melting point of 63–66°, but could not be brought to the melting point (68°)[8] of pure *cis*-1-methylcyclohexanediol-1,2. Its configuration was checked by the preparation of a sodium borate complex, of which 1.57 g. was obtained from 1 g. of the diol, following the procedure of Maan.

Summary

The chlorohydrin formed by addition of methylmagnesium bromide to 2-chlorocyclohexanone is a geometrical isomer of the one made by addition of hypochlorous acid to 1-methylcyclohexene; whereas the latter reacts with aqueous alkali at room temperature to give methylcyclohexene oxide, its isomer is unaffected by this treatment, but is converted by hot alkali into methylcyclopentyl ketone.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

The Influence of Native Proteins on the Activity of Yeast Invertase

BY J. M. NELSON AND EVERETT L. SAUL

While examining the influence of varying hydrogen-ion concentration on the activity of yeast invertase in the presence of normal and anti-invertase sera, obtained from rabbits, Mrs. Schubert[1] noticed that on the acid side of the enzyme's optimum *p*H (4.5–5.0) both sera caused an increase in the rate of hydrolysis of sucrose. She also found that egg albumin exerts a similar influence under these conditions. Fine[2] reported that the presence of high concentrations of blood serum increases the activity of yeast invertase in the *p*H ranges 3–5 and 7–8. Increases in enzymic activity, due to the presence of added proteins, has been observed also in the case of lipases.[3,4,5]

The results given in the present paper were obtained in continuing the study of the influence exerted by proteins on the activity of yeast invertase preparations. As a brief summary, it may be stated that not only the purity (degree of activity of the enzyme preparations), and the *p*H, but also the condition of the protein, native or denatured, were found to be factors in determining the influence of the latter on the enzyme's activity. The procedure followed was to compare the rates of hydrolysis of sucrose in the presence and absence of added protein. Egg albumin, pepsin, edestin, serum, globin and gelatin were used, and when possible in the crystalline form, in order to minimize as much as possible any effect due to extraneous matter. Increases in the activity of the enzyme were observed, provided the *p*H of the hydrolyzing sucrose solutions were less than 4.5, the protein added being in the native condition,

(1) Marcelle V. Schubert, "Dissertation," Columbia University, 1933.

(2) J. Fine, *Biochem. J.*, **24**, 1282 (1930).

(3) O. Rosenheim and J. A. Shaw-Mackenzie, *J. Physiol.*, **40**, xii (1910).

(4) B. S. Platt and E. R. Dawson, *Biochem. J.*, **19**, 869 (1925).

(5) K. G. Falk, *J. Biol. Chem.*, **96**, 53 (1932).

TABLE I

DATA SHOWING THE INCREASE IN ACTIVITY OF YEAST INVERTASE IN THE PRESENCE OF NATIVE PROTEIN AT *p*H 3

Twenty-five cc. of highly active invertase preparation RaaDKDADADSD (time value 0.35 minute at 25°) was added to 200 cc. of sucrose solution, containing added protein as indicated below. Final concentration of sucrose 10%, *p*H 3, citrate–phosphate buffer about 0.15 *M*, temperature of hydrolysis 25°.

A. 20 mg. of native edestin per 225 cc. of solution

Time in minutes	Change in rotation in degrees
2	..
19	0.28
27	.40
45	.70
57	.88
Mean rate[a]	.0162

B. 10 mg. of native egg albumin per 225 cc. of solution

2	..
25	0.33
35	.47
45	.62
57	.78
82	1.15
Mean rate	0.0143

C.

2	..
30	0.35
52	.60
72	.87
91	1.09
109	1.32
Mean rate	0.0124

Increased rate of A over C = 30%. Increased rate of B over C = 15%. Similar increases in the enzyme's activity were observed when ox-serum, native pepsin and native globin were used.

[a] Rate = change in rotation per minute, obtained by dividing change in rotation by the corresponding time interval.

TABLE II

DATA SHOWING THAT DENATURED PROTEIN IS WITHOUT INFLUENCE ON THE ACTIVITY OF YEAST INVERTASE AT *p*H 3.0

Same procedure followed, and highly active invertase preparation used as in Table I, the only difference being that the protein was denatured before it was added to the sucrose solutions.

A. Ten mg. of denatured egg albumin per 225 cc. of solution

Time in minutes	Change in rotation in degrees
2	..
13	0.27
24	.54
34	.79
44	1.02
Mean rate[a]	0.0245

B. Same as A except no protein added

Time in minutes	Change in rotation in degrees
2	..
15	0.32
25	.56
35	.81
50	1.17
Mean rate	0.0245

Similar results were obtained when denatured edestin, denatured globin, denatured pepsin and gelatin were used. No effect on the enzyme's activity was observed when gum arabic, glycylglycine or tryptophan were added to the hydrolyzing sucrose solutions.

[a] Defined in Table I.

TABLE III

COMPARISON OF THE INFLUENCES OF NATIVE PROTEINS ON CRUDE AND HIGHLY ACTIVE INVERTASE PREPARATIONS

Twenty-five cc. of enzyme preparation (kind indicated below) were added to 200 cc. sucrose solution (final concentration of sucrose 10%) and 0.5 cc. of ox serum, except in controls which contained no added protein; citrate-phosphate buffer about 0.15 *M*; *p*H 3.00; temperature of hydrolysis 25°. Rate defined in Table I.

A. Crude invertase preparation RD. Time value 17.3 minutes at 25°

Time in minutes	Change in rotation in degrees
2	..
16	0.39
35	.90
50	1.32
65	1.74
Mean rate	0.0276

C. Highly active invertase preparation RaDK-DKD. Time value 0.34 minutes at 25°

Time in minutes	Change in rotation in degrees
2	..
24	0.56
45	1.10
61	1.50
79	1.90
Mean rate	0.0260

B. Same as A except no serum

2	..
20	0.50
32	.82
45	1.17
60	1.62
Mean rate	0.0275

Rate in A same as in B.

D. Same as C except no serum

2	..
19	0.35
44	.88
76	1.15
97	1.93
Mean rate	0.0205

Rate in C 27% faster than in D

and the enzyme preparation highly active, *i. e.*, free from most of the extraneous matter present in crude preparations. Denatured proteins, gelatin, gum arabic, glycylglycine and tryptophan under the same conditions produced no effects on the rates of hydrolysis of the sucrose.

This accelerative influence due to native protein on the activity of invertase at *p*H 3, raises the interesting question as to what there is about the condition of protein in this state that endows it with this peculiar property. At the present, the most widely accepted view as to the chemical nature of an enzyme such as invertase is that they are proteins or at least protein is essential to their activity. Any condition which tends to denaturize proteins, such as acids, alkalies, heat, etc., also tends to inactivate enzymes. The reversal of denaturation of proteins as described by Anson and Mirsky[6] seems also to have its counterpart in the reactivation of inactivated enzymes. Thus Herriott[7] has shown that yeast invertase inactivated by acid can be reactivated by following Anson and Mirsky's procedure of allowing a solution of the inactivated enzyme to stand for an hour or so at *p*H 6. Northrop[8] reports a similar experience with inactivated pepsin.

Herriott was only able to reactivate fairly crude invertase preparations, *i. e.*, preparations which still retained a considerable portion of the extraneous yeast proteins and gums. The highly active or highly purified preparations of the enzyme failed to respond to the reactivation treatment. In contrast to Herriott's experience, in the present investigation it was found that only highly purified invertase preparations respond to the accelerative influence of added native proteins. In other words, crude preparations already contain an excess of protein so that the addition of more seems to be without effect. The idea thus suggests itself that there may be a relation between the reactivation of invertase preparations containing relatively large amounts of extraneous protein and the accelerative effect on the activity of highly purified preparations by added protein.

At present, enzymes such as yeast invertase are usually regarded as colloidal, and since the stability of a colloid depends primarily upon the particle size and a surface charge it may be argued that the change in hydrogen-ion concentration of the enzyme solution, originally at the optimum *p*H 4.5–5, to *p*H 3, may convert the enzyme particles into a more unstable form of a suspensoid nature and in this way alter the degree of dispersion and lower the hydrolytic efficiency.

(6) M. L. Anson and A. E. Mirsky, *J. Gen. Physiol.*, **14**, 597 (1931).
(7) R. M. Herriott, "Dissertation," Columbia University, 1932.
(8) J. H. Northrop, *J. Gen. Physiol.*, **14**, 713 (1931).

The above-mentioned accelerative effect caused by the addition of native protein could then be attributed to a protective action by the protein. This argument, however, becomes less convincing when substances such as denatured protein, gelatin and gum arabic are added instead of native protein to the sucrose–invertase solutions at *p*H 3, since in none of these instances were any accelerations of the enzyme's activity at *p*H 3 observed. Neither do the added proteins exert a protective influence on the activity of the enzyme at *p*H 3, since it was found that by allowing the highly active invertase solutions, one containing added edestin and the other none, to stand for one hour at *p*H 3, no difference in activity of the enzyme in the two solutions could be detected when these enzyme solutions were subsequently added to sucrose solutions at *p*H 4.5, the *p*H at which the protein is without influence.

The possibility of the formation of more enzyme from a possible zymogen contained in the enzyme preparation was also considered, since there is an actual increase in the invertase activity at *p*H 3 induced by the added native protein. The method used to test this was simply to bring the purified enzyme solution, known to be accelerated by native protein, to *p*H 3 in the presence of native egg albumin. A hydrolysis was then conducted at *p*H 4.5 at which *p*H it has been found that no acceleration occurs. If more enzyme had been formed, then a greater rate of hydrolysis of sucrose should have occurred than in the control experiment in which all conditions were the same except, of course, the absence of added protein. The results obtained indicate therefore that no more enzyme was formed than was originally present.

The fact that this accelerative influence due to the presence of native protein only occurs on the acid side of the enzyme's optimum *p*H 4.5–5.0, is at the present difficult to understand. Von Euler and Josephson[9] have suggested that an enzyme such as invertase may be looked upon as a complex composed of a hypothetical active group, of unknown composition, associated in some way with protein, the latter not only serving as a colloidal carrier, as also claimed by Willstätter and co-workers,[10] but also being essential for the active group to function catalytically. If this view is provisionally adopted, and the decrease in the enzyme's activity on the acid side of its optimum *p*H is due to the tendency of the enzyme complex to dissociate, just as hemoglobin dissociates in acid solution to heme and globin, then the accelerating influence due to the added protein may be due to repressing this dissociation by the addition of more protein to the solution.

The fact that the added protein must be in the native condition in order to bring about this accelerative effect on the enzyme's activity at *p*H 3 suggests that it may be related to the necessity for the globin in hemoglobin being in the native condition when the latter is oxygenated to oxyhemoglobin. The native condition of the globin seems to confer upon the hemoglobin the peculiar property of taking up or giving off molecular oxygen as the pressure of the latter is varied. Similarly the addition of native protein to the invertase solutions may cause more of the former to be tied up in some way with a catalytically active group and just as in the case of the globin in hemoglobin confer in this way a higher catalytic activity to the complex or active group.

The terms "native or undenatured" and "denatured" for describing the conditions of the proteins have been used in the following sense. First, a protein soluble at its isoelectric point in dilute aqueous solution of salt was considered undenatured, while if insoluble under the same conditions then it was considered denatured. In order to decide whether the undenatured protein added to the sucrose–invertase solution at *p*H 3 remained in the native condition during the hydrolyses, pepsin, edestin, sera, egg-albumin and globin were allowed to stand for one hour at *p*H 3 in citrate–phosphate buffered solutions at 25°. The conditions were then similar, except for the absence of the sucrose and a higher concentration of protein, to those obtaining in the hydrolysis of sucrose by the invertase, in the presence of undenatured protein. Since denatured protein is precipitated at its isoelectric point, the above described protein solutions at *p*H 3 were carefully titrated with alkali, the isoelectric points of all the proteins used being above *p*H 3. In every case, with the exception of globin, no coagulation of denatured protein was observed, although a slight turbidity developed in the solutions containing edestin. The absence of protein coagulation in the solutions under these conditions was taken as evidence that the protein remained for the most part in its native state, during the time in which it influenced the activity of the enzyme.

(9) H. v. Euler and K. Josephson, *Ber.*, **59**, 1129 (1926).

(10) R. Willstätter, K. Schneider and E. Wensel, *Z. physiol. Chem.*, **151**, 1 (1926).

Experimental Details

Egg albumin was crystallized according to the method of Sörensen.[11]

Denatured egg albumin was prepared by heating an aqueous solution of the crystalline protein to 70° for about ten minutes. In order to prevent the formation of clots, the solution was made slightly alkaline previous to heating and stirred well during the heating. Dilute acetic acid was then carefully added to precipitate the denatured protein. The precipitate was centrifuged off, washed with water and dissolved in dilute hydrochloric acid at *p*H 3.0 and used in the hydrolyses.

Edestin was twice crystallized according to a slight modification of Osborne's method.[12] In a few words, this consists of an extraction of ground hemp seed, freed from oil by petroleum ether, with a 10% aqueous sodium chloride solution at about 65°. The extract after filtration was allowed to cool slowly at room temperature and finally at 5–10°. The crystalline protein was filtered off and washed with 10% sodium chloride solution, then redissolved and reprecipitated in the same manner as outlined. Denatured edestin was prepared by allowing a 10% aqueous sodium chloride solution of the protein to stand at about *p*H 2 (made acid with hydrochloric acid) for several hours at 25°. Ammonium hydroxide was carefully added to flocculate the denatured protein, which was then dissolved in a citrate–phosphate buffer solution (final *p*H 3.0), ready to be used in the hydrolyses.

Globin, which was shown to be undenatured according to the tests described above, was given by Dr. A. E. Mirsky of the Rockefeller Institute for Medical Research, New York City. Globin, which is readily denatured in acid solutions, was simply allowed to remain for five hours at *p*H 3 in a citrate–phosphate buffered solution. The latter was then used as in the case of the above-mentioned denatured proteins.

Blood sera were prepared from freshly obtained lamb or steer blood. The blood was transported from the slaughter house to the laboratory in paraffin lined bottles, kept cool by ice. After clotting and standing in a refrigerator for several days, a clear yellow serum could be decanted off.

In order to maintain these proteins in the undenatured condition as long as possible, crystalline edestin was kept under a 3% sodium chloride solution as suggested by Dr. D. I. Hitchcock of Yale University; crystalline pepsin was kept in half saturated magnesium sulfate, while globin and crystalline egg albumin were suspended in half saturated ammonium sulfate. All protein suspensions and sera were maintained at a low temperature in a refrigerator.

Ruppert's brewery yeast was used as the source of the invertase. The yeast was washed well with water, then autolyzed by mixing with water and stirring into the mass some toluene and allowed to stand for several weeks at room temperature. After filtering off the insoluble residue, the autolysate was treated as follows. First an alcohol precipitation was conducted, and the precipitate obtained extracted with water and dialyzed against running tap water. The dialyzed solution was then fractionally adsorbed to kaolin and eluted by means of disodium phosphate solution and again dialyzed against running tap water. This dialyzed solution was then adsorbed to alumina, eluted by means of disodium phosphate and dialyzed. In one or two instances the dialyzed solutions from the alumina treatment were concentrated by placing the liquid in Visking sausage casings and suspending the latter in a current of air supplied by an ordinary electric fan.[13] In this way it is possible to concentrate yeast invertase solutions usually with very little loss of activity. The concentrated solutions were then saturated with ammonium sulfate and part of the invertase was precipitated, while part was soluble.[13] The two kinds, both after removal of the ammonium sulfate by dialysis, were found to respond to the accelerative influence of native protein at *p*H 3.

The following serves as a key to the labels used in describing the enzyme preparations mentioned in the tables.

- R = yeast from Ruppert's Brewery.
- a = precipitation of the invertase at *p*H 4.5 in 50% alcohol at 0–5°. The precipitate after filtration was eluted overnight with an equal volume of distilled water.
- K = fractional adsorption of invertase to kaolin at *p*H 3.5–4.0, and eluted in part by disodium phosphate at *p*H 8.
- A = fractional adsorption of invertase to alumina at *p*H 6 and eluted by disodium phosphate solution at *p*H 8.
- D = dialysis of the invertase solution against running tap water or distilled water in a rocking dialysor.
- S = saturation of the invertase solution with ammonium sulfate. After filtration the mother liquor contained a certain variable fraction of soluble invertase.

The time value is defined as the number of minutes required for 50 mg. of an invertase preparation to invert, at 15.5°, 4 g. of cane sugar to the point of zero rotation with respect to sodium light, the total volume of solution, containing 1% phosphate, being 25 cc.

The relative activities of the invertase preparations were determined by means of initial rates of hydrolysis of 10% sucrose solutions under the conditions mentioned in the text. When the percentage of sucrose hydrolyzed by means of yeast invertase is plotted against time, the curve obtained up to 10% hydrolysis of the sucrose is practically a straight line, or in other words, the initial rates of hydrolysis during the first 10% hydrolysis are practically constant, and when the changes in rotation in degrees per minute are plotted against time, the straight line connecting the points intersects the origin.

All sucrose solutions were prepared by dissolving 28.125 g. of sucrose in citrate–phosphate buffered solution, and adjusting the final volume to 250 cc. at 25°. Two hundred cc. of the sucrose solution was delivered into a reaction flask, placed in a thermostat at 25.00 ± 0.01°. Twenty-five cc. of an invertase solution previously brought to the same temperature was then added by means of a pipet delivering in seven seconds, and the time at which the addition was made recorded. The reaction mixture was

(11) S. P. L. Sörensen, *Compt. rend. trav. lab. Carlsberg*, **12** (1916).

(12) T. B. Osborne, THIS JOURNAL, **24**, 39 (1902).

(13) J. G. Lutz, "Dissertation," Columbia University, 1934

shaken thoroughly to ensure homogeneity of the solution. By making up solutions in the manner outlined, the resultant sucrose concentration of the reaction mixture was 10%.

At recorded time intervals, twenty-five cc. of the reaction mixtures were delivered from a pipet into bottles containing saturated alkali in sufficient[14] amount to stop the hydrolysis. After allowing about five to fifteen minutes for mutarotation, the rotation of the solutions in the bottles was determined.

The buffered solutions were prepared by mixing, in definite proportions by volume $M/10$ citric acid and $M/5$ disodium phosphate solutions, the actual proportions depending upon the pH desired. It is obvious that the salt concentrations varied with the pH (0.12 M at pH 3 and 0.18 M at pH 6.5), but since control hydrolyses were always conducted under the same conditions of hydrogen-ion concentration, temperature, etc., and since only relative differences of activity were significant, this factor was disregarded.

If protein was to be present in the reaction mixture, it was usually added to the sucrose solution before adjusting the latter to standard volume. However, it was shown that no difference, within experimental error, could be detected if protein was added first to the enzyme solution or to the sucrose solution.

Concentrations of protein greater than 50 mg. in 225 cc. were deliberately avoided because of the increased viscosity, and the tendency to foam made the drawing of samples by means of a pipet difficult, if air bubbles were to be excluded from the sample when drawn up into the pipet.

(14) When citrate–phosphate buffer solutions at pH 3 were used it was necessary to add five drops of saturated sodium hydroxide, while at pH 4.6, only three drops were required. However, since all samples removed from the reaction mixture were treated in the same way, for any given pH it is clear that the reaction rates were not affected.

The change in the hydrogen-ion concentration of the buffered sucrose solutions when 10 to 50 mg. of protein were added, was at most one or two hundredths of a pH unit, so that the effect on the rate of reaction of such a difference is within experimental error. Finally the pH, as measured by a hydrogen electrode, of the reaction mixture was demonstrated to remain unaltered during the course of hydrolysis, by making such determinations before and after hydrolysis of the sucrose.

Before using undenatured protein, a solution of the protein was always examined to determine the presence of denatured protein. If more than a faint opalescence was observed in the solution, the protein was recrystallized or an entirely new quantity prepared.

Summary

1. Undenatured egg albumin, edestin, pepsin, globin and serum accelerate the activity of purified yeast invertase preparations at pH 3 but not at 4.5.

2. In the undenatured state edestin does not affect the activity of invertase at the pH optimum of yeast invertase or at the pH values 5.88 and 6.51.

3. Gum arabic, gelatin, glycylglycine and tryptophan do not affect the activity of purified invertase preparations at pH 3.

4. Undenatured serum and edestin did not affect the activity of several crude invertase solutions at pH 3.

5. Denatured edestin, pepsin, egg albumin or globin do not affect the activity of highly purified invertase preparations at pH 3.

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[CONTRIBUTION FROM STANDARD ALCOHOL CO.]

The Reaction of Propylene, Pentene-1 and Pentene-2 with Sulfuric Acid

BY BENJAMIN T. BROOKS

Kharasch and his co-workers have indicated that the presence of organic peroxides influences the addition of hydrogen bromide to olefins of the type $RCH{=}CH_2$, peroxides favoring the formation of primary halides, and in the case of propylene reported the formation of large proportions of normal propyl bromide.[1] Ingold and Ramsden[2] reported the formation of 24.8% normal propyl bromide in propane solution and 2.8% in a water solution. Sherrill, Mayer and Walter[3] have recently shown that pentene-1 and heptene-1, on treating with hydrogen bromide in glacial acetic acid, hexane or carbon tetrachloride, give exclusively 1-bromopentane and 1-bromoheptane, respectively, and that 48% aqueous hydrobromic acid gave only the 2-bromo derivatives from these olefins. Ingold and Ramsden[2] have also shown that in water solution only 2-bromopentane is formed from pentene-1.

In view of these facts and since the reaction of these and other olefins with sulfuric acid, followed by hydrolysis to alcohols, is carried out industrially on a large scale, it was of interest to compare the manner of the addition of sulfuric acid to ole-

(1) Kharasch, McNab and Mayo, THIS JOURNAL, **55**, 2531 (1933).
(2) Ingold and Ramsden, *J. Chem. Soc.*, 2752 (1931).
(3) Sherrill, Mayer and Walter, THIS JOURNAL, **56**, 926 (1934).