indicated that enzyme systems exist in animal tissues which are capable of synthesizing phospholipids by such a process (Kornberg & Pricer, 1953; Kennedy, 1954; Dawson, 1954*a*). Ansell & Norman (1953) found that rat-brain GPE had a higher specific radioactivity than phosphatidylethanolamine at various times between 66 and 1455 min. after the injection of labelled phosphate. They concluded, however, that the magnitude of this difference was too small to allow GPE to act as a major precursor of cerebral phosphatidylethanolamine.

The relationships between the specific radioactivities of GPC and phosphatidylcholine and GPE and phosphatidylethanolamine indicate that these water-soluble phosphate esters exist in liver tissue solely as intermediaries in the catabolism of the phospholipids. An enzyme system capable of removing two fatty acids from phosphatidylcholine and phosphatidylethanolamine has not so far been reported in liver. Lecithinase *A* which removes a single unsaturated fatty acid has been found in rabbit liver (Francioli, 1934). Contardi & Ercoli (1935) obtained evidence that a compound which was probably GPC was formed on prolonged incubation of rice-bran extracts with lysolecithin. Schmidt *et al.* (1945) isolated GPC from autolysing ox pancreas, while Hayaishi & Kornberg (1954) recently described the accumulation of GPC when heat-treated extracts of *Serratia plymuthicum* were incubated with phosphatidylcholine.

SUMMARY

1. The specific radioactivities of the free glycerylphosphorylcholine and glycerylphosphorylethanolamine in rat liver have been measured by isolating them chromatographically at various times after the injection of labelled phosphate.

2. A comparison of their specific radioactivities with those of liver phosphatidylethanolamine and phosphatidylcholine has suggested that they cannot act as major isotopic precursors of these phospholipids.

3. Liver phosphatidylcholine obeys the correct radioactive criteria for being the precursor of glycerylphosphorylcholine, while similarly liver phosphatidylethanolamine fulfils the isotopic conditions necessary for being the precursor of glycerylphosphorylethanolamine.

The author is indebted to Sir Rudolph Peters, F.R.S., for his valuable advice.

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The Sulphatase of Ox Liver

4. A NOTE ON THE INHIBITION OF SULPHATASES *A* AND *B*

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(*Received* 8 *June* 1954)

The literature contains many conflicting reports on the influence of electrolytes on the activity of sulphatases, the early work being particularly difficult to interpret, as in many cases clear distinctions were not drawn between the enzymes obtained from different sources, such as mammalian liver, molluscan viscera, and *Aspergillus oryzae*. Recent work on mammalian liver sulphatase has also been unsatisfactory, as in all cases except that of previous work from this laboratory the enzymes used were unpurified preparations containing a number of different sulphatases, or even homogenates of tissues which had been treated with histological fixatives (Seligman, Chauncey & Nachlas, 1951). The present situation is extremely confused: sodium chloride has been reported to inhibit sul-

phatase in tissue sections, but to be without action on homogenates (Rutenburg, Cohen & Seligman, 1952) yet potassium chloride has been claimed (Dodgson, Spencer & Thomas, 1954*a*) to activate the sulphatase in homogenates. Magnesium chloride has similarly been claimed both as an inhibitor (Hommerberg, 1931) and an activator (Seligman *et al.* 1951). It has previously been shown that both sulphatases *A* and *B* are inhibited by sulphate ions (Roy, 1953*b*, 1954*a*), yet both Seligman *et al.* (1951) and Dodgson *et al.* (1954*a*) report the activation of crude sulphatase preparations by sulphate ions. This effect would appear very improbable on theoretical grounds alone.

The present paper describes an investigation of the effect of electrolytes on both crude ox-liver sulphatase and partly purified preparations of sulphatases *A* and *B*. Various other possible enzyme inhibitors have also been studied, and the results are reported below.

EXPERIMENTAL

The general experimental methods have already been described (Roy, 1953*a*, *b*, 1954*a*), the enzymic activity being followed by the colorimetric estimation of the 4-nitrocatechol liberated on hydrolysis of dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate). Sulphatases *A* and *B* were prepared as before, and in the present investigation were purified to stages *A*-3 and *B*-2 respectively.

The 5% (w/v) dispersion of ox liver in water used as the source of unfractionated enzyme was prepared by treating the tissue for 30 sec. in an Atomix blender at room temperature. The liver was used as soon as possible (usually about 1 hr.) after the slaughter of the animal.

Both in the case of the unfractionated enzyme and of sulphatases *A* and *B* an enzyme concentration was chosen such that approximately 100 μg. nitrocatechol were liberated under the experimental conditions described below.

Sulphatase *A* activity was estimated at pH 5·0 and a substrate concentration of 0·003 M nitrocatechol sulphate, and sulphatase *B* at pH 5·9 and 0·06 M nitrocatechol sulphate. The buffers used were sodium acetate, final concentration 0·15 M. The electrolytes were dissolved in the appropriate buffer and mixed with the substrate before the addition of the enzyme, so that the enzyme was treated simultaneously with the substrate and the electrolyte under investigation. Routine adjustments of pH were made with N acetic acid, not HCl as in previous work, since chloride ions have been found to have an influence on the activity of sulphatase *B*.

Since some of the other types of inhibitor were known to react only slowly with proteins, the buffered enzyme (0·25 M acetate, pH 5·0 and 5·9 for sulphatases *A* and *B* respectively) was treated with the appropriate concentration of inhibitor for 1 hr. at room temperature; then the inhibitor–enzyme solution was mixed with the appropriate substrate solution. Attempts to reverse the inhibition were carried out by adding the appropriate reagent to the enzyme–inhibitor mixture 10 min. before adding the substrate.

RESULTS

Influence of electrolytes

Unfractionated enzyme. Fig. 1 shows the effect of 0·025 M sodium sulphate and 0·08 M potassium chloride on the sulphatase activity of a 5% (w/v) dispersion of ox liver. In confirmation of the results of Dodgson *et al.* (1954*a*) it is obvious that at certain pH values both these compounds considerably enhanced the sulphatase activity of such a preparation. At other pH values an inhibition was noted in both cases. This effect was not shown by 0·001 M sodium sulphite, which inhibited strongly at all pH values. As these results were at variance with those previously reported (Roy, 1953*b*, 1954*a*) an investigation of the action of electrolytes on the activity of sulphatases *A* and *B* was undertaken.

Dodgson, Spencer & Thomas (1953), in their study of unfractionated rat-liver sulphatase, reported that the use of 0·2 M phosphate buffers in place of 0·5 M acetate buffers caused a shift in the pH optimum for the hydrolysis of *p*-acetylphenyl sulphate from pH 7·0 to 7·75. When phosphate buffers were used in the present study of the unfractionated ox enzymes no activity could be detected between pH 6·5 and 8·0. This discrepancy is resolved by the fact that the assay method used by Dodgson *et al.* (1953) estimates only the insoluble or microsomal enzyme (Dodgson, Spencer & Thomas, 1954*b*) which is not inhibited by phosphate (Dodgson & Spencer, private communication) and does not estimate the soluble enzymes derived from mitochondria (Dodgson *et al.* 1954*b*) which are inhibited by phosphate and which are essentially the enzymes assayed in the methods used in this

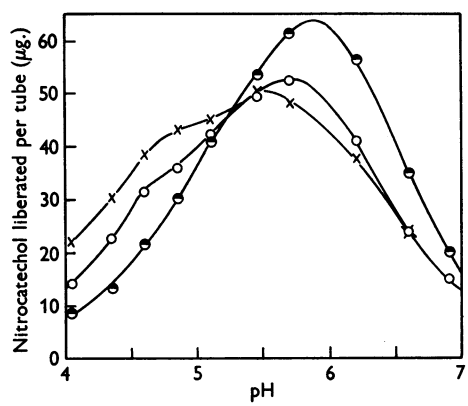

Fig. 1. Effect of electrolytes on the sulphatase activity of an untreated dispersion of ox liver. Assays in 0·15 M acetate buffers, substrate concentration 0·03 M nitrocatechol sulphate, total volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme solution. Incubated 1 hr. at 37°. ○—○, control; ●—●, 0·025 M-Na_2SO_4; ×—×, 0·08 M-KCl.

laboratory (Dodgson *et al.* 1954*b*; Roy, 1954*b*). Although this microsomal enzyme occurs in ox liver (Dodgson *et al.* 1954*b*) the amounts present would be too small to be detected by the present method so that no activity would be expected in the presence of phosphate. It is now proposed to refer to this microsomal sulphatase as sulphatase *C* until it is possible to name the various sulphatase fractions systematically: this decision has been made in agreement with Drs Dodgson and Spencer. The different effect of phosphate obtained in the two laboratories is therefore due to the use of different substrates and to the substrate specificity of the sulphatases (Dodgson *et al.* 1954*b*).

Sulphatase A. The effect of various electrolytes on the activity of sulphatase *A* is shown in Fig. 2. It should be noted that in these experiments a high concentration of enzyme was used so that the pH optimum was approximately pH 5·1 (Roy, 1953*b*). The most striking effect is obviously that of sulphate ions: in their presence the pH optimum is shifted from pH 5·1 to 6·2. Because of this shift in the position of the optimum there is an apparent activation of sulphatase *A* by sulphate ions on the alkaline side of the pH optimum of the untreated enzyme. It must be stressed that this phenomenon is not an activation in the true sense, as the activity of the sulphate-treated enzyme at the appropriate pH optimum is always less than that of the untreated enzyme under its optimal conditions (Fig. 2).

No comparable effect was noted with any of the other ions studied, suggesting that the mechanism of inhibition by sulphate is different from that of the other anions shown in Fig. 2. It is of interest that 0·08 M potassium chloride causes a slight (10 %) inhibition of sulphatase *A* at the pH optimum and on the alkaline side thereof: 0·005 M magnesium acetate caused a 10 % activation throughout the entire pH range studied. Previously reported experiments using high concentrations of magnesium chloride were presumably complicated by the simultaneous and opposing effects of magnesium and chloride ions.

Sulphatase B. In this case no comparable activation by sulphate ions was noted (Fig. 3), but chloride ions had a definite activating effect. It was reported previously (Roy, 1954*a*) that the pH optimum of sulphatase *B* was pH 5·7–5·8; these experiments were unfortunately carried out in the presence of variable amounts of chloride, and it has now been found that in the absence of chloride ions the pH optimum is in the region of pH 6·0 (Fig. 3). It can be shown by the use of chloride-free substrate solutions that chloride ions cause a pronounced activation on the acid side of the pH optimum with the net result of a broadening of the pH optimum into the acid region.

The influence of a number of other ions on sulphatase *B* activity is also indicated in Fig. 3. In contrast to sulphatase *A*, sulphatase *B* was uninfluenced by 0·005 M magnesium acetate.

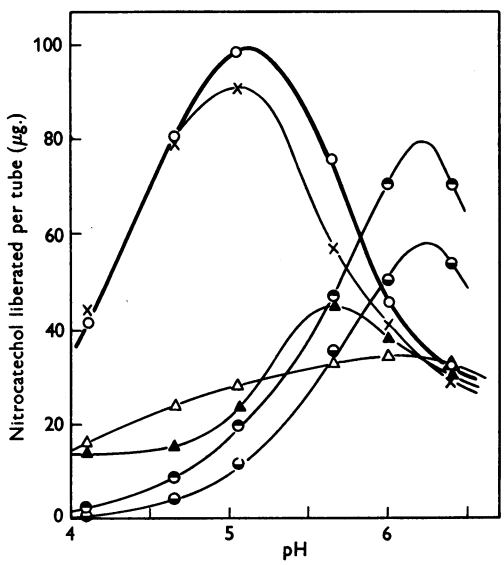

Fig. 2. Effect of electrolytes on the activity of purified sulphatase *A*. Conditions as in Fig. 1, except that the substrate concentration was 0·003 M nitrocatechol sulphate. ○—○, control; ×—×, 0·08 M-KCl; △—△, 0·0005 M-NaH_2PO_4; ▲—▲, 0·00001 M-Na_2SO_3; ◒—◒, 0·025 M-Na_2SO_4 or K_2SO_4; ◒—◒, 0·05 M-Na_2SO_4.

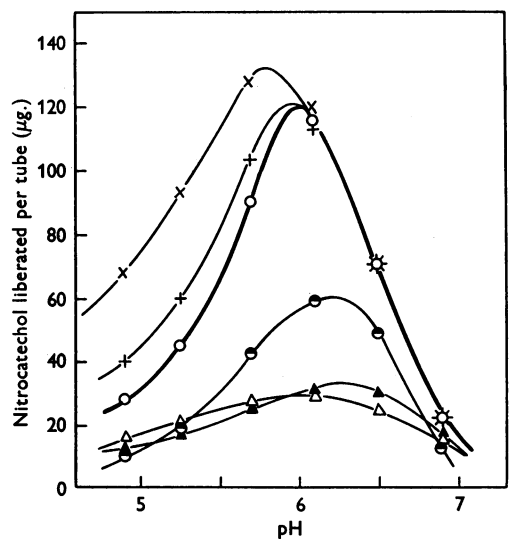

Fig. 3. Effect of electrolytes on the activity of purified sulphatase *B*. Conditions as in Fig. 1, except that the substrate concentration was 0·05 M nitrocatechol sulphate. ○—○, control; ×—×, 0·08 M-KCl; +—+, 0·01 M-KCl; △—△, 0·0025 M-NaH_2PO_4; ▲—▲, 0·0001 M-Na_2SO_3; ◒—◒, 0·1 M-Na_2SO_4.

Table 1. *The action of sulphydryl compounds and of* SH *reagents on sulphatases* A *and* B

Enzyme solutions treated for 1 hr. at room temp. with the inhibitors in 0·25 M acetate buffers, pH 5·0 and 5·9 for sulphatases *A* and *B* respectively. Reversal of inhibition by SH reagents attempted by addition of sulphydryl compound 10 min. before addition of the substrate.

Results of two experiments given, each being the mean of duplicate estimations, with an accuracy of ±2%.

Reagent	Concn. (M)	Activity (relative to control = 1)			
		Sulphatase *A*		Sulphatase *B*	
Iodoacetate	10^{-2}	0·27	0·30	0·05	—
p-Chloromercuribenzoate	10^{-4}	0·31	0·33	1·09	1·07
Cysteine	5×10^{-3}	1·01	1·08	1·25	1·19
Glutathione	5×10^{-3}	0·91	0·88	1·02	0·97
p-Chloromercuribenzoate 10^{-4} + cysteine 5×10^{-3}		0·98	1·05	1·25	1·19
p-Chloromercuribenzoate 10^{-4} + glutathione 5×10^{-3}		0·73	0·81	1·01	0·99

Miscellaneous inhibitors

There has been no report in the literature of any compound which might be regarded as a specific inhibitor of mammalian sulphatases, nor indeed of any sulphatase, except for the report by Robinson, Smith, Spencer & Williams (1952) that taka-sulphatase is inhibited by potassium 4-hydroxy-3-nitrophenyl sulphate. This ester is, however, hydrolysed by both sulphatases *A* and *B*. Torda (1943) claimed that the sulphatase of cat muscle was inhibited by 5% (w/v) cocaine and activated by 5% (w/v) yohimbine.

No result of any interest was obtained in the study of various alkaloids on sulphatases *A* and *B*, neither of which was affected by yohimbine (10^{-2} M), eserine (10^{-3} M), nor the methiodide of *m*-dimethylaminophenyl methyl carbamate (10^{-3} M). Cocaine (10^{-2} M) had likewise no effect on sulphatase *B*, but slightly inhibited sulphatase *A*; higher concentrations of cocaine strongly inhibited sulphatase *A*, but this effect would seem to be of no significance.

The effect of SH reagents is of some interest, and the results are shown in Table 1. Sulphatase *A* was strongly inhibited by iodoacetate and *p*-chloromercuribenzoate: the inhibition of the latter was reversed partially by glutathione and completely by cysteine. Cysteine alone had no effect on sulphatase *A*, but glutathione inhibited slightly. Some specimens of glutathione were very powerful inhibitors of sulphatase *A*: this effect varied from specimen to specimen, although all were more than 90% reduced, as judged by iodine titration, and were free from copper. It is likely that the inhibition was due to decomposition products, as the most inhibitory samples had a pronounced sulphurous smell. Sulphatase *B* was likewise inhibited by iodoacetate, but was slightly activated by *p*-chloromercuribenzoate; a sample of glutathione giving a 10% inhibition of sulphatase *A* was without action on sulphatase *B*, while cysteine activated strongly. The activation by *p*-chloromercuribenzoate was unexpected, as the other results in Table 1 would suggest that sulphatase *B*, like sulphatase *A*, was an SH enzyme. The explanation may be that the *p*-chloromercuribenzoate inhibited some agent in the enzyme preparation which tended to inactivate the sulphatase. In this connexion it should be noted that the available preparations of sulphatase *B* are probably much less pure than those of sulphatase *A*, the protein concentration in the reaction mixture being approximately 250 μg./ml. in the case of *B* and 20 μg./ml. in *A*.

The results, however, indicate that sulphatase *A* is an SH enzyme and are not incompatible with the view that sulphatase *B* is also an SH enzyme, although they do not prove the latter point.

DISCUSSION

The results resolve many of the conflicting reports in the literature and stress the need for using at least partly purified preparations in the study of enzyme inhibition. The activation of the sulphatase of a whole dispersion of ox liver by both sulphate and chloride ions under the appropriate conditions is further evidence for the view that such a preparation contains both sulphatases *A* and *B* which are therefore not artifacts.

In the case of the purified enzymes, one of the most interesting effects is the very powerful inhibition of sulphatases *A* and *B* by sulphite ions, the affinity of both enzymes for sulphite being approximately 100 times their affinity for sulphate and for nitrocatechol sulphate (Roy, 1954*a*). This perhaps reaches the extreme case in taka-sulphatase which, although strongly inhibited by sulphite (Robinson *et al.* 1952) is unaffected by sulphate. No explanation of this can be offered, but it is unlikely that the effect is due simply to the reducing power of sulphite ions, as cysteine is not an inhibitor. Neither does it seem probable, owing to the reversible nature of the inhibition by sulphite, that it is caused by the sulphite reacting with an aldehyde

grouping in the enzyme, as postulated by Rosenfeld & Ruchelman (1940) in the case of taka-sulphatase. The striking shift in the pH optimum of sulphatase *A* in the presence of sulphate is very interesting: it would seem that this must be due to combination of the sulphate with the enzyme, probably the active centre, as the inhibition of sulphatase *A* by sulphate is competitive (Roy, 1953*b*). This might be taken as further evidence that sulphatase *A* contains a positively charged grouping in its active centre (Roy, 1954*a*).

The general problem of the function of these enzymes remains obscure. The possibility that sulphatase might play a part in the *in vivo* synthesis of sulphuric acid esters by acting as a transferase with the 'activated sulphate' of Bernstein & McGilvery (1952) as substrate has been considered briefly by Dodgson *et al.* (1954*a*). The possibility of the occurrence of this reaction *in vivo* cannot be assessed, but it is clear that it could not be occurring in the *in vitro* experiments of Bernstein *et al.* (1952), as these workers used isotonic phosphate buffers, pH 6·5, under which conditions both sulphatases *A* and *B* are completely inactive, and sulphatase *C* only slightly active. It is likewise impossible to assess the possible hydrolytic activities of sulphatases *in vivo* as, according to Gamble (1947), the intracellular fluid contains 100 m-equiv./l. of phosphate, mostly organic. *In vitro* experiments indicate that sulphatases *A* and *B* would be strongly inhibited in such a medium, both enzymes being inhibited by phosphate esters. It is not possible to give quantitative figures owing to the difficulty of obtaining such esters completely free from inorganic phosphate, a very powerful inhibitor.

The multiplicity of sulphatases (Roy, 1953*a*; Dodgson *et al.* 1954*b*) and their widespread distribution throughout animal tissues (Rutenburg *et al.* 1952) might be taken to indicate that these enzymes are fulfilling some important metabolic role, Haldane (1954) even suggesting that such a multiplicity of enzymes might be an evolutionary adaptation of some advantage to the organism concerned. No such metabolic function is as yet obvious, the bulk of the evidence indicating a surprising inertness of sulphatases *in vivo* (Hawkins & Young, 1954). This situation invites comparison with that of β-glucuronidase which also exhibits this multiplicity (Mills, Paul & Smith, 1953) and apparent inertness *in vivo* (Garton & Williams, 1949). It may well be that both groups of enzymes play related roles in the metabolism of mucopolysaccharides, as suggested by the work of Meyer, Linker & Rapport (1951) for β-glucuronidase, and that their respective activities *in vivo* are only distantly related to the commonly used assay methods. In the case of sulphatases at least this would be borne out by the peculiar affinity of these enzymes for highly unphysiological substrates.

SUMMARY

1. Unfractionated preparations of ox-liver sulphatase are activated both by chloride and sulphate ions.

2. Sulphatase *A* is activated by sulphate ions on the alkaline side of the pH optimum owing to a shift of the position of the pH optimum in the presence of sulphate ions. A slight inhibition of sulphatase *A* is brought about by chloride ions at and above the pH optimum.

3. Sulphatase *B* is activated by chloride ions on the acid side of the pH optimum.

4. The effects of various other anions on sulphatases *A* and *B* are given.

5. Sulphatases *A* and *B* are uninfluenced by sodium and potassium ions; magnesium ions are without action on sulphatase *B*, but activate sulphatase *A* to a small extent.

6. Sulphatase *A* is shown to be an SH enzyme. In the case of sulphatase *B* the evidence is conflicting.

7. The significance of the influence of ions on sulphatases is discussed in relation to the possible activity of these enzymes *in vivo*.

The author is grateful to Dr and Mrs Stedman for generous gifts of alkaloids.

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