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The Enzymic Hydrolysis of Acid Anhydrides

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According to recent investigations (Burgen, 1949; Wilson & Bergmann, 1950) tetraethyl pyrophosphate (TEPP) and related inhibitors of esterases are enzymically split into a phosphonium ion, which combines with the nucleophilic group in the active surface, and a negative ion, e.g. the diethyl phosphate ion. The combination 'enzyme-P(OR)₂' is

thus analogous to the complex 'enzyme-C—R',

which we assumed as intermediate in the hydrolysis of esters (Bergmann, Wilson & Nachmansohn, 1950). The main difference between the two combinations is that the enzyme-acyl complex is spontaneously split by the solvent, regenerating the active form of the enzyme, whereas the enzyme-phosphonium compound is stable, at least for the duration of a laboratory experiment.

The superiority of TEPP over trialkyl phosphates in the inhibition of esterases (Aldridge & Davison, 1952) shows that the anhydride linkage is split faster by esterases than the ester group in derivatives of phosphoric acid. It should therefore be expected that anhydrides of carboxylic acids will also be easily attacked by these enzymes. The experiments described in the present investigation substantiate this prediction.

MATERIALS AND METHODS

Substrates and inhibitors. The fatty acid anhydrides were commercial samples and were purified by fractionation *in vacuo*. Dibenzyl acetyl phosphate was prepared essentially as described by Bentley (1948), but the reaction with keten was carried out in the absence of solvent, the liquid end product serving as solvent for dibenzyl hydrogen phosphate. Tetrabenzyl pyrophosphate was synthesized by the method of Kenner, Todd & Weymouth (1952).

Enzyme preparations. Liver esterase was prepared from dog's liver by ammonium sulphate fractionation, as described earlier (Bergmann & Shimoni, 1952). The yellow solution, obtained after two subfractionations, contained 16.6 mg. protein/ml., as determined by precipitation with 20% (w/v) trichloroacetic acid. When diluted 1:100 and with diacetin 0.43 M as substrate, this enzyme liberated 5.3 μ moles CO₂/ml./hr.

Acetylcholine esterase was prepared from the electric organ of *Torpedo marmorata*. The final preparation, when diluted 1:40 000 and with acetylcholine 3.3 $\times 10^{-3}$ M as substrate, hydrolysed 6 μ moles/ml./hr. For some of our experiments the enzyme from *Electrophorus electricus* was used, which in a dilution of 1:1200 hydrolysed 6.6 μ moles acetylcholine/ml./hr.

Manometric methods. The buffer used as medium for these experiments contained 0.1 M-NaCl, 0.026 M-NaHCO₃ and 0.04 M-MgCl₂. The pH was adjusted to 7.3. The gas phase consisted of 95% air and 5% CO₂. In the experiments with toluene as the organic phase, the solution of anhydride in this solvent (0.1–0.3 ml.) was placed in the side bulb of a

Warburg vessel and tipped into the main compartment at zero time. For a rapid attainment of constant manometer readings before mixing, it was found advantageous to add a small quantity (0.02 ml.) of toluene to the main vessel. The anhydrides were finally used in the following dilutions: acetic 1:100, propionic 1:100 and butyric 1:10. The relatively high concentration of the last compound was possible because butyric anhydride undergoes only slow spontaneous hydrolysis when its solution in toluene is brought into contact with bicarbonate buffer. In every case care was taken that the amount of buffer was in excess of that required to neutralize the total amount of acid to be expected after complete hydrolysis of substrate, and the pH of the mixtures was checked at the end of the experiments.

A certain difficulty in quantitative evaluation arises from the fact that the anhydride concentration in the organic phase decreases faster in the presence of enzyme than in the control, i.e. the absolute concentrations, which are equilibrated between the two layers, are equal only at zero time. This, however, makes the positive results obtained in our experiments the more significant. It usually takes a few minutes before equilibrium is established. Therefore it is sometimes observed that enzymic hydrolysis increases during the initial period.

In view of the instability of the carboxylic acid anhydrides in buffer of pH 7.3, it was not feasible to measure the distribution coefficient for the system toluene:water. The amount present in the aqueous phase at a given moment is thus unknown, and no quantitative comparison between different substrates can be applied.

Colorimetric methods. For the colorimetric determination of choline esters the method of Hestrin (1949) was used. Since, in spite of precautions, the samples taken from the two-phase reaction system always contained a small amount of toluene, it was found necessary to gas the solution with air for 1 min. before the addition of hydroxylamine, in order to transfer all anhydride into the aqueous layer and to cause its hydrolysis.

RESULTS

Enzymic hydrolysis of carboxylic acid anhydrides. Enzymic hydrolysis could not be measured when acetic or propionic anhydride was added directly to the aqueous phase, since the spontaneous decomposition of the substrate was too rapid. We therefore devised a new technique, in which the anhydride is dissolved in toluene and delivered gradually to the aqueous phase. In this two-phase technique spontaneous hydrolysis is proceeding at a relatively low rate. Even this improvement did not enable us to demonstrate unequivocally the enzymic splitting of acetic anhydride, but in the case of the higher homologues clear evidence for enzymic catalysis was obtained (Figs. 1 and 2). Although a strictly quantitative comparison is impossible, our results indicate that the order of reactivity is probably analogous to that of the corresponding esters. For example, butyric anhydride is not attacked by cholinesterase, but is a good substrate for liver esterase. It may also be mentioned that cholinesterase does not attack an aqueous solution of ethyl propionate when the substrate concentration

is equal to that which would be obtained from the corresponding anhydride if the latter could pass quantitatively into the aqueous phase. For acetylcholinesterase, propionic anhydride is clearly a better substrate than ethyl propionate.

Fig. 1. Enzymic hydrolysis of propionic anhydride at 23°. The anhydride was dissolved in toluene, 1:100, and 0.2 ml. of this solution put into the side bulb of a Warburg vessel. The thermobarometer contained toluene only. The main compartment received either buffer alone, or a buffered enzyme solution, 2.9 ml. x—x, spontaneous hydrolysis; ●—●, hydrolysis by *Torpedo* esterase, 1:2500; ⊔—⊔, hydrolysis by liver esterase, 1:60.

Fig. 2. Enzymic hydrolysis of butyric anhydride at 23°. The anhydride was dissolved in toluene, 1:10, and 0.3 ml. of this solution put into the side bulb, whereas the main compartment contained 2.8 ml. of buffer or buffered enzyme. x—x, spontaneous hydrolysis; ●—●, hydrolysis by *Torpedo* esterase, 1:2500; ⊔—⊔, hydrolysis by liver esterase, 1:60.

Enzymic synthesis of choline esters from anhydrides. It was shown first by Hestrin (1950) that cholinesterase is able to synthesize esters from choline and fatty acids, and similar experiments on the system choline-fatty acid ester have been carried out by Wilson, Bergmann & Nachmansohn (1950). Applying our two-phase technique, and using the colorimetric method of analysis, we could demonstrate enzymic synthesis of propionyl- and butyryl-

choline from the respective anhydrides (Figs. 3 and 4). Comparison of the rates of ester synthesis from propionic anhydride and propionic acid shows that the former substrate reacts much faster, thus excluding the possibility that the anhydride is hydrolysed before condensation with choline. Thus Hestrin (1950) used both choline and propionate in about 1.5M solution, and acetylcholinesterase in a concentration sufficient to hydrolyse 2 g. acetylcholine/ml./hr. under optimum conditions. He obtained, however, not more than about 0.1 μ mole propionylcholine/ml./hr. In the experiment of Fig. 3 we worked with an enzyme concentration which hydrolysed 27 mg. acetylcholine/ml./hr.; using 0.15M choline, we observed the formation of 8 μ moles of propionylcholine/ml./hr. This is evidence that the enzyme catalyses directly the condensation of the anhydride with choline.

Fig. 3. Enzymic synthesis of propionylcholine. The aqueous phase of each Erlenmeyer flask, 8 ml., contained *Torpedo* esterase, 1:1600, and choline, 0.15M, in bicarbonate buffer (see under methods). Propionic anhydride was dissolved in toluene, 1:50, and 4 ml. of this dilution were shaken with the aqueous layer at 23°. Controls were run with choline and toluene, and with choline and the substrate solution, but without enzyme. All experiments were carried out in duplicate. Samples of 0.5 ml. were withdrawn from the aqueous layer after 5, 10, 15 and 20 min. They were freed from small amounts of toluene by bubbling air for 1 min. and diluted with water to 1 ml. Then 2 ml. of Hestrin's (1949) hydroxylamine mixture were added, and, after 1 min. incubation, the solution was acidified with 1 ml. of 3.5N-HCl and the coloured complex was developed with 1 ml. of 0.74M-FeCl₃, dissolved in 0.1N-HCl. Readings were taken with a Klett-Summerson photoelectric colorimeter, using filter no. 54. x—x, control; o—o, enzymic reaction; Δ—Δ, difference between enzymic and spontaneous reaction.

Fig. 4. Enzymic synthesis of butyrylcholine. Conditions as for Fig. 3. Butyric anhydride was dissolved in toluene 1:10. Samples were withdrawn after 25, 50, 77, 100 and 140 min. x—x, control; o—o, enzymic reaction; Δ—Δ, difference between enzymic and non-enzymic esterification.

The behaviour of a mixed anhydride towards cholinesterase. On the basis of these results it could be predicted that a mixed phosphoric-carboxylic anhydride would also be split by esterases. This type of substrate appeared of special interest since, depending on the preferred direction of hydrolysis, it could serve either as substrate or as inhibitor. We found that dibenzyl acetyl phosphate, at a concentration of 4×10^{-5} M, produced 50% inhibition of acetylcholinesterase, and that this effect was irreversible. However, when the spontaneous hydrolysis of the mixed anhydride was followed by the method of Lipmann & Tuttle (1945), it was found that after complete hydrolysis in Warburg buffer at pH 7.3 (about 20 min.) the inhibitory effect was practically unaltered, indicating that a second compound with a lower rate of hydrolysis may be responsible for the inhibition. The observations of Sheehan & Frank (1950) on related anhydrides suggest that this compound is probably tetrabenzyl pyrophosphate, formed by disproportionation of dibenzyl acetyl phosphate upon standing. Indeed, tetrabenzyl pyrophosphate is

several hundred times more effective than the mixed anhydride, its I_{50} value being about 1×10^{-7} M. A rather small degree of disproportionation would therefore be sufficient to explain our results. Therefore we cannot sustain the claim made by us in a preliminary communication (Bergmann, Wurzel & Shimoni, 1953) that dibenzyl acetyl phosphate is itself an inhibitor of esterases.

DISCUSSION

Our experiments support the view that various derivatives of phosphoric and carboxylic acids are treated principally in the same way by esterases. The demonstration of enzymic hydrolysis of carboxylic acid anhydrides met with great difficulties until the two-phase technique was introduced. It has been claimed recently (Wilson, 1952) that enzymic splitting of acetic anhydride could be demonstrated in the aqueous phase by sampling up to 30 sec. after addition of the substrate. All our own attempts during the last 2 years to measure the hydrolysis of anhydrides by the direct method have failed.

The hydrolysis of carboxylic acid anhydrides by specific and unspecific esterases supports the common mechanism of enzymic ester hydrolysis, which we proposed earlier (Bergmann *et al.* 1950),

namely that the electrophilic carbon of the acylium part (RCO^+) of anhydride or ester combines with the nucleophilic group of the active surface, while the rest of the molecule is removed by combination with a hydrogen atom (probably derived from the active surface). Any compound capable of acylating the enzyme should therefore serve as substrate, e.g. acid chlorides. This question is now under investigation.

SUMMARY

1. Enzymic hydrolysis of carboxylic acid anhydrides can be demonstrated by a two-phase technique, whereby a toluene solution of the substrate is shaken with the aqueous enzyme phase.

2. Acetylcholinesterase catalyses the esterification of choline with carboxylic acid anhydrides. This was shown by applying the two-phase technique, and measuring colorimetrically the amount of ester formed.

3. These results give additional evidence for the mechanism of ester hydrolysis by esterases: the enzyme is acylated and this intermediate is split by the solvent.

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