in vacuo at 50°. Water was added to the residue and then distilled out at reduced pressure. The process was repeated. Sixty cc. of 2 *N* hydrochloric acid was added and the solution was heated under reflux for four hours. The resultant solution was treated with Norite and then concentrated to dryness under nitrogen *in vacuo* at 40°. The residue was taken up in 20 cc. of water. About 3–5 g. of solid sodium acetate was added and followed by saturated sodium carbonate until the *p*H was 4.5–5. The solution was cooled in an ice-bath and 5 g. of potassium cyanate in 15 cc. of water was added. The *p*H was adjusted to 6–6.5 with 5 *N* sodium hydroxide. It was warmed to 40° and allowed to stand one-half to one hour at room temperature. The solution was then adjusted to *p*H 4 by the addition of dilute hydrochloric acid and cooled in an ice-bath. The product separated as a thick mass which was filtered and washed with ice water. The pressed precipitate was dissolved immediately in a minimum of hot absolute alcohol and poured into about 10 volumes of water. After cooling in the refrigerator, it was filtered and dried *in vacuo* over phosphorus pentoxide at room temperature.

The product was hygroscopic and usually had some color (weight, 4.2–4.8 g., 50–56%). When placed in a bath at 158°, it melted at 168° with decomposition.

Anal. Calcd. for $C_{10}H_{16}O_3N_2$: N, 13.20. Found: N, 12.85.

The product gave a characteristic purple-colored solid when treated with bromine in carbon tetrachloride.

Desthiobiotin Isomers, VII.—A 5% sodium bicarbonate solution was added to 10 g. of the imidazolone until a *p*H of 7.5 was attained. The solution was filtered and diluted to 120 cc. with water. Approximately 10 cc. of Raney Ni catalyst was added. The mixture was placed in a 300-cc. bomb and shaken at 2300 lb. pressure and 100° for thirty-six hours. The solution was filtered from the catalyst and the *p*H (11.3) was reduced to 8.5 with 10 *N* sulfuric acid. This was decolorized with Norite and then acidified to Congo red with 10 *N* sulfuric acid. The product separated as colorless crystals. These were washed with ice water and dried in a desiccator over phosphorus pentoxide (weight, 6.3 g., m. p. 136–137°). On cooling, the mother liquor deposited 1.2 g. of rosets, m. p. 135–139°. On concentration of the mother liquor a further crop of 0.78 g., m. p. 130–136°, was isolated. The analyses indicated desthiobiotin had been formed by the reduction.

Anal. Calcd. for $C_{10}H_{18}O_3N_2$: C, 56.03; H, 8.46; N, 13.07. Found: C, 56.14; H, 8.37; N, 12.99, 12.84, 13.22.

By assay with yeast, the first crop of crystals was found to have 30–35% of the activity of an equal weight of biotin. The second was assayed at 20–25%.

As a further check the structure of the product was established by conversion to diaminopelargonic acid by the action of barium hydroxide.[4] The diamino acid was converted to the dibenzoquinoxaline derivative by the action of phenanthrenequinone. This derivative was shown to be identical with the derivative obtained from desthiobiotin prepared from biotin by comparison of its physical properties. A mixture of the two dibenzoquinoxaline samples showed no depression of the melting point.

Due to the presence of the two asymmetric carbons, the synthetic desthiobiotin might be expected to consist of a mixture of the two racemic forms. A sample of the desthiobiotin isomers showed little change in microbiological activity or in melting point with repeated crystallization from water. Some indication of fractionation was obtained. One hundred milligrams of the mixture was dissolved in 10 cc. of water and passed over 10 cc. of Permutit. The effluent solution was concentrated to obtain 31 mg. of product melting at 151–154°. After recrystallization from water, 25 mg. was obtained which melted at 157–159° and showed 55–60% of the activity of an equal weight of biotin on yeast. The mother liquors yielded 41 mg. of crystalline material, m. p. 138–140°, assaying at 35–40% of biotin.

Summary

A total synthesis of *dl*-desthiobiotin has been reported. The product obtained appeared to be a mixture of the two possible racemic forms. This mixture is one-third as active as biotin in promoting the growth of yeast in a medium deficient in biotin.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

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

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

Selective reagents which react with free groups of proteins have become increasingly important in protein chemistry. They are of special value in the study of proteins which have characteristic activities such as certain hormones and enzymes. By careful choice of such reagents, and of the conditions, it is often possible to block or to alter one or more of the free groups of a protein without affecting the others, and, when there is characteristic activity, to ascertain which, if any, of these groups is connected with or essential to its activity. The groups which have been studied most often are the free amino,[3] the free tyrosine[4] and the free sulfhydryl groups[5] of proteins.

The present investigation deals with a study of the influence of a number of selective reagents upon the activity of β-amylase from barley and

(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) (a) J. E. Little and M. L. Caldwell, *J. Biol. Chem.*, **142**, 585 (1942); (b) J. E. Little and M. L. Caldwell, *ibid.*, **147**, 229 (1943); (c) A. M. Pappenheimer, Jr., *ibid.*, **135**, 20 (1938); (d) C. H. Li, M. E. Simpson and H. M. Evans, *ibid.*, **131**, 259 (1939); (e) T. R. Wood and W. F. Ross, *ibid.*, **146**, 59 (1942).

(4) (a) R. M. Herriot and J. H. Northrop, *J. Gen. Physiol.*, **18**, 35 (1934–1935); (b) R. M. Herriot, *ibid.*, **19**, 283 (1935–1936); (c) K. G. Stern and A. White, *J. Biol. Chem.*, **122**, 371 (1937–1938); (d) J. St. L. Philpot and P. A. Small, *Biochem. J.*, **32**, 542 (1938).

(5) (a) L. Hellerman, M. E. Perkins and W. M. Clark, *Proc. Nat. Acad. Sci.*, **19**, 855 (1933); (b) L. Hellerman and M. E. Perkins, *J. Biol. Chem.*, **107**, 241 (1934); (c) C. V. Smythe, *ibid.*, **114**, 601 (1936); (d) A. K. Balls and H. Lineweaver, *Nature*, **144**, 513 (1939); (e) L. Hellerman, F. P. Chinard and V. R. Dietz, *J. Biol. Chem.*, **147**, 443 (1943).

from malted barley. This amylase has been highly purified and found to be a protein.[6] Because of its distinctive action on starches,[7] it seemed of special interest to ascertain whether any relationship could be established between free groups of the protein and its amylase activity and also how such essential groupings, if present, would compare with those already established for another starch splitting enzyme, namely, pancreatic amylase.[3a,b]

Experimental

Highly purified preparations of β-amylase were obtained from barley and from malted barley by the method of Caldwell and Doebbeling.[6] These exerted no measurable α-amylase activity. These preparations were used wherever feasible. In some cases when relatively large volumes of amylase solutions were required, less highly purified solutions were used. These were treated to inactivate α-amylase,[7a] and were also found to exert no measurable α-amylase activity.

The influence of each reagent upon the amylase activity was judged by direct comparisons of the activity of the amylase solution under observation with that of an aliquot of the same amylase solution which had been treated, in an otherwise identical manner, except for the reagent concerned. These aliquots are referred to as controls in the text and table. None of the reagents was found to influence the activity measurements in the concentrations usually employed. Exceptions with larger concentrations were taken care of by the use of suitable blanks.

The amylase activities were all measured under the same specified conditions[6] with 1% soluble potato starch adjusted to 0.01 molar acetate and *p*H 4.5. The saccharogenic activity refers to the milligrams of maltose formed in thirty minutes at 40° by one milligram of the enzyme under these conditions when the concentrations of amylase are selected so that the maltose formed is linearly proportional to the enzyme used.

Results

Experiments with Nitrous Acid.—In addition to its oxidizing action, nitrous acid reacts with free aliphatic amino groups and with free tyrosine groups of proteins. The latter two reactions may be distinguished from each other by the fact that the former is much more rapid than the latter[4d] and by the further characteristic that the reaction with amino groups is third order and becomes second order in the presence of excess nitrite while the reaction with tyrosine is second order and becomes first order in the presence of excess nitrite.[4d]

The data given in the first section of Table I show that β-amylase is inactivated by nitrous acid. When compared with that obtained with pancreatic amylase,[3a,b] the inactivation of β-amylase by nitrous acid is found to be relatively slow. Thus, β-amylase lost only 26% of its activity in sixty minutes under conditions which had caused pancreatic amylase to lose 65% of its activity in thirty minutes. This relatively slow reaction indicates that the inactivation of β-amylase is probably not due, like that of pancreatic amylase,[3a,b] to a loss of amino groups. Moreover, the inactivation of β-amylase did not follow the course of a first or of a second order reaction which would be expected if amino or tyrosine groups were primarily involved.[4d] The inactivation of β-amylase, while slow compared with that of pancreatic amylase,[1a,b] was much more rapid in the first sixty minutes than in the later stages of the reaction: 26% in one hour, 32% in two hours and 41% in four hours. These results also indicated that reactions other than those with amino and tyrosine groups were involved and suggested that oxidation of some essential grouping such as the sulfhydryl might account for the rather sharp initial loss of β-amylase activity.

This point was investigated by the use of hydrogen sulfide. If sulfhydryl groups had been oxidized to disulfide by the nitrous acid, they might be expected to be restored by the action of hydrogen sulfide, whereas the reactions of nitrous acid with amino and with tyrosine groups are irreversible. It seemed, therefore, that it might be possible to distinguish between irreversible and reversible losses of β-amylase activity and thus to measure the magnitude of the effect of the oxidation of sulfhydryl groups. Typical data on this point are also given in Table I.

The data summarized in Table I show that β-amylase which has been inactivated by nitrous acid may be completely reactivated in the early stages of the reaction by subsequent treatment with hydrogen sulfide. After several hours of reaction with nitrous acid, a slow irreversible inactivation of the amylase also becomes apparent.

TABLE I

INACTIVATION OF β-AMYLASE BY NITROUS ACID AND SUBSEQUENT REACTIVATION WITH HYDROGEN SULFIDE

Reaction with nitrous acid,[a] hours	After inactivation, Activity Units[b]	% Control	After reactivation, Activity Units[b]	% Control
0	4220	100	4340	100
1	3100	74	4340	100
2	2850	68	4340	100
4	2480	59	4170	95
6	..	..	3720	86
8	..	..	3420	79
12	..	..	2980	69
16	..	..	2460	59
25	..	..	1860	43
Control	4220	100	4340	100

[a] Enzyme solution held at 0° in presence of 1.0 *M* nitrite, 0.25 *M* acetate at *p*H 4.6. [b] 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.

The reversible inactivation suggests that sulfhydryl groups are essential to the activity of beta-amylase. If the possibility of drastic oxidations which might break up the protein molecule is disregarded, the irreversible inactivation might be taken to indicate that either tyrosine or amino

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

(7) (a) E. Ohlsson, *Z. physiol. Chem.*, **189**, 17 (1930); (b) C. S. Hanes, *Can. J. Res.*, **B13**, 185–208 (1935); (c) G. A. Van Klinkenberg, *Z. physiol. Chem.*, **212**, 173 (1932).

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.

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