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Essential Groups of Porcine Pancreatic Amylase and of Taka Amylase. Acetylation with Acetic Anhydride[1]

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RECEIVED DECEMBER 15, 1958

Crystalline porcine pancreatic amylase and crystalline taka amylase, each has been reacted with acetic anhydride under conditions that, in themselves, do not cause inactivation of the amylase. The results confirm and extend the findings of Little and Caldwell that free primary amino groups of porcine pancreatic amylase are essential to its enzymic action and show, in addition, that they also are essential to the activity of taka amylase. Moreover, the evidence reveals that only approximately 50% of the free primary amino groups of the protein are essential to the activity of either enzyme and, in addition, that in both cases the α-amino or N-terminal amino groups are more important in this respect than the ϵ-amino groups of lysine. Thus, although taka amylase and porcine pancreatic amylase differ markedly in their action on the same substrates, these two α-amylases both require free primary amino groups of the protein for their catalytic activity and both differ in this respect from β-amylase. The results reported here also confirm and extend the indications obtained by Little and Caldwell that the phenolic hydroxyl groups of tyrosine in porcine pancreatic amylase are not involved in its enzymic activity.

Introduction

The chemical modification of biologically active proteins such as enzymes, viruses and hormones with specific reagents has proven useful in determining the nature of the groups responsible for their biological action. Such studies with β-amylase from a number of different sources including barley,[3] malted barley[3] and sweet potatoes (*Ipomea Batatas*)[4,5] have shown that free sulfhydryl groups of the protein and free phenolic hydroxyl groups of its tyrosine are essential to its amylase activity but that free primary amino groups have little if any importance to its catalytic action. On the other hand, free primary amino groups of the protein are essential to the catalytic action of the α-amylase, porcine pancreatic amylase,[6a,7] while free phenolic hydroxyl groups of its tyrosine are of little if any importance to its activity.[6b]

In addition, the accumulated evidence indicates that β-amylase from different sources has the same action on its substrates,[8-14] whereas α-amylases from different sources often differ markedly in their action on the same substrates.[8-10,15-19] Moreover, a direct comparison of several α-amylases[19] has shown that porcine pancreatic amylase and taka amylase represent the two extremes so far observed in the action of typical α-amylases on their substrates. Therefore, it was of interest to determine, if possible, whether the differences observed in the action of these two α-amylases are correlated with differences in the groups essential to their activities.

Accordingly, the present paper reports the results of an additional study of the active groups of porcine pancreatic amylase and of an investigation to determine whether taka amylase resembles porcine pancreatic amylase[6,7] in this respect.

In 1942, Little and Caldwell,[6a] working with highly purified preparations of porcine pancreatic amylase, reported that free primary amino groups in the protein are essential to its amylase activity. This essentiality was demonstrated by the relationship between loss of amylase activity and loss of primary amino groups when the latter were reacted with ketene, phenyl isocyanate, formaldehyde or nitrous acid.

Since the work of Little and Caldwell,[6] crystalline pancreatic amylase[20,21] has become available in larger amounts,[22] and its properties[21-30] and

(1) This investigation was supported, in part, by research grants from the Williams Waterman Fund; in part, by research grants from the National Institutes of Health, Public Health Service. Presented, in part, before the Division of Biological Chemistry, American Chemical Society, April, 1957.

(2) The data reported here are taken, in part, from a dissertation submitted by Ildiko Radichevich in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry under the Faculty of Pure Science of Columbia University.

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those of crystalline taka amylase[31-36] have been studied extensively. In addition, more specific reagents for primary amino groups have been reported[37-40] and milder conditions established for their use.[41-44]

The present paper reports the results of an investigation of the reaction of crystalline porcine pancreatic amylase[22] and of crystalline taka amylase[32] with acetic anhydride,[37,41] carried out under conditions that, in themselves, cause little, if any, inactivation of the amylase.[22,27,32] The results confirm and extend the findings of Little and Caldwell[6] that free primary amino groups of the protein are essential to the enzymic activity of porcine pancreatic amylase and show, in addition, that they also are essential to the enzymic action of taka amylase. Moreover, the evidence reveals that only approximately 50% of the free primary amino groups of the protein are essential to the activity of either enzyme and, in addition, that the α-amino groups, or the N-terminal groups, are more important in this respect with each amylase studied than the ϵ-amino groups.

The results reported here also confirm and extend the indications obtained by Little and Caldwell[6] that the phenolic hydroxyl groups of porcine pancreatic amylase are not involved in its catalytic centers. This better supported conclusion is made possible because more specific quantitative procedures for following the loss of phenolic hydroxyl groups have been reported[45,46] since the work of Little and Caldwell.[6]

I. Investigation of Porcine Pancreatic Amylase

A. Experimental

1. **Amylase.**—Three times crystallized porcine pancreatic amylase, obtained by a procedure developed in this Laboratory[22,47] was used for all of the work with pancreatic amylase. This crystalline amylase had been found to be homogeneous by electrophoresis[22] and by a number of other criteria[22] and to be free from extraneous amino acids and lower peptides by chromatography.[48] It had an amylase activity of 13,000 to 14,000 maltose units.[49]

2. **Acetylation.**—The primary amino groups of the protein were blocked by acetylation with acetic anhydride under conditions that had been modified only slightly from those recommended by Fraenkel-Conrat, Bean and Lineweaver[41] and found satisfactory by subsequent investigators.[50-55] This reaction was chosen because acetic anhydride had been reported to be specific for primary amino groups,[37,38,42] and because the conditions recommended for its use[41] could be adapted with slight modifications to conditions that, in themselves, would cause little, if any, inactivation of the amylase.[22,27]

Under the modified conditions, porcine pancreatic amylase, dissolved in ice-cold 0.0008 *M* phosphate buffer[22] at *p*H 7.0 and containing 0.014 *M* sodium chloride[22] was poured into an equal volume of ice-cold saturated sodium acetate and then reacted with 0.03 to 13.00 ml. of acetic anhydride per gram of protein. For the reaction, the mixture was held at 0° and stirred mechanically for intervals that ranged from 10 to 150 minutes. Portions of the acetylated solution then were removed and measured simultaneously for loss of amylase activity,[49] for loss of primary amino groups,[56,57] and for possible loss of phenolic hydroxyl groups.[45,46]

The extent of acetylation of the primary amino groups was measured both by a modified ninhydrin method[57] and by the Van Slyke procedure.[56] Possible acetylation of phenolic hydroxyl groups of tyrosine in the amylase was measured spectrophotometrically according to Schlögl, *et al.*,[45] and verified by the use of the Folin phenol reagent as recommended by Fraenkel-Conrat and Singer.[46]

In all cases, measurements also were made with control solutions of the unacetylated amylase that had been held under identical conditions and the extents of acetylation, if any, were calculated by difference.

3. **Ninhydrin Procedure.**—The colored products formed by reaction of the primary amino groups of the protein with ninhydrin were measured spectrophotometrically at 570 mμ in a Beckman spectrophotometer.[57]

It was found necessary to modify the procedure recommended by Moore and Stein,[57] in order to minimize the precipitation of the amylase which was a problem under their conditions. The modified procedure included the replacement of the 4 *N* acetate buffer at *p*H 5.5 with a 0.1 *M* phosphate buffer at *p*H 6.0 and the addition of this buffer directly to the acetylated enzyme solution instead of to the ninhydrin–hydridantin reagent. It also was found necessary to reduce the time of heating from the 15 minutes recommended by Moore and Stein[57] to 10 minutes. Thus, the data in Table I show that, with this protein, the length of the heating period in the ninhydrin procedure may influence the results markedly. It is evident that the time of heating should be kept constant and as short as possible. Apparently, the N-acetylated groups of the amylase are subject to more or less hydrolysis by this process. Therefore, a period of 10 minutes was selected as preferable for the work with this protein to the 15 minutes recommended by Moore and Stein.[57]

In order to obtain good color with little, if any, turbidity, it was found best to adjust the final concentration of the amylase to 0.025 mg. per ml. in the reaction mixture of 8 ml.

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TABLE I

INFLUENCE OF TIME OF HEATING IN NINHYDRIN PROCEDURE UPON INCREASE IN FREE PRIMARY AMINO GROUPS IN SOLUTIONS OF ACETYLATED PANCREATIC AMYLASE

Acetic anhydride per amylase, ml./g.	Time of heating in min. 5	10	15	20
	Optical densities at 570 mμ			
0.000	0.228	0.280	0.298	0.304
.026	.214	.237	.274	.293
.049	.194	.224	.255	.299

When used with individual amino acids, this method was found to give relative absorbance ratios at 570 mμ for phenylalanine, tyrosine and lysine that agree well with the relative values reported by Moore and Stein.[57]

The modified ninhydrin procedure was used to derive a calibration curve for the crystalline amylase. Replicate determinations gave a linear relationship between amylase concentration and absorbance at 570 mμ.

4. **Van Slyke Procedure.**—Solutions containing 3.0 to 6.0 mg. of the untreated and of the acetylated amylase at *p*H 7.0 were reacted simultaneously with nitrous acid according to the Van Slyke procedure,[56] and the extent of acetylation was calculated by difference. Periods of 3 minutes and also of 30 minutes were used in attempts to distinguish between the reaction of the α- and the ε-amino groups.[56] For comparisons with the ninhydrin values, the results of the 30 minute measurements were used.

The Van Slyke procedure gave theoretical values for the primary amino nitrogen of glycine, glutamic acid and lysine and values for crystalline egg albumin that agreed well with those of Van Slyke.[56]

5. **Additional Criteria of Extent of Purification of Amylase.**—Both the ninhydrin and the Van Slyke procedures can be used to detect the presence of extraneous amino acids and peptides in an amylase preparation.

As already stated, a linear relationship was observed between the concentration of recrystallized pancreatic amylase and the optical densities of the products formed by its treatment with ninhydrin. In addition, it was found that equal weights of different preparations of porcine pancreatic amylase gave different optical densities after the ninhydrin procedure and that these optical densities depended upon the amylase activities of the preparations. Thus, for example, 0.05 mg. of a preparation of crystalline pancreatic amylase having an amylase activity[49] of only 10,000 gave an optical density of 0.101 after the ninhydrin treatment, while the same weight of a preparation of the three-times recrystallized enzyme with an amylase activity of 13,000 gave an optical density of 0.054. These results indicate that the less active amylase preparation was contaminated with ninhydrin-positive impurities such as free amino acids or peptides. This conclusion was verified in several cases by chromatography.[48]

Similarly, the values for primary amino nitrogen obtained by the Van Slyke procedure for amylase preparations were found to depend upon the extent of their purification.

6. **Possible Acetylation of Phenolic Hydroxyl Groups of Tyrosine in Porcine Pancreatic Amylase.**—Although acetic anhydride had been reported[37,41] to react specifically with the primary amino groups of proteins, it seemed wise to investigate the possible reaction of the free phenolic hydroxyl groups of tyrosine in the amylase with this reagent under the slightly modified conditions used here.

The concentration of the free phenolic hydroxyl groups of tyrosine in the untreated and in the acetylated protein were determined spectrophotometrically, by measurements of the optical densities[45] of the solutions at 280 mμ. This procedure is based upon the report by Schlögl, *et al.*,[45] that free phenolic hydroxyl groups of tyrosine can be distinguished from its O-acetylated groups because the latter do not show any absorbance at 280 mμ while the former do.

The results obtained with pure tyrosine showed that its phenolic hydroxyl groups are subject to more or less acetylation[45,46] under the conditions used here to acetylate the amylase.

B. Results with Porcine Pancreatic Amylase and Discussion

1. **Selection of Conditions for the Acetylation of Porcine Pancreatic Amylase.**—In order to correlate the loss of activity of the amylase with its acetylation, it was necessary to make certain that the conditions used for the reaction did not contribute to the inactivation and that maximum acetylation of the amylase would be attained. Several factors were investigated.

(a) ***p*H Values of the Reaction Mixture during the Acetylation of Porcine Pancreatic Amylase.**—The *p*H values of the amylase solutions were measured before and after the acetylation of the amylase with increasing concentrations of acetic anhydride, from 3 to 17 ml. per gram of amylase, acting for periods of 60 to 90 minutes. Under these conditions, the *p*H values of the reaction mixtures remained well within the range found by Caldwell and Kung[27] to favor the stability of pancreatic amylase in solutions of similar concentrations and *p*H values when held at 2° for much longer periods of time, including 1440 minutes. It is evident that under the conditions used here, the *p*H values of the reaction mixtures during the acetylation were not factors in the inactivation of the amylase.

As would be expected, it also was found that the *p*H values of the well buffered starch substrates and hydrolyzates[22,27,58] used for the measurements of amylase activity were not influenced appreciably by the addition of portions of the solutions of the acetylated enzyme. Even when the higher concentrations of acetic anhydride were used to acetylate the enzyme, the starch hydrolyzates remained well within the range of *p*H values that permit the amylase to exert its optimal activity.[58]

(b) **Relation between Time of Reaction, Concentration of Acetic Anhydride and Extent of Acetylation of Porcine Pancreatic Amylase.**—In order to determine the best conditions for the acetylation of pancreatic amylase, a series of experiments was carried out to study the relation between the time of acetylation, the concentration of acetic anhydride and the extent of acetylation of the amylase.

The data obtained showed that, under the conditions used here, maximum acetylation of pancreatic amylase was attained in 20 to 30 minutes with concentrations of acetic anhydride that ranged from 0.04 to 13.00 ml. per gram of amylase and that prolonging the time of the reaction did not increase the extent of acetylation of the amylase. Therefore, 30 minutes was selected for the acetylation studies.

The data given in Figs. 1 and 2 show that the acetylation of porcine pancreatic amylase and the loss of its amylase activity each progresses along a well defined course when the amylase reacts, under otherwise comparable conditions, for 30 minutes, with increasing concentrations of acetic anhydride. The loss of amylase activity reaches 13% (Fig. 2) when the acetic anhydride is equivalent to the primary amino groups of the protein,[28] 0.04 ml. of acetic anhydride per gram of amylase, and 92% when 5 ml. of acetic anhydride per gram of amylase are used. The data also indicate (Figs. 1 and 2) that concentrations of acetic anhydride higher than 5 ml. per gram of amylase cause only

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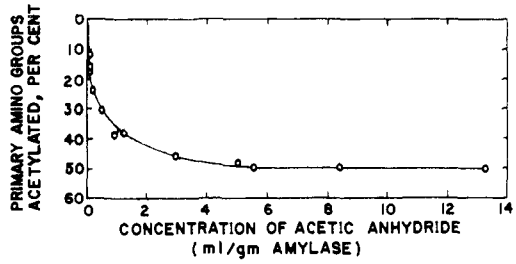

Fig. 1.—Influence of increasing concentrations of acetic anhydride upon extent of acetylation of primary amino groups of porcine pancreatic amylase.

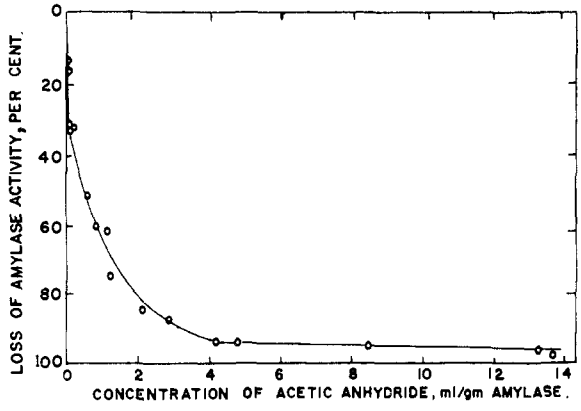

Fig. 2.—Loss of amylase activity when porcine pancreatic amylase reacts with increasing concentrations of acetic anhydride.

negligible increase in its acetylation. Therefore, this concentration was deemed sufficient when maximum acetylation of the amylase was desired.

2. Influence of Loss of Primary Amino Groups of Porcine Pancreatic Amylase upon its Amylase Activity.—The data given in Fig. 3 show the relation between the loss of primary amino groups in pancreatic amylase and the loss of its amylase activity when the amylase reacted under otherwise comparable conditions for 30 minutes with increasing concentrations of acetic anhydride. Curve 1 represents the loss of primary amino groups as measured by the ninhydrin reaction; Curve 2 gives the results obtained with the Van Slyke procedure.

The data show a consistent loss of amylase activity with the disappearance of primary amino groups from the protein. This loss reaches 50% when approximately 25% of the amino groups have been acetylated and 100% when approximately 50% of the amino groups have reacted. The results obtained by the ninhydrin and the Van Slyke methods are in good agreement until the later stages of the acetylation have been reached. It is probable that the ninhydrin values are slightly high because the evidence given in Table I indicates that slight but measurable hydrolysis of the N-acetyl groups of the protein takes place during the heating period in the ninhydrin procedure.

When considered in the light of its amino acid composition,[28] the evidence that porcine pancreatic amylase loses all of its amylase activity when approximately 50% of its primary amino groups have been acetylated indicates that both the α- and the ϵ-amino groups of the protein are involved in its

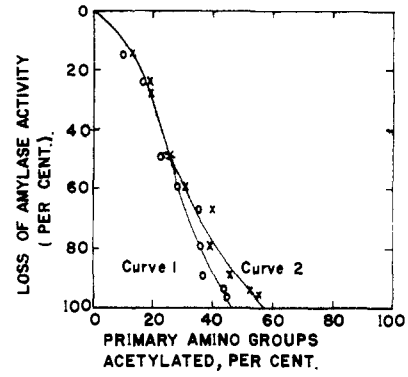

Fig. 3.—Influence of loss of primary amino groups of porcine pancreatic amylase upon its amylase activity.

enzymatically active centers. On the other hand, the pronounced loss of amylase activity in the early stages of the acetylation of the protein, as shown in Fig. 3, indicates that the α-amino groups are more important than the ϵ-amino groups to the amylase activity. Thus, several investigators, including Reid[44] and Liener and Wada,[51] have reported that the α-amino groups of proteins are acetylated preferentially with limited concentrations of acetic anhydride.

3. Evidence of Partial Hydrolysis of N-Acetyl Groups of Acetylated Porcine Pancreatic Amylase upon Standing in Aqueous Solution at 0 to 5°.—Additional support is found in the following evidence for the conclusion that α-amino groups of porcine pancreatic amylase are of primary importance to its amylase activity.

Observations made from time to time showed that solutions of acetylated pancreatic amylase tend to increase in amylase activity upon being held at 0 to 5°. The increases observed ranged from 8 to 18% and appeared to depend upon the extent of acetylation and upon the length of time the solution had stood. Increases in the ninhydrin values of the solutions corresponding to the increased amylase activities also were observed in the solutions.

These findings lead to the conclusion that certain N-acetyl groups of acetylated pancreatic amylase are hydrolyzed with ease and that these susceptible primary amino groups are important to the enzyme activity of the amylase. In accord with the observations of Reid[44] and of Liener and Wada,[51] it is probable that these readily hydrolyzed N-acetyl groups represent α-amino groups in the amylase.

4. Attempt to Determine the Relative Importance of α- and ϵ-Primary Amino Groups of Protein to the Activity of Porcine Pancreatic Amylase; Dinitrophenylation of Porcine Pancreatic Amylase and of Acetylated Porcine Pancreatic Amylase.—The dinitrophenylation method of Sanger[59] was used to determine, if possible, the extent to which the α- and the ϵ-primary amino groups of porcine pancreatic amylase are involved in its acetylation and in the resulting loss of amylase activity.

The method was carried out as follows. Solutions of untreated and of acetylated pancreatic

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amylase, containing 4.8 to 10.7 mg. of amylase, were adjusted with sodium carbonate and sodium bicarbonate[60,61] to *p*H 8.6. An excess of dinitrofluorobenzene was added, 0.2 ml. per 10 mg. of amylase, and the mixture was shaken mechanically for 3–4 hr. at room temperature. The precipitation of the DNP-amylase or of the DNP-acetylated amylase was completed by acidifying the reaction mixture with 1 *N* HCl. The precipitate was washed with water, absolute alcohol and anhydrous ether. The dry DNP-amylase or DNP-acetylated amylase then was hydrolyzed with constant boiling hydrochloric acid for 16 hr.[61] at 105°. The hydrolyzate was extracted with ether. Both the ether and the acid fractions were evaporated to dryness.

The ether fraction was taken up in acetone and developed for 25 to 30 hr. on Whatman No. 4 paper[62] that previously had been buffered to *p*H 6.05 with phthalate buffer. The developing solvent was *t*-amyl alcohol that had been saturated with phthalate buffer at *p*H 6.05.[60]

The acid fraction was dissolved in acetone containing a few drops of 1 *N* hydrochloric acid and developed for 25 to 30 hr., as above.

For quantitative studies, the spots of the individual DNP-amino acids were cut out of the paper and dissolved in 1% sodium bicarbonate.[60] The optical densities of the solutions then were read at 360 mμ.[63] Thus, the concentrations of the DNP derivatives from the untreated and from the acetylated amylase could be compared.

The quantitative results of the dinitrophenylation studies are summarized in Table II. When a low concentration of acetic anhydride was used

TABLE II

COMPARISON OF EXTENTS OF ACETYLATION OF α- AND ε-PRIMARY AMINO GROUPS OF PORCINE PANCREATIC AMYLASE BY ACETIC ANHYDRIDE

Acetic anhydride to amylase, ml./mg.	Loss of amylase activity, %	Extents of acetylation of —primary amino groups, %— Alpha	Epsilon	Total
0.78	62.8	36.3	22.3	26.2
1.46	78.7		37.7	39.0
1.64	83.0	70.8	40.6	41.4
5.00	95.0	89.1	38.6	59.5

(0.78 ml./g. amylase), the pronounced loss in amylase activity of 63% corresponded to the acetylation of 36% of the α-amino groups and to only 22% of the ε-amino groups. On increasing the concentration of acetic anhydride to 1.64 ml. per gram amylase, 83% of the amylase activity was lost, and the acetylation of the α-amino groups had increased to 71% while that of the ε-amino groups had gone up only to 40%. When maximum acetylation was reached, with 5.0 ml. of acetic anhydride, and 95% of amylase activity had been lost, the α-amino groups acetylated had increased to 89% while the percentage of the ε-amino groups acetylated had remained essentially unchanged, at 40%.

Even allowing for the possible accumulation of errors because of the series of manipulations and calculations involved, it is consistently shown in the different stages of acetylation that there is a preferential acetylation of the α-amino groups as compared to the ε-amino groups. Further, this ready acetylation of the α-amino groups appears to go hand in hand with the pronounced loss in amylase activity, especially in the early stages of the acetylation.

The calculations from the dinitrophenylation studies also show that the maximum loss of amylase activity corresponds to an acetylation of 60% of the total amino groups of the amylase. This value agrees reasonably well with the results obtained independently by the ninhydrin and by the Van Slyke procedures.

All the evidence accumulated here indicates that free primary amino groups are necessary for porcine pancreatic amylase action and that its free α-amino groups are more readily acetylated and play a more important part than its ε-amino groups in the structures responsible for the biological action of this enzyme.

5. Acetylation of Individual Free Amino Acids.—Because of the evidence that the primary amino groups of pancreatic amylase and of other proteins differ in susceptibility to acetylation,[44,51] it was of interest to study the acetylation of individual amino acids present in porcine pancreatic amylase.[28] The acetylation of the amino acids was carried out with increasing concentrations of acetic anhydride under the conditions developed for the acetylation of the amylase.

Although a 100-fold excess of acetic anhydride, calculated as mole of reagent per mole of amylase,[28] was required to cause the complete acetylation of the amylase, it was found that five moles of the reagent per mole of individual amino acid gave extensive acetylation of each of the free amino acids studied. This difference indicates that the primary amino groups of the amylase are not as readily available to acetylation as the primary amino groups of the free amino acids present in the amylase. On the other hand, whereas the N-acetyl groups of the acetylated amylase tend to undergo partial hydrolysis upon standing in aqueous solution even at 0°, no evidence of such hydrolysis was observed with the acetylated individual amino acids studied. Lysine, as expected, was acetylated less readily than the other amino acids investigated.

6. The Acetylation of Tyrosine and of the Phenolic Hydroxyl Groups of Porcine Pancreatic Amylase by Acetic Anhydride.—The data given in Table III show that free tyrosine is subject to more or less acetylation by acetic anhydride under the conditions used here to acetylate the amylase and that the extent of the acetylation depends upon the concentration of the reagent and upon the length of time of reaction. The data also show good agreement between the results of the two methods used to detect this acetylation.[45,46]

The data given in Table IV show that the phenolic hydroxyl groups of porcine pancreatic

(60) S. Blackburn and A. G. Lowther, *Biochem. J.*, **48**, 126 (1951).

(61) K. Heyns and G. Wolff, *Z. physiol. Chem., Hoppe-Seylers*, **304**, 200 (1956).

(62) Whatman No. 1 paper was not as satisfactory because the DNP-amino acids moved on it very slowly.

(63) H. Fraenkel-Conrat, J. Ieuan Harris and A. L. Levy, "Methods of Biochemical Analysis," Vol. II, Interscience Publishers, Inc., New York, N. Y., 1955, p. 366.

TABLE III

ACETYLATION OF TYROSINE BY ACETIC ANHYDRIDE

Acetic anhydride to tyrosine, moles/mole	Time of acetylation, min.	Phenolic groups acetylated, % Ninhydrin procedure	Folin reaction
1.03	10	2.4	2.9
	20	4.6	4.9
	30	3.9	2.3
5.40	30	8.8	9.3

amylase, also, undergo more or less acetylation by acetic anhydride under the conditions used here. However, this acetylation of its phenolic hydroxyl groups appears to have little, if any, influence upon the amylase activity of this enzyme. Thus, while the acetylation of the phenolic hydroxyl groups of the protein goes up from 3.0% at 30 minutes of

TABLE IV

COMPARISON OF THE ACETYLATION OF THE PRIMARY AMINO AND OF THE PHENOL GROUPS OF PANCREATIC AMYLASE BY ACETIC ANHYDRIDE

Reaction time, min.	Loss of amylase activity, %	Primary amino groups acetylated, %	Phenolic groups acetylated, %
30	15.9	8.4	3.0
60	15.1	10.5	7.3
90	12.9	9.6	8.0
120	12.3	9.5	15.2

reaction to 15.2% at 120 minutes of reaction, the amylase activity of the acetylated protein and also the extent of acetylation of the primary amino groups of the protein remain essentially unchanged. These data confirm and extend the findings of Little and Caldwell[6b] that the free phenolic hydroxyl groups of porcine pancreatic amylase are not essential to its catalytic action.

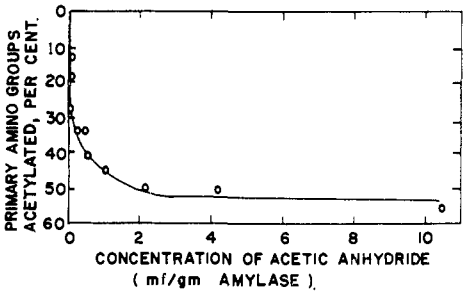

Fig. 4.—Influence of increasing concentrations of acetic anhydride upon extent of acetylation of primary amino groups of taka amylase.

II. Investigation of Taka Amylase

A. Experimental

1. **Amylase.**—Recrystallized taka amylase[32] prepared essentially by the method of Fischer and de Montmollin[31a] was used for all of this work.[64] This crystalline amylase had been found to be homogeneous by electrophoresis[32b] and by several other criteria.[32b] It had an amylase activity of 2200 to 2400 maltose units.[49]

2. **Acetylation.**—The primary amino groups of the protein were reacted with acetic anhydride under the conditions recommended by Fraenkel-Conrat, Bean and Lineweaver[41] and found satisfactory by subsequent investigators.[43,44,50-55] There was no need to modify these conditions, as had been found necessary with porcine pancreatic amylase because they provide *p*H values that permit taka amylase to retain its full amylase activity, that prevent its inactivation.[32b]

(64) The authors wish to thank the Takamine Division of the Miles Laboratories for the concentrated Taka diastase from which this crystallized amylase was prepared.

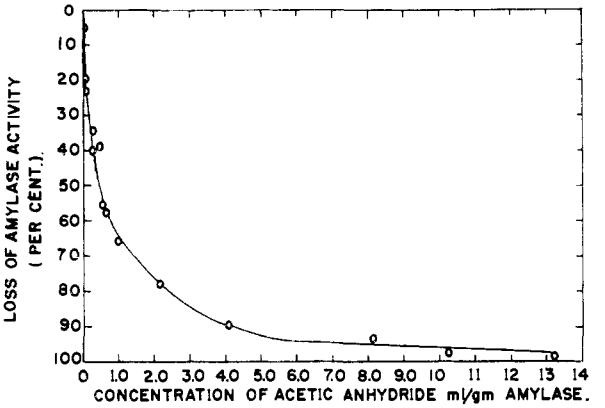

Fig. 5.—Loss of amylase activity when taka amylase reacts with increasing concentrations of acetic anhydride.

For the acetylation, the reaction mixtures, containing 2.0 mg. of amylase per ml. and 0.051 to 13.16 ml. of acetic anhydride per gram of amylase, were held at 0° and stirred mechanically for intervals that ranged from 10 to 150 minutes. Portions of the acetylated solutions then were removed and measured simultaneously for loss of amylase activity[49] and for the loss of primary amino groups by the ninhydrin procedure of Moore and Stein.[67] Control solutions of the unacetylated amylase held under identical conditions, likewise were subjected to ninhydrin and amylase activity measurements; the extent of acetylation and the loss in amylase activity then were calculated by difference.

The ninhydrin procedure was found to give values for the free primary amino groups of glycine and of lysine that agree well with those reported by Moore and Stein for these amino acids.[67]

As was the case with pancreatic amylase, replicate determinations by the ninhydrin method showed a linear relationship between the concentrations of recrystallized taka amylase and the resulting absorbance at 570 mu.

B. Results with Taka Amylase and Discussion

1. **Selection of Conditions for the Acetylation of Taka Amylase.** (a) ***p*H Values of the Reaction Mixtures during the Acetylation.**—The *p*H values of the amylase solutions, measured before and after the addition of increasing concentrations of acetic anhydride, were found to remain well within the range established as favorable to the stability of taka amylase.[32b] Therefore, the *p*H values of the reaction mixtures during the acetylation were not contributing factors to the inactivation of the amylase.

The *p*H values of the well-buffered starch substrates and hydrolyzates[32b] used for the measurements of amylase activity, also, were not influenced appreciably by the addition of the acetylated amylase but remained well within the range of *p*H values that permit taka amylase to exert its optimal activity.[32b]

(b) **Time of Reaction.**—It was found that prolonging the time of acetylation from 30 to 90, to 180 and even to 2900 minutes had only negligible influence upon the extent of acetylaton of taka amylase even when limited concentrations of acetic anhydride per gram of taka amylase were used. Therefore, periods of 90 minutes were selected as satisfactory for the maximum acetylation of the amylase.

(c) **Influence of Concentration of Acetic Anhydride upon Extent of Acetylation and upon Loss of Amylase Activity of Taka Amylase.**—The data given in Figs. 4 and 5 show that when taka amylase

is reacted under otherwise comparable conditions with increasing concentrations of acetic anhydride, the extent of the acetylation of the amylase and its loss of amylase activity both increase with increasing concentrations of acetic anhydride until 5 ml. per gram of amylase have been used. Higher concentrations of the reagent have little influence upon the acetylation of the amylase or upon its loss of activity.

2. Influence of Loss of Primary Amino Groups of Taka Amylase upon its Amylase Activity.—The data given in Fig. 6 show the relation between the loss of primary amino groups in recrystallized taka amylase and the loss of its amylase activity when the enzyme is reacted under otherwise comparable conditions for 90 minutes with increasing concentrations of acetic anhydride. The data show that half of the amylase activity had been lost when approximately 39% of the primary amino groups of the protein had been acetylated and that all of the activity had disappeared when 55% of the primary amino groups had reacted.

Taka amylase has been reported[34] to contain 22 moles of lysine per mole of protein and one N-terminal amino acid, that of alanine.[35] Based on this information, the finding that 55% of the primary amino groups of the protein have been acetylated when taka amylase loses all of its amylase activity leads to the conclusion that both the α- and the ϵ-amino groups of the enzyme are involved in the active structures responsible for its catalytic action.

The pronounced loss of amylase activity shown in Fig. 6 for the early stages of the acetylation of taka amylase and the finding of several investigators[44,51] that the α-amino groups of proteins are acetylated preferentially with limited concentrations of acetic anhydride suggest that with this amylase, also, the α-amino groups of the protein may be of greater importance than its epsilon amino groups to its amylase activity.

3. Evidence of Partial Hydrolysis of N-acetyl Groups of Acetylated Taka Amylase upon Standing in Aqueous Solution at 0 to 5°.—Solutions of acetylated taka amylase that had lost 35 to 90% of their original amylase activity and 20 to 50% of their primary amino groups upon being acetylated were found to recover 1 to 17% of their amylase activity and 1–5% of their primary amino groups on standing for several days at 0 to 5°. In view of the reports[44,51] that the primary α-amino groups of proteins are more easily acetylated and, therefore, presumably more labile than their ϵ-amino groups, this evidence of the partial hydrolysis of N-acetyl groups of taka amylase with the accompanying recovery of amylase activity may be taken as an additional indication that with taka amylase, also, the α-amino groups are of greater importance than its ϵ-amino groups to its amylase activity. Dinitrophenylation studies of the acetylated and unreacted enzyme will be required to clarify this suggestion.

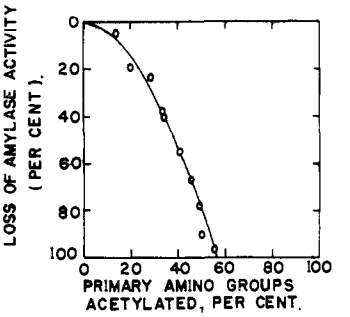

Fig. 6.—Influence of loss of primary amino groups of taka amylase upon its amylase activity.

Conclusion

The results of this investigation show that porcine pancreatic amylase and taka amylase both require free primary amino groups for their catalytic action. Moreover, in both cases, the α-amino groups of the protein appear to be of more importance in this respect. Thus, these two α-amylases resemble each other and differ from β-amylase[3-5] in the active groupings needed for their biological action.

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