equally, mechanical disintegration in the tissue disintegrator or the Waring Blendor resulted in complete destruction of individual particles and thus did not produce complete activation in the present experiments until nearly all the enzyme had been brought into solution. The release of enzyme activity from insoluble material by the surface-active agents Triton X-100 and Teepol XL was difficult to control in degree, but the figures given in Table 6 show that when this process was complete the enzyme was fully active.

Since in the assay the incubation with substrate was carried out in acetate buffer, in which activation of the enzyme must have occurred, the figures given in Table 6 for the total glucuronidase activity of homogenates are subject to error. This consideration does not apply to the figures for soluble enzyme

activity, this fraction of the enzyme being already fully active.

The error arising from the activation of the enzyme during the incubation with substrate is illustrated in Fig. 4 by velocity curves for the hydrolysis of phenolphthalein glucuronide in acetate buffer by mouse liver homogenized in 0.17M-sodium chloride. When the homogenate (2.5%,

activity was slower, and full activity was only reached after 90 min. incubation. This concentration of sodium chloride did not inhibit fully activated preparations of the enzyme.

Similar results were obtained in experiments with homogenates in isotonic sucrose solution, except in that activation proceeded more slowly than in sodium chloride solution. Sucrose in a final

Table 5. *Variations in the glucuronidase activity of adult mouse liver with the homogenate concentration taken for assay*

(All results refer to a single sample of tissue; activities were measured in acetate buffer before and after conversion of all the enzyme into a soluble form by treatment for 20 min. in the tissue disintegrator.)

Concentration of homogenate* (g. tissue/100 ml. homogenizing fluid)	Glucuronidase units/g. liver			
	Homogenate in 0.17M-NaCl		Homogenate in 0.25M-sucrose	
	Before treatment	After treatment	Before treatment	After treatment
40	1320	3200	1200	3240
20	1680	3200	1560	3200
10	2200	3200	2120	3240
5.0	2400	3280	2240	3200
2.5	2380	3240	2200	3240
1.0	2300	3200	2100	3240

* 0.5 ml. of homogenate of the concentration shown was incubated with 0.5 ml. substrate solution and 3 ml. buffer in the usual way.

Table 6. *Effects of various measures on the total and soluble glucuronidase activity in homogenates (2.5%, w/v) of mouse liver in isotonic NaCl or sucrose solution*

(Activities measured in acetate buffer, and results expressed as percentages of the activity of the whole homogenate after 10 min. treatment in the tissue disintegrator.)

Treatment		Homogenate in 0.17M-NaCl		Homogenate in 0.25M-sucrose	
Agent	Extent	Total activity	Soluble activity	Total activity	Soluble activity
None	—	75	12	66	8
Tissue disintegrator	1 min.	82	50	79	55
	2 min.	91	72	88	78
	5 min.	100	98	100	97
	10 min.	100	100	100	100
Waring Blendor	0.5 min.	88	68	87	66
	1 min.	94	85	95	83
	3 min.	100	100	101	100
	5 min.	99	99	101	103
Triton X-100	0.1 %	101	101	102	102
Teepol XL	0.4 %	101	98	101	97
Water	$\frac{1}{4}$ vol.	82	14	75	17
	1 vol.	90	23	86	29
	3 vol.	100	47	97	51
Incubation in acetate buffer, pH 5.2	1 hr.	87	26	83	18
	2 hr.	92	41	91	30
	4 hr	100	80	100	69

w/v) was added to the usual buffer-substrate mixture, the rate of hydrolysis gradually increased until, after about 45 min., it reached the value obtained with a fully activated sample of the same homogenate. When the buffer-substrate mixture was made 0.17 M with respect to sodium chloride, so as to prevent dilution of the homogenizing medium in the enzyme preparation, the change in enzyme

concentration of 0.25M was found to cause about 10 % inhibition of fully activated glucuronidase preparations.

Replacing acetate by citrate buffer during the incubation at pH 5.2 not only slowed down the transfer of enzyme activity from insoluble material to the solution (see above), but also decreased the rate of activation of homogenates in isotonic media.

The rate of activation in citrate buffer became even slower when 0.17M-sodium chloride was present, and slower still when this was replaced by 0.25M-sucrose. Velocity curves for the hydrolysis of phenolphthalein glucuronide in sucrose-citrate (pH 5.2) by homogenates of adult and infant mouse liver in 0.25M-sucrose solution are shown in Fig. 5.

Fig. 4. Rate of hydrolysis of phenolphthalein glucuronide (0.00125M) in acetate buffer (final concentration 0.075N, pH 5.2) by a homogenate of adult mouse liver in 0.17M-NaCl solution. Final tissue concentration 0.3% (w/v). ●—●, buffer and substrate made 0.17M with respect to NaCl; ○—○, no NaCl added to buffer or substrate; —x—, assay of homogenate after treatment for 10 min. in the tissue disintegrator.

This graph illustrates the difficulties which would attend attempts to make comparative measurements of mouse-liver glucuronidase activity in homogenates in isotonic media without preliminary activation. It can be seen, however, that in untreated preparations the activity of the infant tissue was at all stages higher in terms of µg. phenolphthalein liberated/g. liver than that of the adult tissue (cf. Levvy *et al.* 1948).

The nature of the inactive enzyme

Table 7 shows the effect of varying the substrate concentration on the activity, expressed as glucuronidase units, of different preparations of the same tissue added to incubation mixtures of varying composition. When a homogenate in 0.17M-sodium

Fig. 5. Rate of hydrolysis of phenolphthalein glucuronide (0.00125M) in citrate buffer (final concentration 0.075N) by homogenates of adult and infant mouse liver in 0.25M-sucrose solution. Final tissue concentration 0.3% (w/v). Substrate and buffer made 0.25M with respect to sucrose. ○—○, adult; ●—●, infant (4-days-old); —x—, assay of adult liver after treatment of homogenate for 10 min. in the tissue disintegrator. —△—, assay of infant liver after treatment of homogenate for 10 min. in the tissue disintegrator.

chloride solution was incubated with increasing concentrations of phenolphthalein glucuronide in acetate buffer, inhibition of glucuronidase activity by excess substrate was seen, whether or not the enzyme preparation had been subjected to prior activation. Considerable activation of the untreated preparation must of course have occurred during the assay in acetate buffer. When a homo-

Table 7. Effect of varying substrate concentration on the rate of hydrolysis of phenolphthalein glucuronide in 0.1N-acetate or citrate buffer by homogenates of adult mouse liver in 0.17M-NaCl or 0.25M-sucrose solution

(All results refer to a single sample of tissue; g.v./g. liver measured at pH 5.2 before and after treatment for 10 min. in the tissue disintegrator.)

Substrate concentration (M)	Saline homogenate in acetate buffer		Sucrose homogenate in citrate buffer		Sucrose homogenate in sucrose-citrate buffer	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
0.00125	2640	3600	2000	3600	1320	3200
0.00438	2320	3120	1960	3040	1440	2640
0.00875	2000	2480	2000	2400	2080	2240

genate in 0.25M-sucrose solution was added to citrate buffer containing the same concentration of sucrose and a varying concentration of substrate, increase in the latter caused a rise in the net activity of the untreated homogenate, but a fall in the activity of the activated preparation. Whatever the factor may be that is responsible for the low activity of glucuronidase in mouse liver homogenized in isotonic media, it can thus to some extent be overcome by the use of a substrate concentration in itself high enough to inhibit the enzyme. These two opposing effects of increasing the substrate concentration on the net activity of an untreated preparation were exactly balanced when a sucrose homogenate was incubated with a mixture of citrate buffer and phenolphthalein glucuronide made up in water alone.

The results shown in Table 7 are entirely explicable on the view that the enzyme in a homogenate of mouse liver in isotonic media, but not in water, is subject to the action of an endogenous competitive inhibitor. An alternative explanation must, however, be considered, namely that the enzyme associated with the insoluble particles is surrounded by material which the substrate only penetrates with difficulty.

DISCUSSION

Accepting that the localization of an enzyme in the intact cell can be inferred from its distribution amongst the components of an isotonic homogenate, it is concluded from the present study that all or almost all of the glucuronidase activity of mouse liver is associated *in vivo* with subcellular particles, especially the large and small cytoplasmic granules.

The enzyme can be irreversibly released from the particles by various treatments which produce either swelling or fragmentation of the particles. The small amount of the enzyme which is present in a soluble form in isotonic preparations may be derived from the particles when they are liberated into the medium or it may arise from damage to the particles in the homogenizer. The amount of this soluble fraction is, however, not reduced either by employing hypertonic media which preserve better the intracellular appearance of the particles (Hogeboom *et al.* 1948) or by a very gentle grinding of the tissue. As the medium is made increasingly hypotonic there is a transfer of enzyme activity from the particles to the solution till, at low sucrose or saline concentrations, the distribution characteristic of a water homogenate is approached. It may be significant that the release of enzyme activity which occurs on prolonged incubation of a homogenate in an isotonic medium also ceases when the soluble activity reaches the figure for a water homogenate.

The distribution of glucuronidase in infant and regenerating liver is similar to that in normal adult liver, and the higher enzyme activity of these tissues is spread over particles of all sizes. In actively proliferating as compared with resting tissue, a greater proportion of the enzyme in all fractions of the sedimentable material is released in water. This agrees with the observation of Kerr & Levvy (1951) who found that in water homogenates the increase in the soluble activity accounted entirely for the higher overall activity of proliferating tissues.

As enzyme is released from the particles in homogenates in isotonic media, there is an increase in the activity of the enzyme remaining bound to insoluble material. Two explanations have been suggested for the initially low glucuronidase activity of these homogenates, namely the existence of a competitive inhibitor in the homogenate, or of material which hinders access of the substrate to the enzyme in the insoluble particles. The latter explanation is considered to be the more satisfactory since it covers the behaviour of water homogenates. In hypotonic media, a change in the particle occurs, permitting the escape of some of the enzyme from the particles. This change presumably permits much easier access of the substrate to the enzyme remaining within the particles. The existence of a competitive inhibitor is hard to imagine since it would have to be very selective in its action, affecting the insoluble enzyme in homogenates in isotonic media, but neither the enzyme in completely soluble preparations, nor the insoluble enzyme fraction in water homogenates.

Various factors have been found to influence results obtained in the assay of glucuronidase associated with insoluble material. It is clear that reliable comparative measurements of tissue activity can only be made after complete solution or complete activation of the enzyme. The physiological significance of results thus obtained is, however, another matter. If one accepts the premise that the state of an enzyme in homogenates in isotonic media approximates to its state in the intact tissue (Schneider & Hogeboom, 1951), the possibility must be considered that factors such as those encountered in the present work have effects on the activity of the enzyme in living tissues, which may be as important as changes in the active protein content, detected by the use of soluble preparations.

SUMMARY

1. The partition of glucuronidase activity between the subcellular particles and the solution has been studied in homogenates of mouse liver in isotonic sodium chloride or sucrose solution. The particles were sedimented by centrifuging at a high speed, or by centrifuging at a low speed after agglutination with acetate buffer.

2. The two methods of sedimentation gave identical results and 80–95 % of the enzyme activity was found in the precipitate.

3. As the medium was made increasingly hypotonic, a greater proportion of the enzyme activity was released from the particles. Particles of different sizes, separated by fractional sedimentation, behaved comparably in this respect.

4. The distribution of enzyme activity over particles of different sizes was the same in infant liver and in liver regenerating after partial hepatectomy as in the normal adult tissue, but prepara-

tions of the growing or regenerating tissue showed greater release of enzyme activity to the solution on dilution with water.

5. The homogenates in isotonic media did not display their full glucuronidase activity on direct assay, but partial activation occurred under assay conditions. Full activity was displayed when about half the enzyme present was brought into solution.

6. The possible mechanism of activation and its physiological significance are discussed.

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The Chemical Composition and Structure of the Yeast Cell Wall

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The chemical composition of the yeast cell wall was first studied by Salkowski (1894), who investigated the polysaccharide material which remained after intact cells had been digested with dilute sodium hydroxide solution. Zechmeister & Tóth (1934) continued the study, but again disrupted the cells by fairly vigorous chemical action. They suggested, however, that an enzymic method might do less damage to the cell wall, and indeed later they isolated the glucan component of the cell wall by the action of pepsin and amylase on an autolysed yeast suspension (Zechmeister & Tóth, 1936). These chemical and enzymic investigations have indicated that several polysaccharides may be present in the cell wall, and it has been suggested that, besides the glucan, a mannan (Haworth, Hirst & Isherwood, 1937) and possibly a 'glycogen' (Ling, Nanji & Panton, 1925) may form part of the structure; no direct evidence for this has been given. The structure of these polysaccharides has been investigated by

various workers. The general method of isolation has been extraction of whole yeast with 3 % sodium hydroxide solution at 100°, whereby the mannan and some glycogen go into solution, whereas the glucan and most of the glycogen remain as the insoluble material, which is seen under the microscope to retain the shape of the cell, and is therefore assumed to be part of the cell wall. The glucan was shown to contain only glucose with β -1:3 linkages between the radicals, by Zechmeister & Tóth (1934) and Hassid, Joslyn & McCreedy (1941); more recently Bell & Northcote (1950) found that this polysaccharide was highly branched and determined its average chain length. The structure of the mannan has been studied by Haworth *et al.* (1937), Haworth, Heath & Peat (1941) and Lindstedt (1945), but the constitution of the glycogen, and especially its possible existence in two forms (Ling *et al.* 1925; Daoud & Ling, 1931) has received little attention.