groups or both are necessary to the amylase activity. The extreme slowness of this irreversible inactivation, however, favors the idea that tyrosine rather than amino groups are involved. This conclusion was strengthened by further study. When the log fraction of the amylase activity which remained after inactivation by nitrous acid and subsequent reactivation with hydrogen sulfide was plotted against time, the irreversible inactivation was found to be first order with respect to the amylase. Furthermore, the constant obtained for this reaction was of the same order of magnitude as that obtained under similar conditions by Little and Caldwell[3b] for the formation of the azo compound with pure tyrosine. In addition, the same constant was obtained when the inactivation and reactivations were repeated with another concentration of the amylase.

These results appear to rule out any appreciable influence either of deaminazation or of drastic oxidations and lead to the conclusion that free tyrosine is essential to the activity of beta-amylase.

Acetylation with Ketene.—The investigation was continued by the use of ketene. This reagent is efficient for the acetylation of primary amino groups, sulfhydryl groups and phenolic hydroxyl groups of proteins[8a,b,c] but has been reported[8c] not to react appreciably with the guanidino groups or with the aliphatic hydroxyl groups of amino acid and proteins. Moreover, the reaction with primary amines is much more rapid than that with phenolic hydroxyls.[8a]

A number of experiments with different solutions of β-amylase showed that treatment with ketene caused a much more rapid loss of amino nitrogen than of amylase activity. Thus, in a typical experiment, when purified β-amylase in 0.25 *M* disodium phosphate solution was held at 0° and treated with ketene for fifteen minutes, a loss of 37% of the amino nitrogen was accompanied by a loss of only 10% of the amylase activity.

These results with β-amylase are in marked contrast to those previously obtained with pancreatic amylase[3a] and strengthen the conclusion reached as a result of the experiments with nitrous acid that free amino groups probably are not essential to the activity of β-amylase. In addition, since sulfhydryl groups are also acetylated by ketene under the conditions of these experiments, it seems possible and even probable that the small losses of amylase activity, observed during the early stages of the acetylation of β-amylase with ketene, as outlined above, were due, at least in part, to the blocking of sulfhydryl groups of the protein.

Attempts to use the method of Herriot and Northrop[4a,b] to determine whether any part of the loss of the activity of β-amylase upon treatment with ketene was due to the acetylation of tyrosine were not successful as the acid and alkali employed inactivated the amylase.

(8) (a) H. Staudinger, "Die Ketene," F. Enke, Stuttgart, 1912; (b) M. Bergmann and F. Stern, *Ber.*, **63**, 437 (1930); (c) A. Neuberger, *Biochem. J.*, **32**, 1452 (1938).

Summary

A study of the influence of nitrous acid upon the activity of β-amylase from barley and from malted barley has been made.

The influence of acetylation with ketene upon the amylase activity and amino nitrogen of solutions of β-amylase also has been observed.

Consideration of the results leads to the conclusion that free sulfhydryl and free tyrosine groups are essential while free amino groups probably are not essential to the activity of β-amylase from barley and from malted barley.

Additional conclusive evidence of the importance of free sulfhydryl groups to the activity of β-amylase is given in the second paper of this series.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

A Study of the Essential Groups of β-Amylase.[1] II. Sulfhydryl Groups

BY C. EDWIN WEILL[2] AND M. L. CALDWELL

Previous work[3] led to the conclusion that free sulfhydryl groups are essential to the activity of β-amylase from barley and from malted barley. This conclusion rests on the observation that β-amylase activity is lost by treatment with nitrous acid and that it may be completely recovered, in the early stages of the reaction at least, by subsequent treatment with hydrogen sulfide.

The present investigation confirms this conclusion in a number of other ways. A study has now been made of the influence on the activity of β-amylase of other oxidizing agents and especially of reagents which appear to be specific for sulfhydryl groups. Particular attention has been given to those reactions which may be reversed by means of hydrogen sulfide or of cysteine.

(1) Grateful acknowledgment is made to Mr. Robert Schwarz of the Schwarz Laboratories, Inc., and to The Ladish-Stoppenbach Company who kindly furnished the barley and malted barley used in this investigation.

(2) This work is taken from a dissertation submitted by C. Edwin Weill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University. The major portion of this paper was presented at the New York meeting of the American Chemical Society, September, 1944.

(3) C. E. Weill and M. L. Caldwell, THIS JOURNAL, **67**, 212 (1945).

Experimental

Highly purified preparations of β-amylase[4] which showed no measurable α-amylase activity were used. These preparations and the general procedures employed have been described in a previous paper.[3]

Results

The Nitroprusside Reaction.—The nitroprusside reaction is a very sensitive test for sulfhydryl groups. Under certain specified conditions[5] it may be used, in comparison with standard solutions of cysteine, to give fair estimations of the concentrations of sulfhydryl present in protein solutions.

All the solutions of β-amylase examined showed evidence of sulfhydryl groups as determined by the nitroprusside reaction.[5] The concentrations were equivalent to 1×10^{-4} to 1×10^{-3} millimole of sulfhydryl per ml. of amylase solution. It is interesting to note that the amylase activities of the solutions were found to be directly proportional to the concentrations of sulfhydryl thus determined.

Experiments with Iodine.—Iodine has been reported to inactivate many enzymes.[6] Hanes[6c] found that the activity of β-amylase from barley is completely lost upon treatment with dilute iodine solutions.

With certain enzymes, especially those in which sulfhydryl groups are essential to the activity, the inactivation caused by iodine may often be partially reversed by treatment with reducing agents such as cysteine or hydrogen sulfide.[6a,b,e] In such cases, the extent of the reactivation depends upon the particular enzyme and upon the conditions of the inactivation. If the treatment with iodine is too drastic, the inactivation is irreversible. Oxidation of sulfhydryl beyond the reversible disulfide stage,[6e] or the iodination of tyrosine to form diiodotyrosine,[7] are probably important causes of the irreversible inactivation of these enzymes with iodine. A reversible inactivation of an enzyme with iodine appears to constitute fairly good evidence that sulfhydryl groups are essential to the enzyme activity.[6a,b,e]

In view of these considerations, an investigation was made of the inactivation of β-amylase by iodine, and of the possibility of its reversal by hydrogen sulfide. The results of a number of such experiments confirmed the findings of Hanes,[6c] that dilute iodine solutions completely inactivate β-amylase. They showed, in addition, that 10 to 15% of the activity can be recovered by treatment of the inactivated solutions with hydrogen sulfide or cysteine.

As finally developed, the inactivation with iodine was carried out by adjusting the amylase solution to 0.1 *M* acetate, *p*H 4.5, 0.0000776 *M* iodine and 0.02 *M* potassium iodide and holding it at 0° for thirty minutes At the end of this time, one drop of 0.05 *M* sodium thiosulfate was added. Aliquots of the solution were then diluted with equal volumes of water or of a saturated solution of hydrogen sulfide or of cysteine and examined for amylase activity. The influence of the iodine on the amylase was judged not only by the usual thirty-minute activity measurements[3] but also by activity measurements of longer duration. Typical data are given in Fig. 1.

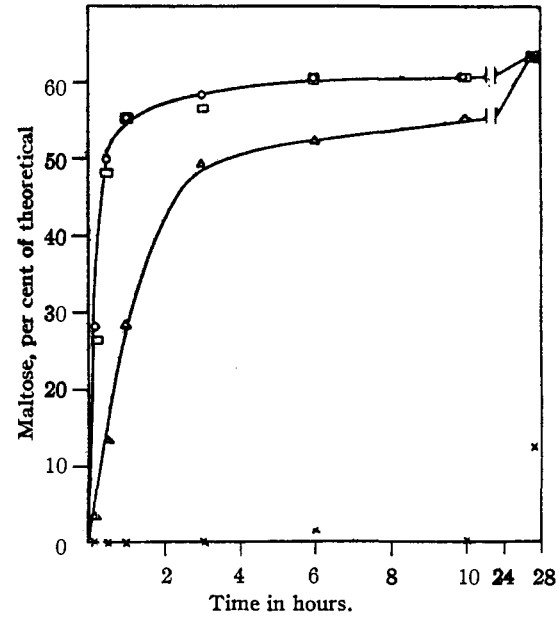

Fig. 1.—Conversion of starch to maltose by β-amylase before and after inactivation with iodine and after reactivation with hydrogen sulfide: ○, control, untreated amylase solution; ×, same solution after treatment with iodine; □, same solution after treatment with hydrogen sulfide but not with iodine; △, same solution after treatment with iodine followed by treatment with hydrogen sulfide. Reducing values of the reaction mixtures are given as per cent. of the maltose which could theoretically be obtained from the starch.

The data given in Fig. 1 show that β-amylase which has been inactivated by treatment with dilute solutions of iodine may be partially reactivated by treatment with hydrogen sulfide. This reactivation is interpreted to mean that sulfhydryl groups, essential to the activity of the amylase, were oxidized reversibly to disulfide by the iodine and reduced back by the hydrogen sulfide.

Confirmation of this conclusion was found in the close correlation obtained between the concentrations of sulfhydryl shown to be present in the

(4) M. L. Caldwell and S. E. Doebbeling, *J. Biol. Chem.*, **110**, 739 (1935), and unpublished work.

(5) M. L. Anson, *J. Gen. Physiol.*, **24**, 399 (1940–1941).

(6) (a) L. Hellerman, M. E. Perkins and W. M. Clark, *Proc. Natl. Acad. Sci.*, **19**, 855 (1933). (b) L. Hellerman and M. E. Perkins, *J. Biol. Chem.*, **107**, 241 (1934). (c) C. S. Hanes, *Dept. Sci. Ind. Res. (Brit.). Rept. Food Invest. Board*, **1937**, 115 (1938). (d) L. Rapkine, *Biochem. J.*, **32**, 1729 (1938); (e) L. Hellerman, *Physiol. Rev.*, **17**, 454 (1937).

(7) R. M. Herriot, *J. Gen. Physiol.*, **20**, 335 (1936–1937).

amylase solutions by the nitroprusside test[5] and the concentrations of iodine found necessary completely to inactivate the amylase.

Experiments with Ferricyanide and Cupric Ions.—The results obtained when β-amylase was treated with low concentrations of ferricyanide ions or of cupric ions, alone or in combination, are illustrated by the data summarized in Table I.

TABLE I

INACTIVATION OF β-AMYLASE BY FERRICYANIDE AND CUPRIC IONS AND SUBSEQUENT REACTIVATION WITH HYDROGEN SULFIDE

Concn. of ferricyanide ions,[1] millimoles per ml.	Concn. of cupric ions,[a] mg. per ml.	After inactivation Activity[b] Units[d]	After inactivation Activity[b] % of control	After reactivation Activity[c] Units[d]	After reactivation Activity[c] % of control
2×10^{-5}	0.02	88	7	755	57
10×10^{-5}	0.02	141	11	1240	87
0	0.02	1180	96	1310	99
20×10^{-5}	0	1080	88	1330	100
0 (Control)[e]	0	1230	100	1330	100

[a] Amylase solution adjusted to 0.1 *M* acetate and *p*H 4.5 reacted with reagent at 0° for thirty minutes. [b] Aliquots of treated solutions diluted with distilled water. [c] Aliquots of treated solutions diluted with a solution of hydrogen sulfide. [d] Milligrams of maltose formed in thirty minutes at 40° by one milligram of enzyme acting on 1% soluble potato starch at *p*H 4.5 in the presence of 0.01 *M* acetate. The concentrations of amylase were adjusted so that the maltose formed was linearly proportional to the concentration of amylase used. [e] Control: Original amylase solution held under identical conditions but without added reagent and diluted either with water or with a saturated solution of hydrogen sulfide.

These data show that low concentrations of ferricyanide ions alone or of cupric ions alone have little if any influence upon the activity of β-amylase under the conditions of these experiments. When used in combination, however, these ions caused marked losses of β-amylase activity. Moreover, significant reactivation was observed upon subsequent treatment with hydrogen sulfide. The concentrations of ferricyanide ions used here were considerably below those employed by Anson[8] to oxidize the sulfhydryl groups of denatured egg albumin. The importance of the presence of both ferricyanide and cupric ions is in accord with the findings of Hellerman, *et al.*,[6a] for certain other enzymes which depend for their activities upon sulfhydryl groups.

Experiments with Other Oxidizing Agents.—Neither cystine nor 2,6-dichlorophenolindophenol had any measurable influence on the activity of β-amylase. These results also are in accord with the findings of other investigators[6b,9] working with other enzymes which are reported to contain active sulfhydryl groups.

Experiments with Special Sulfhydryl Reagents.—Aryl-mercuric compounds react with sulfhydryl groups[6e] and have been found to inactivate urease and papain.[6b,e,9] Reactivation was obtained in each of these cases by treatment with hydrogen sulfide, hydrogen cyanide or cysteine.[6b,e,9]

That these mercury compounds are specific reagents rather than general enzyme inactivators is shown by the fact that they have no significant influence on insulin,[10] arginase[11] or pancreatic amylase.[12]

Typical results showing the influence of two aryl-mercuric compounds upon the activity of β-amylase are given in the first two sections of Table II.

TABLE II

INACTIVATION OF β-AMYLASE BY SPECIFIC SULFHYDRYL REAGENTS AND SUBSEQUENT REACTIVATION WITH CYSTEINE

Treatment Reagent	Millimoles per ml. of enzyme soln.	After inactivation Activity[c] Units[d]	After inactivation Activity[c] % of control	After reactivation Activity[d] Units[d]	After reactivation Activity[d] % of control
Phenylmercuric-chloride[a]	0	930	100	1000	100
	(satd. soln.)	0	0	1010	101
p-Chloromercuri-benzoic acid[a]	0	1330	100	1330	100
	2×10^{-4}	0	0	1110	83
	20×10^{-4}	0	0	1070	80
Iodoacetamide[b]	0	1100	100	1290	100
	0.1	430	39	470	36

[a] Amylase solution adjusted to 0.1 *M* acetate, *p*H 4.5, reacted with reagent for thirty minutes at 0°. [b] Amylase solutions were adjusted to 0.1 *M* phosphate, *p*H 6.8, treated with the reagent and held at 25° for one hundred and twenty minutes. The control was treated in the same way except that no iodoacetamide was added. [c] Aliquots of treated solutions diluted with distilled water. [d] Aliquots of treated solutions diluted with a solution of hydrogen sulfide or of cysteine. [e] Milligrams of maltose formed in thirty minutes at 40° by one milligram of enzyme acting on 1% soluble potato starch at *p*H 4.5 in the presence of 0.01 *M* acetate. The concentrations of amylase were adjusted so that the maltose formed was linearly proportional to the concentration of amylase used.

In each case the buffered amylase solution adjusted to 0.1 *M* acetate and *p*H 4.5 was allowed to react with the reagent for thirty minutes at 0°. Aliquots of the solutions were then diluted either with water or with a solution of hydrogen sulfide or of cysteine. Hydrogen sulfide was used for the reactivation in the case of phenylmercuric chloride while cysteine was used for the reactivation in the case of *p*-chloromercuribenzoic acid.

The results given in the first two sections of Table II show that these aryl mercuric compounds completely inactivate β-amylase and that the amylase activity may be completely or largely recovered by subsequent treatment with hydrogen sulfide or with cysteine. These results are further evidence that free sulfhydryl groups in the protein are necessary to the activity of β-amylase.

Confirmatory evidence of this conclusion was given by the fact that amylase solutions which had

(8) M. L. Anson, *J. Gen. Physiol.*, **23**, 247 (1939–1940).

(9) L. Hellerman, F. P. Chinard and V. R. Dietz, *J. Biol. Chem.*, **147**, 443 (1943).

(10) E. D. Schock, J. Jensen and L. Hellerman, *ibid.*, **111**, 553 (1935).

(11) L. Hellerman and M. E. Perkins, *ibid.*, **112**, 175 (1935–1936).

(12) C. E. Weill and M. L. Caldwell, unpublished.

been inactivated by treatment with either of these aryl-mercuric reagents no longer gave a nitroprusside reaction[5] and by the close agreement found between the concentration of *p*-chloromercuribenzoic acid necessary to inactivate the amylase completely and the concentration of sulfhydryl[5] shown to be present in the untreated amylase solution.

Iodoacetamide is another reagent which reacts with sulfhydryl groups. If the hydrogen-ion activities of the solutions are kept below *p*H 8, the reaction is considered specific for thiol groups.[13] Certain enzymes such as urease[9] are irreversibly nactivated by iodoacetamide while the activity of others such as pancreatic amylase[12] is not appreciably influenced by this reagent.

The data given in the last section of Table II, are typical of the results obtained when β-amylase was treated with iodoacetamide under conditions reported to be specific for sulfhydryl groups.[13] The amylase was markedly inactivated under these conditions and the inactivation was not reversed by subsequent treatment of the solutions with cysteine. β-Amylase resembles urease in this respect.[13] The results with iodoacetamide increase the evidence that free sulfhydryl groups are essential to the activity of β-amylase from barley and from malted barley.

(13) (a) C. V. Smythe, *J. Biol. Chem.*, **114**, 601 (1936). (b) L. Michaelis and M. P. Schubert, *ibid.*, **106**, 331 (1934).

Summary

The results of experiments with a number of oxidizing agents and of other reagents which have been reported to be specific for sulfhydryl groups confirm and extend previous evidence which leads to the conclusion that free sulfhydryl groups of the protein are necessary to the activity of β-amylase from barley and from malted barley.

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Mechanism of Vinyl Polymerization. I. Role of Oxygen[1]

BY CARL E. BARNES[2]

It has been known since the very beginning of polymerization studies that light, heat and air are effective agents for inducing the polymerization of certain vinyl compounds.[3] For example vinyl chloride[4] and vinyl acetate[5] may be polymerized by irradiation with ultraviolet light. Both these substances also polymerize readily upon heating to moderate temperatures, and it has been reported frequently that oxygen, ozone and peroxides have a pronounced catalytic effect upon the rate of this thermal polymerization. In fact, it has been demonstrated repeatedly that in the complete absence of oxygen, many vinyl compounds either fail to polymerize altogether or the rate is very slow.[6] On the other hand it has been established that oxygen acts as a marked inhibitor in the photopolymerization of vinyl acetate.[7] This paper is concerned with the elucidation of this apparent anomaly and its relation to the mechanism of polymerization reactions of the vinyl type.

The mechanism of the catalytic action of oxygen is undoubtedly related to peroxide formation. The presence of peroxides in many vinyl compounds has been observed by various investigators.[8] Moureu and Dufraisse[6,9] clearly established that it is combined oxygen rather than oxygen itself which catalyzes the polymerization of acrolein. Conant[10] concluded that the catalytic effect of oxygen in the pressure polymerization of isoprene is due to the formation of a peroxide.

These conclusions of Moureu and Dufraisse and of Conant, *et al.*, are undoubtedly of a very general nature. During the course of this investigation no compound containing the vinyl or vinylidene group has been found which does not readily form a peroxide upon exposure to air under ordinary conditions; furthermore these peroxides have all been found to act as polymerization catalysts.

Less well established is the nature of the inhibiting action of oxygen in photopolymerization. It seemed to the author that this apparent anomaly might be explained very simply by assuming peroxide formation to occur in preference to polymerization in accordinace with the following scheme

(1) $M + UVL \longrightarrow M^*$
(2) (a) $M^* + O_2 \longrightarrow MO_2$ (preferred reaction)
(b) $M^* + M \longrightarrow MM^* + M \longrightarrow MMM^*$, etc.

where M represents the unactivated monomer molecule; M*, the active center and MO_2, a peroxide. It must be further assumed that the catalytic activity of the peroxide is due to its tendency to generate M* (or similar active center) and that these active centers exhibit the same

(1) The experimental work described here was carried out in 1939 while the author was with the Norton Company, Worcester, Mass., and has now appeared in British Patent 549,234 (1942).

(2) Present address: General Aniline and Film Corp., Central Research Laboratory, Easton, Penna.

(3) Redtenbacher, *Ann.*, **47**, 113 (1843); Baumann, *ibid.*, **163**, 312 (1872).

(4) Baumann, *ibid.*, **163**, 217 (1872).

(5) Staudinger, Frey and Starck, *Ber.*, **60B**, 1782 (1927).

(6) Moureu and Dufraisse, *Bull. soc. chim.*, [4] **35**, 1564 (1924); Staudinger and Schwalback, *Ann.*, **488**, 8 (1931); E. I. du Pont de Nemours & Co., *Ind. Eng. Chem.*, **28**, 1161 (1936).

(7) Staudinger and Schwalback, *loc. cit.*; Taylor and Vernon, THIS JOURNAL, **53**, 2529 (1931).

(8) Staudinger and Lautenschläger, *Ann.*, **488**, 1 (1931); Pratesi and Celeghini, *Gazz. chim. ital.*, **66**, 365 (1936).

(9) Moureu and Dufraisse, *Chemistry & Industry*, **47**, 828 (1928).

(10) Conant and Tongberg, THIS JOURNAL, **52**, 1666 (1930); Conant and Peterson, *ibid.*, **54**, 628 (1932).