[CONTRIBUTION FROM THE DIVISION OF CHEMISTRY, NEW YORK STATE AGRICULTURAL EXPERIMENT (GENEVA) STATION]

Water Relations of Enzymes. II. Water Concentration Required for Invertase Action[1]

BY Z. I. KERTESZ

When plant or animal materials containing enzymes are dehydrated for the purpose of preservation, the desiccation is continued generally to a point lower than that required to prevent the growth of microörganisms. The water content should be low enough to prevent enzyme action and consequent deterioration. The literature contains references to the highest permissible water content of certain products, but no information could be found on the critical water concentration for enzyme action. Investigations on this question were started, therefore, and this second paper of the series on water relations of enzymes deals with the rate of invertase action in the presence of 0 to 20% moisture.

As mentioned in the previous communication[2] Nelson and Schubert[3] found that the concentration of the water is a factor in determining the magnitude of the velocity of invertase action in liquid reaction mixtures in the presence of alcohol and high concentrations of sucrose. In the present experiments a solid reaction mixture was used to which water was added and mixed to reach moisture contents up to 20%. The mixture approximated the composition of dried apples and contained 75% sucrose and 1% of a highly active yeast invertase preparation. The remaining 24% of the mixture was made up from 20% dried apple pomace and 4% phosphate buffer to ensure the approximately optimum *p*H 4.5 for the enzyme. All components were reduced to pass a 40-mesh screen and were well mixed in a mortar. This mixture was not hygroscopic because the pomace used contained only 11.59% sugars of which 7.86% were reducing sugars, mostly fructose. Consequently the final mixture contained only 1.57% reducing sugars and only traces of water were absorbed at 75% humidity at 25°. The samples stored at 100% relative humidity absorbed 12% moisture in eight days, 23% in fourteen days, and 39% in twenty-eight days, and the hydrolysis of the sucrose proceeded rapidly and was complete on or before the twenty-eighth day of storage.

The 2-g. samples used for the determination of the water required for invertase action were mixed thoroughly with the calculated amount of water and stored in small air-tight bottles at −18, 0, +10, 25 and 40° for different periods up to seventy-five days. No antisepticum was applied in the samples because of the high sugar concentrations used and also because previous experiments[4] showed that more than 20% moisture is required in apple pomace to enable the growth of microörganisms. At the time of sampling they were mixed with neutral lead acetate and sodium carbonate solutions, made up to 50 cc. with water and the optical rotation of the solution determined in a polarimeter.

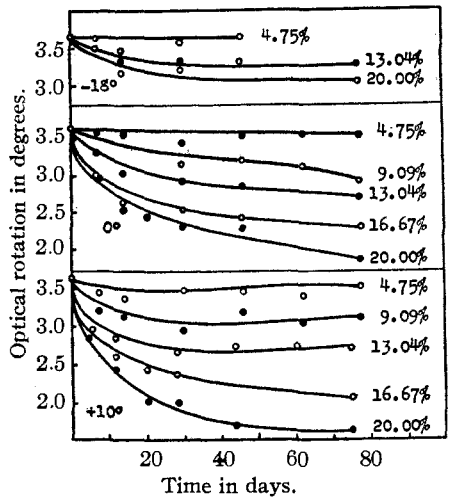

Fig. 1.—Hydrolysis of sucrose by invertase at −18, 0, and 10° in the presence of 4.75 to 20% moisture.

As shown in Fig. 1, at −18° slow but definite action could be observed in the samples having 13 and 20% moisture, while the optical rotation of the sample with 4.75% moisture changed immaterially. The hydrolysis was faster at 0°. In the samples containing 4.75% moisture and stored at 0 and 10° slight hydrolysis appeared to take place during the first few weeks of the experi-

(1) Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 88, March 26, 1935.

(2) Kertesz, THIS JOURNAL, **57**, 345 (1935).

(3) Nelson and Schubert, *ibid.*, **50**, 2188 (1928).

(4) Kertesz, New York State Agr. Exp. Sta. Tech. Bull. No. 179 (1931).

ment, but there was no further decrease after thirty days. The rotation was lowered in both cases by about 0.18° showing that only about 4% of the sucrose was hydrolyzed.

At 25° no change in the sample stored without moisture could be observed, as shown in Fig. 2.

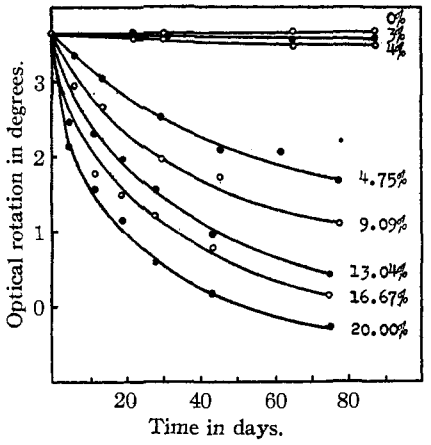

Fig. 2.—Hydrolysis of sucrose by invertase at 25° in the presence of 0 to 20% moisture.

In the samples containing up to 4% moisture there was no definite hydrolytic action, the changes being within the experimental error. The samples containing 4.75% moisture or more showed a strong and steady invertase action, which if sufficient time were allowed for the reaction would have resulted in the complete hydrolysis of the sucrose present. When this set of experiments at 25° was repeated using the same mixture, except that a commercial invertase preparation (invertase scales containing melibiase, Wallerstein Laboratories) was used, the results obtained were essentially the same. No hydrolysis occurred in 130 days in the samples containing 0 to 4% moisture and only very slight action was observed in the sample with 5% moisture. Definite and steady hydrolysis was found in the samples containing 6% or more of moisture. The extent of hydrolysis increased with higher moisture content, but it was always less for the same period of storage than in the case of the yeast invertase preparation of higher activity.

At 40°, as shown in Fig. 3, the hydrolysis proceeded more rapidly for some fifteen days than at any other temperature used, but later the enzyme became completely inactivated. At the five degrees of humidity studied, 8, 16, 25, 34 and 45%, respectively, of the sucrose present was hydrolyzed before the enzyme became inactivated.

To be certain that the hydrolyses observed were catalyzed by the enzyme, reaction mixtures without enzyme and with heated enzyme and different moisture contents were prepared, stored and analyzed. Although occasionally slight changes in the polarimeter readings could be observed, they never showed progressive changes and never exceeded the variation introduced by taking samples from the mixture used.

These experiments establish the fact that invertase might be active in the presence of 5% moisture. This water content is far below the concentration usually advocated as necessary for any enzyme action. The lowest concentration of water used by Nelson and Schubert was 48%. Assuming an approximately linear relationship between the water concentration and the velocity of hydrolysis not only in the range studied by Nelson and Schubert but also below 48% water content, the termination of hydrolysis could be predicted at some 20 to 25% moisture. The limit found in this work is much lower. One is justified in predicting, therefore, a considerable flattening off of the curve presented by Nelson and Schubert at low water concentrations. Naturally the accompanying materials, qualitatively as well as quantitatively, must have some influence on the rate of the hydrolysis of sucrose.

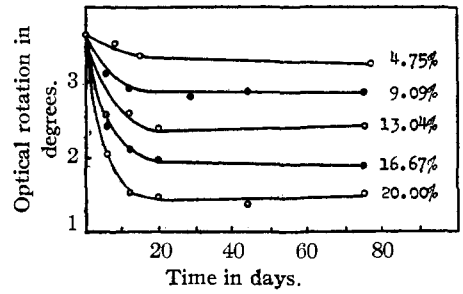

Fig. 3.—Hydrolysis of sucrose by invertase at 20° in the presence of 4.75 to 20% moisture.

Attempts to evaluate the results of the hydrolyses by the use of any of the common formulas failed and for this reason the optical rotations of the samples are presented as observed without further calculation. In the mixture used the invertase action may have been influenced by a number of factors as the different mutual solubilities of the ingredients, the different affinity of the sucrose, potassium phosphate, hydrophilic colloids, etc., to water at different temperatures,

etc. No sufficient knowledge of these factors is available to warrant any speculation about the causes of the changes observed.

Summary

In reaction mixtures approximating the composition of dried apples and containing invertase this enzyme has been found active in the presence of 4.75–5% moisture at 25°. No definite enzyme action could be found at lower water concentrations. Raising the water content above 5% resulted in quicker and more regular hydrolyses.

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[CONTRIBUTION FROM THE NICHOLS LABORATORY, NEW YORK UNIVERSITY]

The Preparation of Some β-Amino Acids

BY JOHN V. SCUDI

In analogy with the addition of piperidine to benzalmalonic ester[1] $C_6H_5CH{=}C(COOC_2H_5)_2 + HNC_5H_{10} \longrightarrow C_6H_5CH(NC_5H_{10})CH(COOC_2H_5)_2$ it has been found that ammonia reacts similarly, the amino group entering the β-position, with a 47% yield as compared with a yield of 19% obtained by the preparation of this substance by the interaction of benzaldehyde, ammonia and malonic ester.[2] Hydrolysis and decarboxylation produce β-amino-β-phenylalanine. Benzalmalonic acid reacts with ammonia, yielding β-amino-β-phenylalanine directly. Piperidine reacts with benzalmalonic acid to give the salt and not the β-piperidyl-β-phenyl α,α-dicarboxylic ethane structure previously assigned[3] to the same product obtained by the condensation of benzaldehyde, piperidine and malonic acid. Goldstein[1] has shown the β-amine to be unstable.

Experimental Part

β-Phenyl-β-amino-α,α-dicarbethoxyethane (I).—Benzalmalonic ester[4] (15 g.) in 20 cc. of 10% alcoholic ammonia (1.5 moles) was evaporated over a period of one hour on a steam-bath and from the ether extract of the residue 8.5 g. (47% yield) of the hydrochloride separated on saturation of the solution with hydrogen chloride. After recrystallization from an equal part mixture of alcohol–ether, the hydrochloride was identified through comparison with an authentic sample (m. p. 159–160°). From the ethereal filtrate 3.4 g. of benzalmalonic ester, 1.2 g. of benzaldehyde and 2.1 g. of diethyl malonate were recovered. Doubling the time of heating and the amount of alcoholic ammonia gave a 45% yield of I.

β-Phenyl-β-aminopropionic Acid.—One gram of the above amino ester (I) was boiled for one hour in 10 cc. of concentrated hydrochloric acid and 20 cc. of water, and evaporated to dryness on a steam-bath. The residue recrystallized from alcohol–ether solution melted at 220–222° and showed no depression when mixed with an authentic sample; yield, 0.43 g. (70%).

Addition of Ammonia to Benzalmalonic Acid.—Benzalmalonic acid (8.5 g.) in 35 cc. of 10% aqueous ammonia when evaporated to dryness on a steam-bath gave 5.6 g. of residue from which 2.7 g. of β-phenyl-β-alanine was extracted with dilute hydrochloric acid, leaving 1.9 g. of cinnamic acid undissolved.

Piperidinium Acid Benzalmalonate.—Adding 0.5 g. of piperidine to 1.0 g. of benzalmalonic acid in 5 cc. of alcohol immediately gave 1.5 g. of the product (m. p. 163–164°) obtained by the condensation process.[3] Treatment with dilute acids precipitated benzalmalonic acid quantitatively from aqueous solutions of the salt.

Summary

Ammonia has been added to the double bond of benzylidene-malonic acid and its ester, producing beta amino derivatives under conditions approximating those used to produce these amines by the Knoevenagel method.

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(1) J. Goldstein, *Ber.*, **29**, 814 (1896).

(2) W. M. Rodionow and A. M. Fedorova, *ibid.*, **60**, 805 (1927).

(3) W. M. Rodionow and Holmogorzeva, THIS JOURNAL, **51**, 851 (1929).

(4) E. Knoevenagel, *Ber.*, **31**, 2591 (1898).