sodium is not directly responsible for the acetoacetic ester condensation, but serves only to generate sodium ethoxide, which is the active condensing agent. In further support of this contention another important observation should be emphasized, *viz.*, that sodium ethoxide produces practically the same amount of condensation (Runs 3 and 5) and just as readily (Run 5) as does metallic sodium. This observation is not in agreement with the findings of some of the earlier investigators of the reaction, but it is in accord with the results recently reported by Kutz and Adkins.[13]

Summary

A study of the reaction of sodium and ethyl acetate has been made in which the amounts of the reaction products, acetoacetic ester, alcohol and hydrogen have been determined. The amount of alcohol found in the reaction mixture is approximately the sum of that produced by the acetoacetic ester condensation and that resulting from the reduction of the ester by the sodium used in the reaction. It has also been shown that sodium ethoxide brings about the condensation just as readily and completely as does metallic sodium. These results lead to the conclusion that the role of sodium in the acetoacetic ester condensation is to generate, by reduction of the ester, sodium ethoxide, which is the real condensing agent.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF COLUMBIA UNIVERSITY, No. 658]

A QUANTITATIVE STUDY OF THE INFLUENCE OF ACETATE AND OF PHOSPHATE UPON THE ACTIVITY OF THE AMYLASE OF ASPERGILLUS ORYZAE

BY M. L. CALDWELL AND M. G. TYLER

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Recent work[1] has emphasized anew the importance of maintaining the optimal hydrogen-ion activity in studies of enzyme action and has also shown that this is not necessarily a fixed value, but may and often does differ with changes in the environmental conditions under which the enzyme acts. Important among the factors which have been found to influence enzyme action is the kind and concentration of electrolyte present. The experiments reported briefly here were undertaken to establish quantitatively the relations of acetate and of phosphate to the saccharogenic activity (formation of reducing sugar, chiefly maltose)[2] of the amylase

[13] Kutz and Adkins, THIS JOURNAL, **52**, 4393 (1930).

[1] (a) Sherman, Caldwell and Adams, *ibid.*, **49**, 2000 (1927); **50**, 2528, 2529, 2535 (1928); (b) Sherman, Caldwell and Boynton, *ibid.*, **52**, 1669 (1930); and other references therein contained.

[2] Sherman and Punnett, *ibid.*, **38**, 1878 (1916).

of *Aspergillus oryzae* and to the hydrogen-ion activities which favor its action.

Experimental

The general plan of this work was: first, to establish the optimal hydrogen-ion activities for the enzyme in the presence of each of several different concentrations of acetate or of phosphate, by a series of parallel measurements of the activity in starch systems in which the hydrogen-ion activities differed at close intervals but in which the concentration of acetate or of phosphate was kept constant and all other conditions were strictly comparable; second, to make direct comparisons of the activity of the enzyme in the presence of the different concentrations of each buffer when the systems were in each case adjusted to the optimal hydrogen-ion activity as previously determined; and, third, to compare the activity of the enzyme in the absence of either buffer, with its activity in the presence of the most suitable concentration of phosphate or of acetate, each solution being adjusted to the most favorable hydrogen-ion activity. In these parallel experiments the control systems containing neither acetate nor phosphate were adjusted to closely graded hydrogen-ion activities by the use of graded concentrations of sodium hydroxide.

All reagents were carefully purified. Merck's soluble potato starch according to Lintner was used. It was repeatedly washed with distilled and finally with redistilled water, air dried, and moisture determined. The salts, sodium acetate and the mono- and disodium phosphates, which were of the best commercial chemically pure grade, were recrystallized twice from distilled and once from redistilled water. They were then air dried and moisture determined. Redistilled water was used for all solutions. The preparation of the amylase was obtained from a commercial product[3] by the method of Sherman and Tanberg.[4] Fresh portions of this preparation were weighed out for each experiment but the concentration was kept constant throughout the work. The activity of the enzyme (the formation of reducing sugar, chiefly maltose,[2] from starch) was determined under accurately controlled constant conditions by a slight modification of the gravimetric copper-reduction method previously described.[5] The enzyme acted for thirty minutes at 40 ± 0.02° upon 2% starch containing the concentrations of acetate or phosphate under consideration. When one concentration of a buffer at different hydrogen-ion activities was being studied, the total concentration of acetate or of phosphate was kept constant but the proportions of equimolar acetic acid and sodium acetate or of equimolar mono- and disodium phosphates were changed, the proportions depending upon previous electrometric titrations of similar starch–acetate or starch–phosphate systems. The hydrogen-ion activities of all solutions were verified by electrometric measurements with a saturated calomel electrode and a hydrogen electrode of the type described by Wilson and Kern.[6]

[3] Courtesy of Parke, Davis and Company.

[4] Sherman and Tanberg, THIS JOURNAL, **38**, 1638 (1916).

[5] Sherman, Kendall and Clark, *ibid.*, **32**, 1073 (1910); Sherman and Walker, *ibid.*, **43**, 2461 (1921).

[6] Wilson and Kern, *Ind. Eng. Chem.*, **17**, 74 (1925).

Results

Interrelation of Hydrogen-Ion Activity, Concentration of Acetate or of Phosphate and Activity of the Amylase of Aspergillus Oryzae.—The average of several determinations of the influence of hydrogen-ion activity upon the amylase activity of this enzyme in the presence of each of several concentrations of acetate or of phosphate gave the following results: the maximum enzymic activity occurs under the observed conditions at hydrogen-ion activities of P_H 5.3 to 5.5, P_H 4.9 to 5.5, P_H 4.7 to 5.3 and P_H 4.8 to 5.2 in the presence of 0.01, 0.03, 0.06 and 0.10 *M* sodium acetate, respectively; and at hydrogen-ion activities of P_H 5.3 to 5.5, P_H 5.0 to 5.3, P_H 4.9 to 5.1 and P_H 4.7 to 5.2 in the presence of 0.01, 0.03, 0.06 and 0.10 *M* phosphate, respectively. In both series of experiments there is a tendency for the maximum activity of the amylase to occur in slightly more acid solutions as the concentration of acetate or of phosphate is increased from 0.01 to 0.10 *M*. Similar results have also been noted with pancreatic and malt amylases.[1,7]

Influence of the Concentration of Acetate or of Phosphate upon the Activity of the Amylase of Aspergillus Oryzae.—When the activities of the amylase in the presence of each of the four concentrations of acetate, with each system adjusted to the optimal hydrogen-ion activity as previously determined, were compared, it was found that there was a consistent but very small decrease in the activity of the enzyme as the concentration of acetate was increased from 0.01 to 0.10 *M*. Thus, the average amylase activity expressed in terms of reducing power calculated to maltose[2] was found, in twelve strictly comparable determinations, to be 211, 209, 207, 206 mg. of maltose in the presence of 0.01, 0.03, 0.06 and 0.10 *M* acetate, respectively.

Similar experiments with the four concentrations of phosphate under optimal conditions of hydrogen-ion activity for each system showed that there was no measurable difference in the activity of the amylase as the phosphate concentration was increased from 0.01 to 0.10 *M*. Thus, the average amylase activity expressed in terms of reducing power calculated to maltose was found in six strictly comparable determinations to be 213, 212, 213, 213 mg. of maltose in the presence of 0.01, 0.03, 0.06 and 0.10 *M* phosphate, respectively.

A Comparison of the Saccharogenic Activity of the Amylase of Aspergillus Oryzae in the Absence and in the Presence of Acetate or Phosphate.—In view of the results discussed above a concentration of 0.01 *M* acetate and of 0.01 *M* phosphate was chosen for the comparison of their influence upon the activity of the enzyme. The amylase acted simultaneously upon 2% starch alone, 2% starch containing 0.01 *M* acetate at the optimal hydrogen-ion activity of P_H 5.5, 2% starch containing

[7] Sherman, Caldwell and Dale, THIS JOURNAL, **49**, 2596 (1927).

0.01 *M* phosphate at the optimal hydrogen-ion activity of P_H 5.3, and upon a series of 2% starch systems to which graded concentrations of sodium hydroxide had been added, resulting in a series of hydrogen-ion activities from P_H 4.4 to 5.5, the optimal amylase activity under these conditions occurring at about P_H 5.1. It was found that under these conditions the activity of the amylase was the same in the presence of 0.01 *M* acetate as in the presence of 0.01 *M* phosphate, that this was consistently higher than its activity in the unbuffered unadjusted aqueous starch systems and slightly lower than the highest activities obtained in the unbuffered systems in which the hydrogen-ion activities were adjusted by the use of sodium hydroxide instead of by acetate or phosphate buffers. The average values of several parallel determinations under these conditions in which the activity of the amylase is expressed in terms of reducing power calculated to maltose, were 196, 203, 203, 207 mg. of maltose, respectively, for the unadjusted unbuffered starch, 0.01 *M* acetate, 0.01 *M* phosphate starch systems and for the average of the highest activities in the systems adjusted only with sodium hydroxide. In the latter unbuffered systems, however, the results were very irregular, probably due to the impossibility of obtaining quantitatively reproducible hydrogen-ion activities.

These results show that if the most favorable conditions for each case are maintained, the saccharogenic activity of the amylase of *Aspergillus oryzae* is the same in the presence of 0.01 *M* acetate or of 0.01 *M* phosphate. While the activity of the enzyme is slightly lower in the presence of these buffers than that which may be obtained in their absence, the differences are very small and may, in our opinion, be considered negligible, as they would be more than offset in usual measurements of amylase activity by the advantage of having well buffered systems so necessary for reproducible and comparable results. For this reason also, acetate is preferable to phosphate for use in the measurements of activity of the amylase of *Aspergillus oryzae*, as it is a much more efficient buffer at the hydrogen-ion activities at which this amylase is most active.

Summary

The optimal hydrogen-ion activities for the action of the amylase of *Aspergillus oryzae* in the presence of different concentrations of acetate or phosphate have been quantitatively established, and found to be dependent both upon the kind and concentration of the buffer used.

The highest amylase activity of this enzyme in the presence of either of these buffers occurs in systems of slightly lower hydrogen-ion activity as the concentration of either is increased from 0.01 to 0.10 *M* (measurements at 40° for thirty minutes in presence of 2% starch).

Increasing the concentration of acetate from 0.01 to 0.10 *M* causes a slight decrease in the activity of the amylase. The decrease in activity

of the amylase in the presence of 0.01 *M* acetate is negligible and is more than offset by the increase in reproducibility of results over those obtained in unbuffered systems.

Changes in the concentration of phosphate from 0.01 to 0.10 *M* do not influence the activity of the enzyme if the hydrogen-ion activities of the systems are suitably adjusted.

If the hydrogen-ion activities of the systems are suitably adjusted, the activity of this amylase is the same in the presence of 0.01 *M* acetate or of 0.01 *M* phosphate.

In measurements of the activity of the amylase of *Aspergillus oryzae*, acetate is preferable to phosphate because of its more efficient buffer effect in the region of hydrogen-ion activities which are optimal for the action of this amylase.

In measurements of thirty minutes at 40° with 2% starch containing 0.01 *M* acetate, the amylase of *Aspergillus oryzae* is most active saccharogenically at hydrogen-ion activities of P_H 5.3 to P_H 5.5.

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[CONTRIBUTION FROM THE INSECTICIDE DIVISION, BUREAU OF CHEMISTRY AND SOILS]

THE "YELLOW COMPOUNDS" RESULTING FROM THE DECOMPOSITION OF ROTENONE IN SOLUTION

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The literature on *Deguelia* (Derris) contains numerous references to "yellow compounds" which are obtained in the process of isolating the active insecticidal principles, or which result from the treatment of rotenone ($C_{23}H_{22}O_6$, m. p. 163°), the most important constituent, with various reagents.[1]

Greshoff's[2] derrid, described as an amorphous material melting at 61°, and decomposing at 160°, was probably a mixture of rotenone and certain yellow materials. The same investigator later reported the melting point of pure derrid as 204°.[3]

Van Sillevoldt[4] obtained from the roots of *Deguelia elliptica* a pale yellow substance melting at 73° which he also called derrid. This, on treatment with an alcoholic solution of hydrogen chloride, gave yellow needles

[1] Clark, *Science*, **71**, 396 (1930), has isolated a yellow crystalline material, toxicarol, from the root of *Deguelia sp.* and it is possible that other yellow compounds are associated with rotenone in this root. This paper, however, deals primarily with such yellow compounds as result from the decomposition of rotenone in solution.

[2] Greshoff, *Ber.*, **23**, 3537 (1890).

[3] Greshoff, *Meded. 'Slands Plant.*, **25**, 47 (1898).

[4] Van Sillevoldt, *Arch. Pharm.*, **237**, 595 (1899).