LXXVII.—*The Enzymic Synthesis of β-Hydroxyethyl Dihydrogen Phosphate.*

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As yet, the number of cases in which the reversible activity of an enzyme * derived from plant or animal tissues has been clearly demonstrated *in vitro* by the isolation in a pure form of the synthesised product of the reaction, is small. The demonstration of such synthetic activity has considerable importance both with regard to the theory of catalysis and in connexion with the rôle played by the particular enzyme in the life processes of the tissue from which it is derived.

Using the very active phosphoric esterase of the intestinal mucosa as a catalyst, I have shown (*Biochem. J.*, 1928, **22**, 855) that sodium glycerophosphate may be readily synthesised at 37° from sodium phosphate and glycerol if the concentration of water in the reaction mixture is kept sufficiently low. The synthesised ester may be conveniently isolated as the alcohol-insoluble barium salt.

The production, by the same esterase, of a phosphoric ester from ethylene glycol will now be described, and details with regard to its composition and properties will be given which show its identity with the hydroxyethyl phosphoric ester derived from the product of the direct combination between ethylene chlorohydrin and phosphoryl chloride (Plimmer and Burch, this vol., p. 284).

EXPERIMENTAL.

Commercial ethylene glycol was twice distilled, the portion of b. p. 193—195° (uncorr.) being used. An extract of the duodenal mucosa of a cat was prepared by carefully washing out the intestine with water, scraping off the mucosa of the duodenum, and grinding

* The first definitely proved case of enzymic synthesis was recorded by Croft Hill (J., 1898, **73**, 634).

a weighed quantity of the wet tissue with washed sand and water containing a little chloroform. The extract was made up to twenty times the original weight of mucosa with water.

Ten g. of disodium hydrogen phosphate crystals were dissolved in 75 c.c. of this extract, and 200 c.c. of the redistilled glycol added. A sample was taken at once from the slightly alkaline reaction mixture, and inorganic and total phosphorus were determined in it. The determinations were repeated at intervals (Table I).

TABLE I.

Rate of synthesis of the ester.

	Mg. of P per c.c. of reaction mixture.		
Time (days).	Inorganic P.	Total P.	Combined (ester) P.
0	3·27	3·31	0·04
3	2·55	3·30	0·75
6	2·38	3·31	0·93
10	2·22	—	1·09
15	2·13	—	1·18
17	2·08	3·31	1·23
21	2·06	3·27	1·21

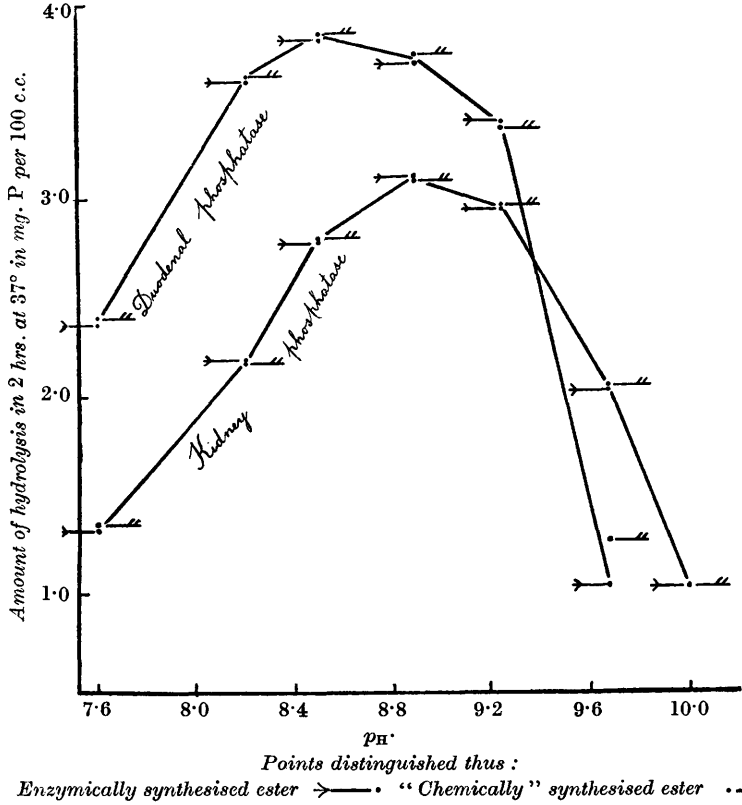
Controls with boiled enzyme showed no synthesis whatever in 21 days.

At this stage the reaction had evidently almost reached equilibrium. The reaction mixture (262 c.c.), containing 317 mg. P of esterified phosphate, was placed in a boiling water-bath for 10 minutes to inactivate the enzyme and then cooled. This treatment produced no rise in the inorganic phosphate. Barium nitrate (8 g. in 50 c.c. of water) was added, followed by barium hydroxide solution until the whole was a faint pink to phenolphthalein. All the inorganic and most of the combined phosphorus were thus precipitated (ppt. A), but there still remained 77 mg. of esterified P in the filtrate. This was precipitated as the lead salt, which was washed twice with water and decomposed by hydrogen sulphide, and the solution after aeration was neutralised with sodium hydroxide. The lead precipitation was repeated once more in dilute acetic acid with lead acetate. After removal of the lead, the aerated filtrate was neutralised with warm barium hydroxide solution, filtered, and treated with 2 volumes of 98% alcohol. The precipitated barium salt was washed with 60% alcohol, dissolved in water containing a little dilute acetic acid, again precipitated by addition of an equal volume of 98% alcohol, washed, and dried at 110° (0·40 g. Product B).

The main amount of esterified phosphate was still in ppt. A. This was ground with dilute acetic acid and filtered: the whole of the combined phosphorus was found in the solution (220 mg.) together with 20 mg. of inorganic phosphate.

20 C.c. of 25% lead acetate solution were added to the faintly acid filtrate, and the precipitated lead salt was washed and decomposed in the usual way. The barium salt was now prepared, and precipitated by alcohol. It was transformed again into the lead salt, and finally the purified barium salt was precipitated twice by an equal volume of alcohol, obtained in micro-crystalline form from warm aqueous alcohol, and dried at 100° over phosphoric oxide

FIG. 1.



Points distinguished thus :

Enzymically synthesised ester ——•—— *"Chemically" synthesised ester* .—//—

(yield, 1·6 g. Product C) (Found in product B: Ba, 50·3; P, 11·25. Found in product C: Ba, 49·7, 49·9; P, 11·3, 11·4. Calc. for barium hydroxyethyl phosphate, $HO{\cdot}C_2H_4{\cdot}O{\cdot}PO_3Ba$: Ba, 49·5; P, 11·2%).

Through the kindness of Prof. R. H. A. Plimmer and Mr. W. T. J. Burch, who provided me with a sample of barium hydroxyethyl phosphate prepared by purely chemical means, I was able to obtain further evidence confirming the identity of the chemically and the

biochemically synthesised substance as follows. The two barium salts were dissolved separately in water and decomposed by the theoretical quantity of sodium sulphate solution. The filtrates were diluted to contain 0·300 mg. P per c.c. Preliminary experiments having shown that the optimum p_H for the enzymic hydrolysis of the glycophosphate was between 8·0 and 9·0, 5 c.c. portions of the two solutions were run into two series of test-tubes, containing 5 c.c. of borate or glycine buffers to cover a p_H range from 7·6 to 10·0.

In one series 0·5 c.c. of a solution containing kidney phosphatase, and in the other 0·5 c.c. of the same duodenal extract which catalysed the synthesis, were then added to each tube, and the amount of hydrolysis of the ester after a period of 2 hours at 37° was determined in each series, the Briggs method (*J. Biol. Chem.*, 1922, **53**, 13) of phosphate determination being used. Adequate controls were made in each series.

The results are shown in Fig. 1, which indicates clearly that the two substrates are hydrolysed (*a*) at the same optimal p_H, (*b*) at the same rate, by the same enzyme solution. (The optimum is not quite the same for the two widely different enzyme preparations.) A solution of sodium β-glycerophosphate submitted to the action of the same enzyme preparations under identical conditions showed a considerably higher rate of hydrolysis.

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