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The Glucuronidase Content of Animal Tissues and the Role of the Spleen in the Metabolism of Conjugated Glucuronic Acids

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It has been recognized for many years that the formation of conjugated glucuronic acids is an important result of the introduction into the body of many foreign organic compounds containing a hydroxyl or carboxyl group. That the main site of formation of these glucuronides is the liver has been established by Hemingway, Pryde & Williams (1934) using perfusion experiments and by Lipschitz & Bueding (1939) using the tissue slice technique.

The enzyme β -glucuronidase, which hydrolyzes glucuronides is found in several tissues (Masamune, 1934; Oshima, 1934, 1936; Fishman, 1938, 1939, 1940). Fishman has concluded, therefore, that the liver is not the sole site of glucuronide formation in the body. Fishman found the spleen to be a rich source of glucuronidase, the liver, ovary, uterus and kidney being relatively good sources. On feeding dogs or mice with menthol or borneol for a few days, Fishman (1940) observed that the glucuronidase content of liver, kidney and spleen increased, whereas that in the uterus remained the same and that in the ovary decreased. When oestrogenic substances (oestrone, oestradiol, etc.) were fed, the uterine glucuronidase increased but the liver glucuronidase remained unchanged (Fishman & Fishman, 1944). They also found that ovariectomy caused a decrease in uterine glucuronidase but not in the liver enzyme. Fishman (1940) concludes on the basis of his experiments that glucuronides are formed in organs other than the liver and that the uterus is the sole site of formation of the oestrogen glucuronides.

Lipschitz & Bueding (1939), on the other hand, found that liver slices were the only tissues to form significant amounts of glucuronides in the presence of menthol and borneol, the spleen being inactive and the kidney only slightly active in this respect.

The question arises, therefore, whether the glucuronidase present in tissues is concerned with the synthesis of glucuronides (Florkin, Crismer, Duchateau-Bosson & Houet (1942), found glucuronidase to synthesize bornylglucuronide *in vitro* to a small extent from borneol and glucuronic acid), or whether it is concerned only with their hydrolysis. In this connexion, it should be mentioned that glucuronides when fed to animals are hydrolyzed in the body, for Quick (1928) has shown that on feeding benzoylglucuronide, about 50% of its benzoic acid is excreted conjugated with glycine in the dog.

That liver and spleen glucuronidase may be different is indicated by the work of Talbot, Ryan & Wolfe (1943) who found that the spleen enzyme prepared according to Fishman (1938) would not hydrolyze pregnadiolglucuronide in urine satisfactorily, whereas a preparation from liver would.

The work of Mitsuba (1927) suggests that the spleen may play a part in the destruction of glucuronides. This worker found that, after splenectomy, the amount of glucuronic acid excreted by rabbits and dogs in response to anthranilic acid was greater than in the intact animal. In view of the fact that Mitsuba's method for estimating glucuronic acid is now considered unreliable and consequently that his experimental findings are inconclusive, it was decided to reinvestigate this problem. At the same time, more detailed figures than were hitherto available for the glucuronidase content of rabbit and rat organs are presented.

METHODS

Enzyme extracts for assay. The animals were killed by a blow on the head and the organs dissected out, minced finely with scissors, ground with silver sand and 3 vol. of water, shaken vigorously for 1 hr. and allowed to stand

overnight at room temperature. The extract was then centrifuged, the solution precipitated with 2 vol. acetone and the precipitate obtained shaken vigorously for 1 hr. with an amount of water equivalent to twice the weight of the original organ. The precipitate was removed on the centrifuge and the solution assayed immediately for glucuronidase.

Glucuronidase assay. One ml. of a suitable dilution of the enzyme extract (1/2 or 1/4) was incubated for 18 hr. at 37° with 2 ml. 0.1 M-acetate buffer, pH 5.2, and 1 ml. 0.025 M-Na *l*-menthylglucuronide solution (adjusted to pH 5.2 with acetic acid). Trichloroacetic acid (4 ml. of 10% (w/v)) was then added and the precipitate removed by centrifuging, the resulting solution poured into 6 × 1 in. pyrex tubes and the free glucuronic acid determined by the method of Miller & Van Slyke (1936) as modified by Fishman (1938), using 0.1% xylene cyanol F.F. (British Drug Houses Ltd.) as indicator in the titration. 1 mg. glucuronic acid liberated under these standard conditions = 1 unit of activity.

1-Menthylglucuronide was prepared according to Quick (1924). The Na *l*-menthylglucuronide was prepared by dissolving the free acid in the requisite amount of 0.1 N-NaOH, adjusting the pH to 5.2 and diluting to a standard volume.

Estimation of glucuronic acid in urine

(i) **Total glucuronides.** The method of Hanson, Mills & Williams (1944) was used. In this method it was found essential, for consistently accurate results, to observe the following four conditions:

(a) the quality of commercial naphthoresorcinol is variable and a fresh calibration curve must be made for each sample; (b) 0.25% naphthoresorcinol solutions are not reliable after 3 days even when stored in the dark at 0°; (c) it is essential to get complete separation of the aqueous and amyl alcohol layers in order to eliminate minute droplets of water in the final amyl alcohol extract, since the presence of water causes variable readings in the 'Spekker' photoelectric absorptiometer; (d) the water-bath must be boiling vigorously during the heating period otherwise variable results are obtained.

(ii) **Ether soluble glucuronides.** 2 ml. filtered urine + 2 ml. 2% (w/v) H₂SO₄ were extracted with ether for 1 hr. in a small continuous extractor. The ether extract was taken to dryness, the residue dissolved in hot water, made up to a standard volume and the glucuronic acid estimated as above. By this method 97–100% of *l*-menthylglucuronide added to urine could be recovered. The ether-soluble glu-

curonide in normal rabbit urine is of the order of 0–50 mg./day, but depends to some extent upon the rabbit.

Animal experiments. Adult rabbits were maintained on a constant diet of 200 g. cabbage and 50 g. crushed rat cubes per day, and the 24 hr. urine samples were collected each morning. After collecting the urine on the second day each animal was given by stomach tube a dose of 1 g./kg. *l*-menthol (British Drug Houses Ltd. [α]_D¹⁵ = -50.7° in absolute ethanol) suspended in water and the urine collected for the following 3 days. All the extra glucuronic acid excreted in response to menthol is eliminated in 36–48 hr. For glucuronic acid estimations the urine was diluted 1/50 on the control days and for the 2 days after feeding menthol, 1/250. If *D*₁, *D*₂, etc., are the excretions of glucuronic acid on days 1, 2, etc., respectively, the extra glucuronic acid in response to menthol = *D*₃ + *D*₄ - (*D*₁ + *D*₂ + *D*₅) × $\frac{1}{2}$.

RESULTS

Glucuronidase in rabbit and rat organs. The glucuronidase content of rabbit and rat liver, kidney and spleen are recorded in Tables 1 and 2.

Table 1. *Glucuronidase content of rabbit organs*

(Averages of seven animals—range in brackets.)

Tissue	Glucuronidase units*/g. fresh tissue	Organ (% of body wt.)	Glucuronidase units/100 g. body wt.
Liver	10.8 (7.6–14.5)	3.61	39.0 (25.0–46.6)
Kidney	1.8 (1.4–2.5)	0.72	1.15 (0.70–1.52)
Spleen	13.7 (11.4–17.2)	0.044	0.60 (0.52–0.64)

* One unit of glucuronidase activity = 1 mg. glucuronic acid liberated in 18 hr. at 37°, from 0.006 M-*l*-menthylglucuronide at pH 5.2 in 0.05 N-acetate buffer.

Table 2. *Glucuronidase content of rat organs*

(Averages of eighteen animals—range in brackets.)

Tissue	Glucuronidase units*/g. fresh tissue	Organ (% of body wt.)	Glucuronidase units/100 g. body wt.
Liver	5.65 (4.0–6.7)	3.81	21.5 (17.0–25.4)
Kidney	8.5 (5.5–11.9)	0.73	6.2 (4.4–8.5)
Spleen	29.1 (23.9–34.7)	0.33	9.7 (8.3–11.3)

* See footnote to Table 1.

Table 3. *Control excretion of glucuronides and percentage conjugation of 1-menthol in normal rabbits*

Group	Rabbit no.	Control excretion of total glucuronic acid (mg./day)	Extra glucuronic acid excretion (calculated as % of <i>l</i> -menthol fed)		Difference (total ether- soluble)
			Ether-soluble	Total	
1	22	254	48.5 : 46.0	68.0 : 65.3	18.1%
	23	238	36.2 : 54.9	59.0 : 70.0	
	28	266	48.0 : 43.0	56.0 : 70.0	
	29	252	39.0 : 46.8	58.0 : 60.9	
		Av. 252	Av. 45.3	Av. 63.4	
2	14	238	71.0 : 69.0	80.5 : 72.0	9.0%
	15	124	40.0 : 60.0	54.0 : 65.0	
			35.5	42.0	
	32	235	63.0 : 68.0	76.0 : 73.0	
	33	250	53.8	60.9	
		Av. 212	Av. 58.6	Av. 67.6	
		Average	52.7	65.6	12.9%

Effect of splenectomy on glucuronide excretion in rabbits. The total and ether-soluble glucuronides were estimated in the urine of normal rabbits before and after feeding *l*-menthol at a level of 1 g./kg. body weight, and the extra glucuronic acid excreted in response to the *l*-menthol, was calculated as a percentage of the menthol fed. The results obtained are shown in Table 3. The rabbits were splenectomized and divided into two groups. The first group was tested over a period of two weeks—starting a few days after splenectomy. The second group was rested for three weeks after splenectomy and then tested over a period of two weeks. The results obtained are shown in Table 4. For the sake

of the organ. When such figures are calculated on the basis of a standard animal weight a different picture is obtained. In the rabbit (Table 1) it will be seen that about 95 % of the total glucuronidase of this organism is contained in the liver and only about 1.5 % in the spleen. In the rat, however (Table 2), these figures are 60 and 25 % respectively. Organs other than the liver, spleen and kidney contribute only about 2 % to the total in the rabbit and about 5 % in the rat. The importance of spleen glucuronidase may, therefore, vary considerably from species to species. It is interesting to note in this connexion that Cannon & McClelland (1929) have shown that there is a species difference in

Table 4. *Excretion of conjugated glucuronides in the rabbit after splenectomy*

Group	Rabbit no.	Time between splenectomy and menthol feeding (days)	Control excretion of total glucuronic acid (mg./day)	Extra glucuronic acid excretion (calculated as % of <i>l</i> -menthol fed)		Difference
				Ether-soluble (%)	Total (%)	
1	23	6	352	65.0	72.5	10.0
	28	6	375	64.5	75.6	
	29	6	338	63.0	74.4	
1	23	13	239	69.0	87.0	22.0
	28	13	282	70.0	89.0	
	29	13	324	50.0	80.0	
2	14	28	193	54	62	8.0
	15	28	113	35	41	
	32	27	189	56	60	
	33	27	250	34	48	
2	14	35	200	65	72	6.0
	15	35	136	49	65	
	32	34	204	57	70	
	33	34	228	60	70	

of comparison, the results recorded in Tables 3 and 4 may be condensed (Table 5), when it will be seen that over the first and second week after splenectomy there is an increased conjugation of menthol, and that by the fourth and fifth weeks the level of conjugation has returned to normal.

Table 5. *Extra glucuronic acid excreted in response to l-menthol in the rabbit. (Calculated as percentage of menthol fed)*

	Intact animal (%)	Av. 1st and 2nd weeks after splenectomy (%)	Av. 4th and 5th weeks after splenectomy (%)
Ether-soluble glucuronides	52.7	63.6	51.0
Total glucuronides	65.7	79.7	61.0
Difference	13.0	16.1	10.0

DISCUSSION

The figures given by Fishman (1940) for the glucuronidase content of animal (dog and mouse) organs are misleading since no account is taken of the size

splenic activity during an infection. According to the interpretation of Oshima (1936) and Fishman (1940) one would expect that the removal of the spleen would be followed by a decrease in the conjugation of menthol with glucuronic acid and since, in the rabbit, the spleen glucuronidase is very small compared with the liver glucuronidase, any alteration in amount of conjugation should be highly significant.

The figures recorded in Tables 3 and 4 show that in the two weeks following splenectomy there is first an increase in the normal output of urinary glucuronic acid and secondly an increase in the percentage conjugation of menthol. Both the increases in normal output of glucuronic acid and the percentage conjugation of menthol have declined again by the fourth and fifth week after splenectomy to the levels before splenectomy. These results are consistent with the view that the spleen plays some part in the breakdown of glucuronides in the body rather than with the view that the glucuronidase in all organs has a synthetic function. It is well known that after splenectomy certain functions of the spleen are taken on by other organs; this may also

apply to the destructive action of the spleen on glucuronides.

The figures recorded in Tables 3 and 4 bring out the interesting point that some of the glucuronide excreted in response to menthol is insoluble in ether. Since *l*-menthylglucuronide is easily soluble in ether, this may mean that a small proportion of the menthol may be transformed into a compound forming an ether-insoluble glucuronide. Possible transformations are either a dehydrogenation to a benzenoid compound containing a phenolic hydroxyl group, it being known that phenolic glucuronides are insoluble or only slightly soluble in ether, or an oxidation of either side chain with the formation of an aliphatic hydroxyl or carboxyl

group. These types of compound also yield glucuronides which may be insoluble or only poorly soluble in ether.

SUMMARY

1. Figures are presented for the glucuronidase content of certain organs of the rabbit and the rat.

2. In the rabbit the normal output of glucuronic acid and the percentage conjugation of *l*-menthol with glucuronic acid are both increased for the first two weeks after splenectomy and then fall back to the level for the intact animal by the fourth week. The function of spleen glucuronidase is discussed in the light of these results.

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The Uptake of Oxygen by Blood *in vitro*

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In some recent experiments (Ramsay, 1944b) it was observed that the oxygen capacity of fresh blood often appeared lower than the value obtained from the same blood after a treatment involving reduction with titanous citrate or tartrate. This confirmed similar results obtained by Ammundsen (1941), who used carbon monoxide instead of oxygen and treated the blood with sodium hyposulphite ($\text{Na}_2\text{S}_2\text{O}_4$) instead of a titanous salt. The present author, however, also observed that if the blood was allowed to stand in air, the apparent oxygen capacity increased until it approached the 'total' value obtained initially after reduction. This communication reports a somewhat fuller investigation of the phenomenon, which resembles that encountered by Kallner (1945), who found that the carbon monoxide capacity also increased during exposure of blood to

the air, but that the initial 'low' value was not observed at all if the pressure of carbon monoxide used for the saturation was 150 mm. instead of the 25 mm. originally recommended by Van Slyke & Hiller (1928).

METHODS

Specimens. Since blood from horses gave the most striking results in the earlier investigation, attention was concentrated on that species, but observations were made on other animals, principally sheep, cattle and pigs, and also man. In most of the animals blood was drawn from the external jugular vein into flasks containing oxalate, and suitable precautions were taken to prevent the access of air until the experiments were begun. Except in the case of the pig bloods, this was usually within 5–10 min., although occasionally the delay was reduced to 3 min. Tests showed, however, that a delay of up to at least 3 hr. did not seem to affect the general trend of the results.