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The Preparation and Properties of β-Glucuronidase

7. ACTION ON ESTER GLUCURONIDES

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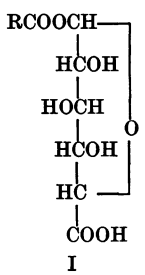
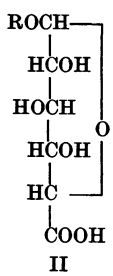
It has long been known that aromatic carboxylic acids can be conjugated with glucuronic acid in the animal body to form ester glucuronides (general formula, I; for review, see Williams, 1949): the best-known example is benzoyl glucuronide (I, R = C_6H_5), but the glucuronide of veratric acid (3:4-dimethoxybenzoic acid) is more easily prepared (Sammons & Williams, 1946). Interest in the ester glucuronides has recently been stimulated by the discovery that the branched-chain fatty acids, α-ethylbutyric acid and α-ethylhexanoic acid, can form derivatives of this type *in vivo* (Kamil, Smith & Williams, 1953). Unlike the ether glucuronides formed by alcohols and phenols (general formula, II), the ester glucuronides in general are unstable in alkaline solution and therefore give a positive reaction in tests for reducing sugars.

The biosynthetic glucuronides of alcohols and phenols are hydrolysed by the enzyme β-glucuronidase, but the action of this enzyme on ester glucuronides has not hitherto been studied, unless the synthetic compound β-glucuronic acid 1-phosphate (II, R = H_2PO_3; Levvy & Marsh, 1952), is regarded as a member of the group. At the suggestion of Professor R. T. Williams, specimens of α-ethylbutyryl, α-ethylhexanoyl and veratroyl glucuronides which he supplied were examined as possible substrates for mouse-liver β-glucuronidase.



I



II

EXPERIMENTAL AND RESULTS

Methods

In general, methods followed those used in earlier papers, in particular, Levvy & Marsh (1952) and Levvy (1954).

Inhibition of the hydrolysis of phenolphthalein glucuronide

α-Ethylbutyryl, α-ethylhexanoyl and veratroyl glucuronides were tested as competing substrates in the hydrolysis of phenolphthalein glucuronide by mouse-liver β-glucuronidase (Levvy & Marsh, 1952). As a test of enzyme specificity, this technique is less dependent on the purity of the enzyme preparation than direct measurements of hydrolysis. In the present instance, the difficulty to be avoided was the possible presence in the liver preparations of an esterase capable of hydrolysing ester glucuronides. To conserve supplies of the ester glucuronides, the microprocedure for enzyme assay described in connexion with baicalinase (Levvy, 1954) was adopted. Incubation was for 1 hr. at 37° and pH 5·2 in sodium acetate–acetic acid buffer, final concentration 0·125 N. The phenolphthalein glucuronide concentration ranged from 0·00125 to 0·000031 M.

All three ester glucuronides depressed the release of phenolphthalein and acted competitively, the percentage depression varying inversely with the phenolphthalein glucuronide concentration. Values for K_i, the dissociation constant for the complex formed between enzyme and competing compound, were determined by the method of Lineweaver & Burk (1934). The mean values obtained were: α-ethylbutyryl glucuronide, $7{\cdot}9 \times 10^{-4}$ M; α-ethylhexanoyl glucuronide, $2{\cdot}2 \times 10^{-4}$ M; veratroyl glucuronide, $3{\cdot}5 \times 10^{-5}$ M. The reciprocals of these figures give an approximate measure of the affinities of the respective ester glucuronides for the enzyme (Levvy & Marsh, 1954). For phenolphthalein glucuronide and mouse-liver β-glucuronidase, the

dissociation constant is $1{\cdot}3 \times 10^{-4}$M (Levvy, 1952). One should mention the corresponding figure for glucuronic acid, 16×10^{-4}M, since this product of glucuronide hydrolysis inhibits the enzyme (Spencer & Williams, 1951): the affinity, however, is too low for its presence to account for the effects of the ester glucuronides.

Hydrolysis of α-ethylhexanoyl glucuronide

It was found possible to extract α-ethylhexanoyl glucuronide from acidified aqueous solutions with ethyl acetate, and thus to follow its enzymic hydrolysis by measuring the glucuronic acid liberated, since this is not extracted. The procedure was similar to that used for following the hydrolysis of flavone glucuronides by mouse-liver β-glucuronidase (Levvy, 1954), terminating in the cerimetric estimation of reducing sugar (Levvy, 1946). α-Ethylhexanoic acid did not interfere in this estimation, but in any case it was extracted by ethyl acetate. The ester glucuronide in pure solution, incidentally, had 60% of the reducing power of an equivalent weight of free glucuronic acid for alkaline ferricyanide.

From the results shown in Table 1, it can be seen that the ester glucuronide was rapidly hydrolysed by a mouse-liver β-glucuronidase preparation, and that hydrolysis was inhibited by boiled saccharate solution (saccharo-1:4-lactone; see Levvy, 1952). In a concentration of $2{\cdot}5 \times 10^{-4}$M, saccharate caused 88% inhibition with $2{\cdot}5 \times 10^{-3}$M substrate. In another experiment, in which the saccharate concentration was doubled and the substrate concentration halved, there was no demonstrable liberation of reducing sugar in presence of the inhibitor. For purposes of comparison, Table 1 shows a parallel enzyme hydrolysis of menthol β-glucuronide. The lower affinity of this compound for the enzyme (dissociation constant 4×10^{-3}M; Fishman, 1939) as compared with α-ethylhexanoyl glucuronide is reflected in the more powerful effect of saccharate.

pH-*Optimum for α-ethylhexanoyl glucuronide*

The measurement of reducing material not extracted by ethyl acetate was applied to determinations of the pH-optimum for hydrolysis of α-ethylhexanoyl glucuronide by a mouse-liver β-glucuronidase preparation. At each point, the enzyme activity was determined in duplicate, and the usual controls were done for the effects of enzyme and substrate on the pH, and for the non-extractable reducing material present after incubation of each alone. Owing to the errors inherent in the method, individual results were not entirely satisfactory. Different experiments were, however, consistent in showing the presence of two peaks in the pH/activity curve, at pH 4·4 and pH 5·1–5·2 (Fig. 1). The curve as a whole thus resembles very closely those obtained for the hydrolysis of glucuronides of alcohols and phenols by β-glucuronidase, with optima at pH 4·5 and pH 5·0–5·2 (Mills, 1948; Kerr, Graham & Levvy, 1948).

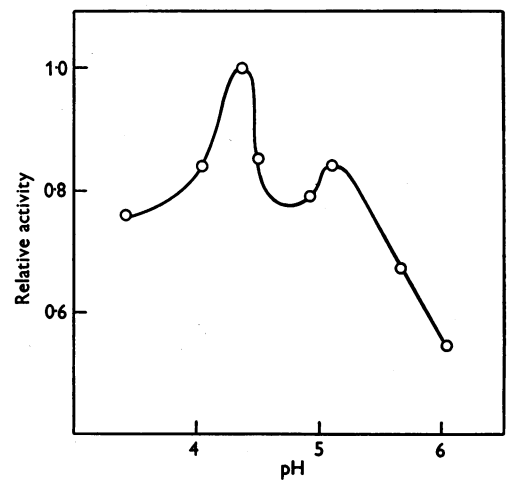


Fig. 1. Effect of pH on the hydrolysis of 0·0025M α-ethylhexanoyl glucuronide in 0·125N sodium acetate–acetic acid buffer after 2 hr. incubation at 37°.

Table 1. *Liberation of reducing sugar from α-ethylhexanoyl glucuronide by mouse-liver β-glucuronidase*

Hydrolysis mixture incubated for 2 hr. at 37° and pH 5·2 in sodium acetate–acetic acid buffer, final concentration 0·125N. Saccharate solution boiled for 30 min. before use.

Substrate	Inhibitor	Glucuronic acid liberated (μg.)*	Hydrolysis (%)	Inhibition (%)
0·0025M α-Ethylhexanoyl glucuronide	None	453	58	—
0·0025M α-Ethylhexanoyl glucuronide	0·00025M Saccharate	55	7	88
0·0025M α-Ethylhexanoyl glucuronide	0·00001M Saccharate	308	40	32
0·01M Menthol β-glucuronide	None	169†	5	—
0·01M Menthol β-glucuronide	0·00001M Saccharate	70†	2	59

* Total in incubation mixture (1·6 ml.).
† Enzyme preparation diluted to one-third.

β-Glucuronidase as an esterase

If β-glucuronidase hydrolysed ester glucuronides, the question arose as to whether it had any general properties as an esterase. A number of common esters were therefore examined as competing substrates for mouse-liver β-glucuronidase in the hydrolysis of 0·00063M phenolphthalein glucuronide in 0·125N-sodium acetate–acetic acid buffer, pH 5·2 (Levvy & Marsh, 1952). All the test compounds were in a final concentration of 1 mg./ml. None of them had any effect, i.e. if it is an esterase, β-glucuronidase is specific for compounds in which the alcohol radicle is glucuronic acid. The compounds tested were: ethyl acetate, methyl oleate, sodium β-glycerophosphate, glyceryl monoacetate, glyceryl diacetate, tributyrin, tricaproin, glyceryl monostearate and glyceryl monoricinoleate.

DISCUSSION

The ester glucuronides of α-ethylbutyric, α-ethylhexanoic and veratric acids had high affinities for mouse-liver β-glucuronidase: in the case of veratroyl glucuronide the affinity was higher than that of phenolphthalein glucuronide. α-Ethylhexanoyl glucuronide was rapidly hydrolysed by partially purified β-glucuronidase preparations, and the peaks on the pH/activity curve coincided with those for ether glucuronides. Saccharo-1:4-lactone caused almost complete inhibition of the hydrolysis of α-ethylhexanoyl glucuronide. From these experiments it may be concluded that β-glucuronidase hydrolyses ester glucuronides. Since the enzyme is specific for β-glucopyranuronides (Levvy & Marsh, 1952), this conclusion supports the generally accepted structure (I) for the ester glucuronides. β-Glucuronidase, however, did not appear to possess general esterase properties.

Evidence for the unity of a group-specific enzyme can never be anything else but circumstantial, subject always to the possibility that the enzyme may be physically separated into components with different specificities. While the ester glucuronides behaved in every respect like any other substrate for β-glucuronidase, one must admit that the experimental findings are open to an alternative explanation. Hydrolysis of the ester glucuronides could have been partially or entirely due to a second enzyme in the β-glucuronidase preparations if the following three assumptions are made: (i) that the pH/activity curve resembles that for β-glucuronidase, (ii) that the second enzyme is powerfully inhibited by saccharo-1:4-lactone, (iii) that ester glucuronides are competitive inhibitors in the general sense for β-glucuronidase. On such assumptions as these, however, a distinct enzyme could be postulated for the hydrolysis of every individual glucuronide.

SUMMARY

1. The ester glucuronides of α-ethylbutyric, α-ethylhexanoic and veratric acids had high affinities for mouse-liver β-glucuronidase, as measured by their effects in depressing the hydrolysis of phenolphthalein glucuronide by the enzyme.

2. α-Ethylhexanoyl glucuronide was rapidly hydrolysed by β-glucuronidase preparations. The pH/activity curve was similar to those for ether glucuronides, and hydrolysis was inhibited by saccharo-1:4-lactone.

3. β-Glucuronidase did not appear to possess general esterase properties.

4. On the basis of these findings it was concluded that ester glucuronides are substrates for β-glucuronidase.

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