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Further experiments upon the purification of pancreatic amylase.

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In recent years much light has been thrown upon the chemical nature of some typical enzymes, both by the direct method of purification and analysis and by other methods which, while indirect, have phoven none the less instructive.

Pancreatic amylase has been purified by the methods developed in this laboratory[1] to a maximum enzymic activity practically constant in many independent purification experiments. This material in the dry state is an amorphous white powder having the ultimate composition, and showing the color reactions of typical proteins. Moreover, the enzyme material showed, on analysis by the Van Slyke method,[2] the same forms of nitrogen as are found in typical proteins, and in typical quantitative proportions. Like the purified malt amylase previously described by Osborne,[3] this pancreatic amylase on heating in water yielded a coagulated albumin and left in solution a proteose or peptone.

Our purified pancreatic amylase preparation has much the highest enzymic activity of any material of which we have found adequate record. In thirty minutes at 40°, this material splits 20,000 times its weight of starch and forms 10,000 times its weight of maltose; and notwithstanding its gradual inactivation in solution, this material has, in sufficiently long experiments, digested as much as 4,000,000 times its weight of starch and formed as much as 2,800,000 times its weight of maltose.[4]

This great enzymic activity was shown at a dilution of 1:100,000,000, whereas the most delicate tests for protein are probably not valid at dilutions greater than about 1:100,000. Thus the failure of protein reactions in solutions enzymically

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[1] Sherman, H. C., and Schlesinger, M. D., *J. Am. Chem. Soc.*, 1911, xxxiii, 1195-1204; 1912, xxxiv, 1104-1111.

[2] Sherman, H. C., and Gettler, A. O., *J. Am. Chem. Soc.*, 1913, xxxv, 1790-1794.

[3] Osborne, T. B., *J. Am. Chem. Soc.*, 1895, xvii, 587-603; 1896, xviii, 536-542.

[4] Sherman, H. C., *Proc. Nat. Acad. Sc.*, 1923, ix, 81-86.

active does not show, as many writers assume, that the enzyme is of other than protein nature, since as in this case, the enzymic activity may constitute a test 1000-fold more delicate than any reaction which can be employed as a test for the presence of protein material.

The investigations of this laboratory upon pancreatic amylase have shown that this enzyme loses its activity in aqueous solutions, that this loss proceeds more rapidly with increase of temperature, and that it is undoubtedly connected with hydrolytic changes taking place in protein material which constitutes either the enzyme molecule itself or an essential part of it. Extensive studies of the conditions influencing the rate of hydrolytic inactivation of the enzyme, especially as affected by added amino acids, all give added evidence of the protein nature of the enzyme.[5, 6]

This is further confirmed by the finding that the antiseptics which act chemically upon proteins are enormously more destructive of the enzyme than are the antiseptics of the lipoid-dissolving type.[7]

The amylase of malt (while more stable than that of the pancreas) has yielded qualitatively similar results; and it has also been possible in working with this more stable enzyme to show that it has a definite isoelectric point or zone and that the material which shows the enzymic activity is an ampholyte, presumably protein.[8]

Thus four different kinds of experimental evidence all point strongly and consistently to the protein nature of the enzyme.

Willstätter and his co-workers[9] have questioned this conclusion. Largely by the use of methods involving adsorption with alumina gel and with kaolin, they have effected what they believe to be an unprecedented degree of purification of pancreatic amylase and have concluded that the enzyme as thus purified is of other than protein nature.

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[5] Sherman, H. C., and Walker, Florence, *J. Am. Chem. Soc.*, 1921, xliii, 2461-2469; 1923, xlv, 1960-1964.

[6] Sherman, H. C., Caldwell, M. L., and Naylor, N. M., *J. Am. Chem. Soc.*, 1925, xlvii, 1702-1709.

[7] Sherman, H. C., and Wayman, M., *J. Am. Chem. Soc.*, 1921, xliii, 2454-2461.

[8] Sherman, H. C., Thomas, A. W., and Caldwell, M. L., *J. Am. Chem. Soc.*, 1924, xlvi, 1711-1717.

[9] Willstätter, R., Waldschmidt-Leitz, E., and Hess, A. R. F., *Z. physiol. Chem.*, 1923, cxxvi, 143-169.

Valuable as is their work upon some aspects of the adsorption phenomena, their conclusions regarding the chemical nature of the enzyme and its quantitative activity as purified by their method are shown by our experiments to be in error. The outstanding source of error in their work is that, having reverted to the use of glycerol for the preservation of the enzyme in solution (which Osborne, and later we, had set aside in favor of procedures capable of yielding a dry product) they determined the activity of their glycerol-water solution of purified amylase and then interposed a long period of dialysis against running water before determining the (residual) solids for the calculation of the enzymic activity and testing them for the chemical nature of the enzyme. As we understand their account of their work, the tests upon which they base their conclusion regarding the nature of the enzyme were made upon material which exhibited no amylase activity. Their assumption that the substance of the enzyme (although inactivated) might be expected to remain in the dialyzer without material qualitative or quantitative alteration, we have found to be invalid.

In the work of this laboratory it has been repeatedly shown that pancreatic amylase in aqueous solution undergoes a relatively rapid hydrolysis into substances which are readily dialyzable and which therefore must have been lost by Willstätter, Waldschmidt-Leitz and Hess[9] when they dialyzed for a week against running water, after making the enzymic test in their solution and before applying tests for the chemical nature of the enzyme, or attempting to ascertain its weight.

Hence it must be concluded that they greatly over-estimated the activity of their purified enzyme material because of greatly underestimating the amount of it present in the glycerol-water solution which they tested, and that their negative findings as to the chemical nature of the enzyme are inconclusive because of the long dialysis which they interposed between the tests for the activity of the enzyme and for its chemical nature had involved such serious losses that their subsequent tests cannot be regarded as having the significance which they attached to them.

We have carefully repeated their method of purification with painstaking precautions to establish and maintain the most favorable conditions for each step. The actual concentration of active enzyme relative to total nitrogen effected by their method in our hands is apparently about one-half that attained previously in this laboratory by the method of Sherman and Schlesinger. We have confirmed and extended many of their observations regarding the adsorption and elution of the enzyme, and, by careful determination of optimal conditions have obtained data which indicate that pancreatic amylase like malt amylase[8] has a definite isoelectric point or zone at essentially the same pH at which it exerts its optimum enzymic activity.

These new experiments, including the direct repetition of the work of Willstätter and his co-workers, confirm the conclusion, reached in our previous investigations in which the problem of the chemical nature of pancreatic amylase has been approached from several different angles, that the enzyme either is of protein nature or contains protein as an essential constituent.

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Further proof of the "bios" character of crystalline bios 223.*

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In our original report[1] on the isolation and properties of Bios 223 we reported failure to obtain either the ninhydrin or mustard oil reaction for a primary amine, but did obtain a reaction with benzene-sulfon-chloride. In pursuit of the chemical structure of the crystals we have since obtained a reaction with HNO_2 and a Van Slyke nitrogen determination that checks our previous value obtained by the Dumas method. These results have not only given us new leads in the study of structure, but also a means of determining definitely whether the yeast growth stimulatory power resides in the crystals of Bios 223, or is due to contamination of the crystals by the active factor. The experimental data by which we have demonstrated that the crystals are responsible for the activity follow and constitute the subject of this report.

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* To avoid confusion we have elected to identify our Crystalline Bios by using the melting point 223° C. in the designation.

[1] Eddy, Kerr, and Williams, R. R., *J. Am. Chem. Soc.*, 1924, xlvi, 2846.