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Concerning Enzymic Reactions in Heavy Water. II. Deuterium and the Hydrolysis of Starch.*

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One of us[1] called attention to the contradictory results reported by writers who have investigated various influences of heavy water upon living organisms. It was suggested that studies of numerous biochemical reactions *in vitro,* supplementing investigations upon complex metabolic processes such as respiration, growth, motion, reproduction, etc., in entire organisms, should indicate possible mechanisms involved. Experiments described in the above-mentioned report showed definitely that no retardation of various enzymic reaction rates occurred, but that on the contrary, certain of such processes, notably the hydrolysis of starch by amylase from the crystalline style of the mussel *Mytilus californianus,* were slightly accelerated in high concentrations of D_2O. Steacie[2] reports that the inversion of cane sugar by saccharase was retarded by 25% in concentrated heavy water, buffered at pH 4.6, but that the breakdown of the glucoside salicin by emulsin was accelerated to an equal degree in the presence of the pure isotope, buffered at pH (pD?) 4.5.

Hornel[3] cites the work of Moelwyn-Hughes, who found that the catalytic influence of acid in the hydrolysis of cane sugar was greater in D_2O than in ordinary water; also the investigations of Schwartz, who observed that both methyl and ethyl acetates were hydrolyzed more rapidly by 50% in acidic solutions of heavy water than in light water under similar conditions. Hornel's own work on the rate of acid hydrolysis of methyl acetate showed that the catalytic coefficients (assuming that complete dissociation of sulfuric acid took place in both kinds of water) were in the ratio, $K_{D_3O^+}/K_{H_3O^+} = 1.86$ at 15°, and 1.68 at 25°.

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1 Fox, D. L., *J. Cell. and Comp. Physiol.,* 1935, **6**, 405.

2 Steacie, E. W. R., *Z. Physik. Chem.,* 1934, **27**, 6; *ibid.,* 1935, **28**, 236.

3 Hornel, J. C., *Nature,* 1935, **135**, 909.

While, in the earlier work of the senior author, it seemed that the rate of production of maltose from starch was at least slightly enhanced in 99% D_2O, it was observed (by the achromic point method) that there was an unmistakable increase in the rate of the first conversion, *i. e.*, that of starch to erythrodextrin, in the same solutions. It was mentioned that further experiments should provide additional information regarding the relative enhancement or retardation of the rate of hydrolysis of "heavy starch", *i. e.*, starch in which the labile, O-linked hydrogens are replaced by deuterium following treatment with heavy water, a process which, according to Brickwedde[4] occurs in such compounds as glucose, sucrose, ethylene glycol, cellulose, etc.

With but a very limited supply of pure heavy water,† on hand, some semi-micro experiments were conducted with a view to illuminating this point. The enzyme was present in the dry pulverized styles of mussels, prepared as described previously (op. cit.).

A series of hydrolyses of hydrated starch by mussel-style amylase, like the chromic (iodo starch) experiments reported in the earlier paper, was repeated, using throughout the series identical quantities of buffer salts (pH 7.0), identical proportions of enzyme, substrate, and water in each system and allowing both enzyme and starch to stand separately, preserved under toluene, in the respective aqueous solutions for an overnight period before mixing and allowing each digestion to proceed at 38°C. The difference in this set of experiments was that the incubations and digestions were carried out in 5 different solutions, *viz.*: 0.0, 24.7, 49.5. 74.2, and 99.2% D_2O instead of only the first and last named, and the rates compared.

Reducing sugars were readily demonstrated with Benedict's reagent in each digest shortly after the beginning.

After incubating for about 2 hours, identical quantities of acidic iodine-potassium iodide reagent were added as a test for residual starch in each system. The most starch (most blue color) appeared in the ordinary water system, the least starch (most brown-purple color, therefore presumably most erythrodextrin) appeared in the 99.2% D_2O system; the other members of the series showed a gradation of intermediate colors in the direction of increasing erythrodextrin and decreasing starch with increasing D_2O concentration of the digestion medium.

The earlier experiments (op. cit.) had revealed that the hydrolysis

[4] Brickwedde, F. G., *J. Wash. Acad. Sci.*, 1935, **25**, 157.

† 99.2% D_2O of certified high purity from the Norsk Hydro-Elektrisk Kvaelstofaktieselskab, Oslo, Norway.

of starch by mussel style amylase in 1% D_2O was not different in rate from the same reaction in ordinary water, all other factors being equal. It was thought possible, however, that if "heavy starch" were first prepared by hydrating the dry material in concentrated heavy water, and allowing sufficient time for labile (*i. e.*, hydroxyl) hydrogen in the starch, to be exchanged for deuterium in the water, this modified polysaccharide might then show a difference in its readiness of breakdown by the enzyme.

Accordingly, 50 mg. of starch were placed into each of a pair of thick-walled Pyrex test tubes containing dry buffer salt from 0.3 cc. of approximately .08 M. phosphate buffer solution of pH 7.0.[5] To one tube was added 0.3 cc. of 99.2% heavy water; into the control tube was placed an equal volume of ordinary twice distilled water. Both tubes were sealed in an oxygen flame and the starch completely hydrated and brought into solution by heating in an oven at 115°C. They were then allowed to stand at room temperature over night. When opened next day, 0.7 cc. of buffer solution was added to each tube to aid in dispersing the gels which had formed on cooling. With both starch and enzyme at 38°C., 5 cc. of a 0.41% style suspension in phosphate buffer solution (pH 7.0) were added to each tube, and the time taken by stopwatch, while the resulting mixtures were allowed to incubate in a glycerine bath maintained at the above temperature; 1 cc. samples were withdrawn from time to time and titrated in the regular manner, using Benedict's quantitative copper reagent, for maltose formed.

Reference to the curves in Fig. 1 shows that the hydrolytic reaction had reached equilibrium far sooner in the case of the "heavy starch" in approximately 5% heavy water than in the control; the 120 minute point was the first in which as great an amount of maltose was measured in the control digest as was found in the D_2O system after the first 10 minutes. The curves would indicate an initial difference in rate of hydrolysis of three or four fold. It will be noted that this experiment, which reveals different results from an earlier one (op. cit.) in which 1% D_2O allowed the hydrolysis of starch to take place simultaneously with the same reaction in the control, was conducted in a very different manner in that, instead of merely incubating the previously hydrated substrate with the dilute D_2O solution as formerly, the substrate was in this case originally hydrated in the *hot concentrated* isotope water. The protium-deuterium exchange between water and certain other substances,

[5] Clark, W. M., The Determination of Hydrogen Ions, 3rd ed., 1928, Williams and Wilkins, Baltimore.

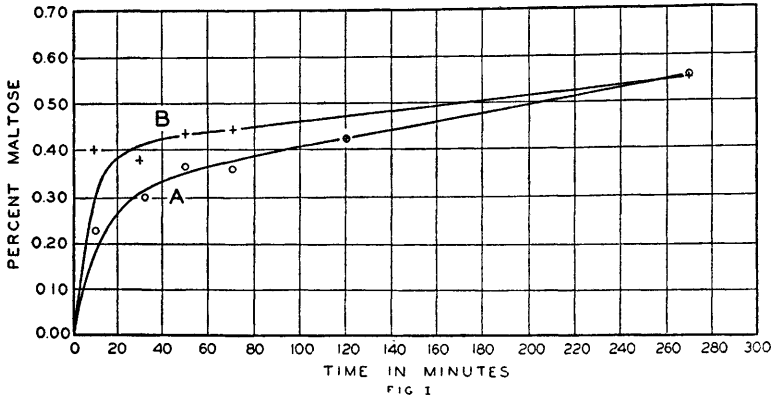

FIG. 1.

Hydrolysis of starch (previously hydrated in respective kinds of water at 115° C.)
Concentration of buffer salts, ca .08 M, pH 7.0; temp. 37-38° C.
Starch concentration 0.83%
Style concentration 0.33%
A (circles); starch hydrated and hydrolized in ordinary water.
B (crosses); starch hydrated in 99.2% D_2O.
hydrolized in 5% D_2O.

according to Brickwedde,[4] occurs readily. See also Polanyi.[6]

The addition of the same quantity of iodine reagent to the residue in each tube at the end of the experiment yielded at first similar appearing brown-red solutions but later showed the deep blue to blue-purple color of residual starch in the control tube and the red-purple of predominating erythrodextrin in the heavy water tube.

From the few experiments permitted by the limited supply of heavy water on hand, it is provisionally concluded that starch which has been allowed to become hydrated with heavy water, or which may have exchanged some of its labile protium for deuterium, is more readily hydrolyzed by this enzyme, other conditions being the same, than is starch of ordinary history.

A very interesting projection of such experiments as these would be a test of the relative rates of starch synthesis (in light) and starch hydrolysis (in darkness) by algae grown in dilute D_2O on the one hand and in ordinary water on the other.

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6 Polanyi, M., *Nature* (Supplement), 1934, **135**, 19.