[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

A Study of the Influence of a Number of Factors upon the Stability and upon the Activity of Pancreatic Amylase[1]

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The stability and the activity of pancreatic amylase are exceedingly sensitive to its chemical environment. This paper reports additional observations concerning some of the factors concerned. The work was carried out with three times crystallized electrophoretically homogeneous amylase. The factors studied include: the influence of the *p*H of its aqueous solutions upon the stability of the amylase at different temperatures; the influence of the concentration of the amylase upon its stability in aqueous solution; the influence of calcium and of chloride ions upon the stability and upon the activity of the amylase. Previous evidence has shown that pancreatic amylase requires certain anions for its action and that chloride ions are outstanding in this respect. These findings have been confirmed and extended. Chloride ions also protect the amylase from inactivation in aqueous solution. On the other hand, while calcium ions also protect pancreatic amylase from inactivation in aqueous solution, they have no influence upon the activity as distinguished from the stability of the amylase. The evidence makes it clear that there is a fundamental difference between the protection of the amylase from loss of activity, a protection which, under the conditions studied is exerted to very nearly the same extent by chloride and by calcium ions, and the activation of the amylase by chloride and by certain other anions. This difference is being investigated.

Introduction

The stability and also the activity of pancreatic amylase are exceedingly sensitive to its chemical environment. The interrelationships involved have been recognized as important and studied by many investigators.[2-11] The present paper reports additional observations concerning factors that influence the stability and the activity of pancreatic amylase and gives information which increases our understanding of the chemical nature of this important enzyme.

TABLE I

INFLUENCE OF *p*H OF ITS AQUEOUS SOLUTIONS UPON STABILITY OF PANCREATIC AMYLASE AT 2°

*p*H	Amylase solution[a] treatment Temp., °C.	Time, min.	Saccharogenic activity[b] remaining, %
7.19	2	0	100
7.19	2	1440	100
8.55	2	0	100
8.55	2	1440	100
6.53	2	0	98
6.53	2	1440	97
5.18	2	0	92
5.18	2	1440	85

[a] Pancreatic amylase, 3 times crystallized; maltase-free and protease-free[11]; 0.044 mg. per ml.; 0.01 *M* phosphate; 0.01 *M* acetate; *p*H as indicated. [b] Mg. maltose equivalents per mg. amylase. Lintner's soluble potato starch, 1%; 0.02 *M* chloride; 0.01 *M* phosphate; *p*H 7.2; 30 minutes at 40°.

Experimental

Amylase.—The pancreatic amylase used in this work was obtained from frozen pancreas glands of hogs[11] and crystallized three times. It had an amylase activity[5b,11] of 16,000. It was maltase-free and protease-free.[11]

Substrate.—The substrate for all activity measurements was 1% Lintner's soluble potato starch, by Merck, adjusted to *p*H 7.2 and, unless otherwise stated, 0.02 *M* chloride, and 0.01 *M* phosphate.[5b] The Lintner's soluble potato starch had been freed as completely as possible from extraneous electrolytes by being washed at least 20 times by decantation with large volumes of cold distilled water and three times with cold redistilled water and air dried.

Activity Measurements.—The hydrolyses were carried out at 40° as described previously.[5b,11] The saccharogenic activities were measured by an iodometric procedure.[12]

Results and Discussion

Factors that Influence the Stability of Pancreatic Amylase

Influence of the *p*H of its Aqueous Solutions upon the Stability of Pancreatic Amylase at Different Temperatures.—The data given in Table I and in Figs. 1 and 2 give comparable amylase activities for portions of the same amylase solution that had been adjusted to different *p*H values at 0° and then held under otherwise the same conditions, for the time intervals indicated, at 2, 25 or 40° before being measured for amylase activity[5b,11] at *p*H 7.2 and 40°.

The data given in Table I show that there is no loss of amylase activity when solutions of three times crystallized pancreatic amylase, containing 0.044 mg. of amylase per ml. of buffer, are held at *p*H 7.19 or at *p*H 8.55 and 2° for 24 hours. Under these conditions, the amylase lost 3% of its activity in 24 hours at *p*H 6.53 and 15% of its activity in 24 hours at *p*H 5.18, the isoelectric point of the amylase.[7,8,11]

The data given in Fig. 1 show that at 25°, solutions of pancreatic amylase lost their activity more rapidly when adjusted to *p*H 8.55 than when adjusted to *p*H 6.53. Again, the loss of amylase activity was most rapid and extensive in solutions adjusted to *p*H 5.18, the isoelectric point of the amylase.[7,8,11] In this case, the data for the inactivation give an S shaped time curve. The lag in the inactivation of the amylase at its isoelectric point may have significance.

(1) This investigation was supported by a research grant from the National Institutes of Health, Public Health Service.

(2) H. C. Sherman, E. C. Kendall and E. D. Clark, THIS JOURNAL, **32**, 1073 (1910).

(3) E. C. Kendall and H. C. Sherman, *ibid.*, **32**, 1087 (1910).

(4) R. Willstätter, E. Waldschmidt-Leitz and A. R. F. Hesse, *Z. physiol. Chem.*, **126**, 143 (1923).

(5) (a) H. C. Sherman, M. L. Caldwell and Jane E. Dale, THIS JOURNAL, **49**, 2596 (1927); (b) H. C. Sherman, M. L. Caldwell and Mildred Adams, *ibid.*, **50**, 2529, 2535, 2538 (1928).

(6) M. L. Caldwell and M. Adams, "Enzymes and Their Role in Wheat Technology," edited by J. A. Anderson, Interscience Publishers, Inc., New York, N. Y., 1946, p. 55.

(7) K. H. Meyer, Ed. H. Fischer and P. Bernfeld, *Arch. Biochem.*, **14**, 149 (1947); *Experientia*, **3**, 106 (1947); *Helv. Chim. Acta*, **30**, 64 (1947).

(8) Ed. H. Fischer and P. Bernfeld, *ibid.*, **31**, 1831 (1948).

(9) Ed. H. Fischer and P. Bernfeld, *ibid.*, **31**, 1839 (1948).

(10) P. Bernfeld, "Advances in Enzymology," Vol. XII, Interscience Publishers, Inc., New York, N. Y., 1951, p. 396.

(11) M. L. Caldwell, M. Adams, J. F. T. Kung and G. Toralballa, THIS JOURNAL, **74**, 4033 (1952).

(12) M. L. Caldwell, S. E. Doebbeling and S. H. Manian, *Ind. Eng. Chem., Anal. Ed.*, **8**, 181 (1936).

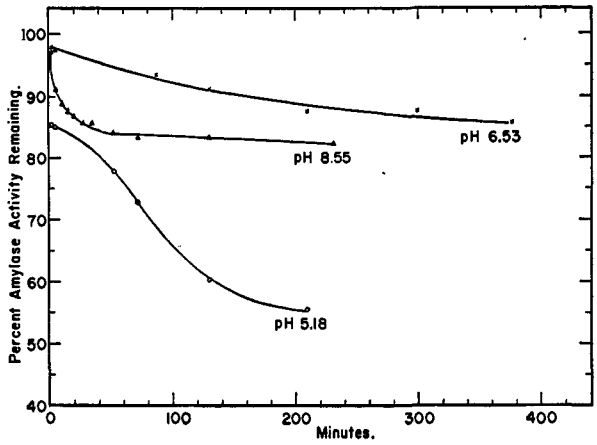

Fig. 1.—Influence of the *p*H of its aqueous solutions upon the stability of pancreatic amylase at 25°. Amylase solutions: 0.044 mg. 3 times crystallized pancreatic amylase per ml.; 0.01 *M* acetate; 0.01 *M* phosphate; held at 25° at the *p*H values and for the time intervals indicated before being measured for activity. Amylase was maltase-free and protease-free.[11] Activity measurements: Lintner's soluble potato starch, 1%; 0.01 *M* phosphate; 0.02 *M* chloride; *p*H 7.2; 30 minutes; 40°.[5]

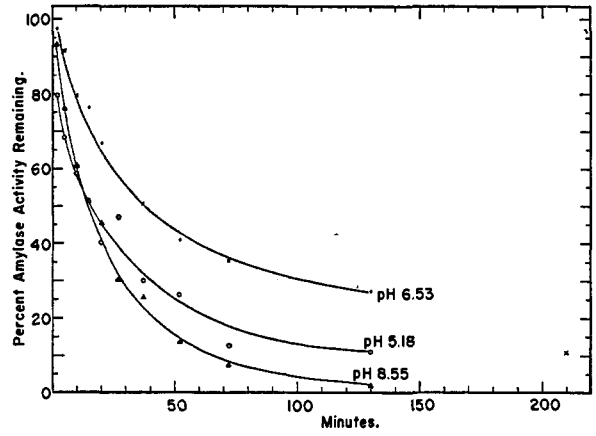

Fig. 2.—Influence of the *p*H of its aqueous solutions upon the stability of pancreatic amylase at 40°. Amylase solutions, activity measurements and other conditions the same as those described for Fig. 1 except that the amylase solutions were held at 40° before being measured for amylase activity.

A comparison of the data given in Figs. 1 and 2 shows that solutions of pancreatic amylase lose their amylase activity more rapidly and more extensively at 40° than at 25°. Again at 40° as at 25°, the losses of amylase activity were lower in the solution adjusted to *p*H 6.53 than in the more acid or more alkaline solutions studied. In the earlier stages, the losses of amylase activity at 40°, like those at 25°, were more rapid in the solution adjusted to *p*H 5.18 than in the solution adjusted to *p*H 8.55, but upon standing for longer periods of time at 40°, the loss of amylase activity was more extensive in the more alkaline solution, at *p*H 8.55. Further study of the comparable data given in Table I and in Figs. 1 and 2 shows that the inactivation of pancreatic amylase is progressively more marked with increasing temperatures at *p*H 8.55 than in the more acid solutions, at *p*H 6.53 or at *p*H 5.18. Additional studies are being made in an attempt to correlate the changes that take place in the protein in these solutions with the losses in its amylase activity.

The data given in Fig. 3 compare the amylase activities of portions of a solution of pancreatic amylase that had been adjusted at 0° to different *p*H values and then held at 40° for 10 minutes before being measured for amylase activity at *p*H 7.2. The data show that at 40° aqueous solutions of pancreatic amylase are most stable, under the conditions studied, between *p*H 6.6 and *p*H 7.2. These *p*H values of greatest stability at 40° had been found previously[5b] also to be most favorable to the action of pancreatic amylase at 40°.

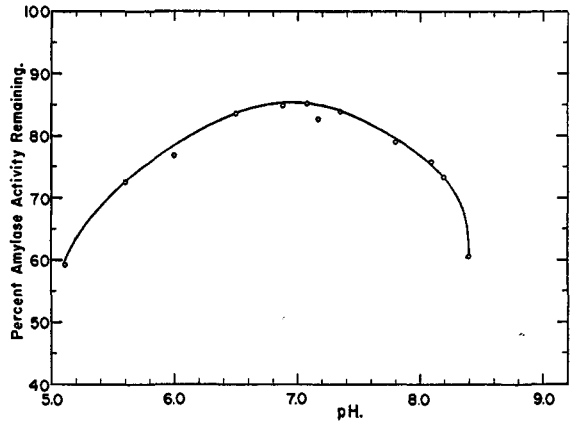

Fig. 3.—Influence of *p*H of its aqueous solutions upon stability of pancreatic amylase when its aqueous solutions, adjusted to different *p*H values, were held at 40° for 10 minutes being measured for activity. Amylase solutions, activity measurements and other conditions the same as those described for Fig. 1.

Influence of Concentration of Pancreatic Amylase upon its Stability in Aqueous Solution.—The data given in Table II illustrate an observation that has been made repeatedly, that the concentration of a protein influences its stability in aqueous solution. Other conditions being the same, pancreatic amylase is more stable in aqueous solution as its concentration is increased. Therefore, statements about its stability in aqueous solution should include its concentration as well as the other conditions. Three times crystallized pancreatic amylase such as that used here has been found to be free from all detectable traces of protease[11] that often accompanies less highly purified preparations and that may tend to make their solutions less stable.[9] When protease-free and reasonably concentrated, aqueous solutions of pancreatic amylase are stable for prolonged periods of time when held at approximately 5° and at favorable *p*H values. Thus, solutions of 3 times crystallized protease-free pancreatic amylase containing 0.01 mg. of amylase per ml. and adjusted to 0.02 *M* sodium chloride; 0.01 *M* sodium phosphate and *p*H 7.2[5b] have been held at approximately 5° for more than 6 months without any measurable loss of amylase activity. Similarly, a solution containing 3.5 mg. of protease-free crystalline pancreatic amylase per

ml. of redistilled water, unadjusted, at approximately *p*H 6.5, showed no loss of amylase activity after being held at approximately 5° for more than one year.

TABLE II

INFLUENCE OF CONCENTRATION OF ITS AQUEOUS SOLUTION UPON STABILITY OF PANCREATIC AMYLASE[a]

Treatment of amylase solution[a]				Amylase activity[b]	
Concn., mg./ml.	Time, min.	Temp., °C.	*p*H	Activ. units[b]	Remaining, %
3.1	0	0	7.2	16,120	100
3.1	30	40	7.2	15,320	95
0.31	30	40	7.2	14,800	92
.031	30	40	7.2	13,800	86
.0031	30	40	7.2	12,500	78
.00062	30	40	7.2	10,450	65
.00031	30	40	7.2	8,680	54

[a] Three times crystallized, protease-free, maltase-free, hog pancreatic amylase.[11] Aqueous solution adjusted to 0.01 *M* phosphate and *p*H 7.2. [b] Mg. maltose equivalents per mg. amylase in 30 minutes at 40° from Lintner soluble potato starch, 1%; 0.01 *M* phosphate; 0.02 *M* sodium chloride; *p*H 7.2.[5b]

TABLE III

INFLUENCE OF CHLORIDE AND OF CALCIUM IONS UPON STABILITY OF PANCREATIC AMYLASE IN AQUEOUS SOLUTION AT 40°

Amylase solution[a] treatment					Saccharogenic activity[b]		
Temp., °C.	*p*H	Time, min.	Cl^-, *M*	Ca^{++}, *M*	Activity	Remaining, %	Loss, %
0	7.2	0	0.02	0.02	15,700	100	0
0	7.2	70	.02	.02	15,700	100	0
40	7.2	70	.02	.02	14,300	91	9
40	7.2	70	.02	0	14,000	89	11
40	7.2	70	0	0.02	13,800	88	12
40	7.2	70	0	0	9,800	62	38

[a] Amylase solution—0.038 mg. 3 times crystallized pancreatic amylase per ml.; 0.04 *M* acetate; *p*H 7.2. Amylase was maltase-free and protease-free.[11] [b] Saccharogenic activity—mg. maltose equivalents per mg. amylase when acting for 30 minutes at 40° on Lintner soluble potato starch, 1%; 0.02 *M* chloride; 0.01 *M* phosphate; *p*H 7.2; 1.3×10^{-3} mg. amylase per mg. starch.

firm and extend previous observations[13] that calcium ions protect pancreatic amylase from inactivation in aqueous solution. In addition, the data given in Table III show that chloride ions also protect pancreatic amylase from inactivation; that the protection of the amylase under the conditions studied is very nearly the same for equivalent concentrations of calcium and of chloride ions; that the presence of both calcium and chloride ions is somewhat more favorable to the stability of the amylase than either calcium ions or chloride ions alone.

Previous evidence[13] has shown that the starch substrate also has marked protective action on pancreatic amylase. It seems probable that the union of the amylase and its substrate may serve to protect the amylase protein at certain vulnerable or labile centers or groups. The stabilizing action of calcium and of chloride ions also may be due to their union with the protein through labile groups. This suggestion is being investigated with the crystalline amylase.

Factors that Influence the Activity of Pancreatic Amylase

Influence of Chloride and of Calcium Ions upon Activity of Pancreatic Amylase.—Previous work[5b] with starch solutions adjusted to *p*H values from *p*H 5.7 to *p*H 7.7 and 0.01 *M* phosphate have shown that pancreatic amylase requires certain anions for its action and that chloride ions are outstanding in this respect. These findings have been confirmed and extended to include three times crystallized pancreatic amylase. The results obtained with starch adjusted to *p*H 7.2 are given in Table IV. Again, pancreatic amylase shows no measurable action unless chloride ions, or certain other anions,[5b] also are present. This failure of amylase activity is a very sensitive property of the amylase. As shown in Table IV, the favorable influence of chloride ions was measurable, under the conditions of these experiments, when 0.00008 *M* chloride ions were introduced into the hydrolyzate with the amylase.

TABLE IV

INFLUENCE OF CHLORIDE AND OF CALCIUM IONS UPON ACTIVITY OF PANCREATIC AMYLASE AT *p*H 7.2

Amylase solution[a]			Starch substrate				Final hydrolyzate		Saccharogenic activity,[b] %
Acetate, *M*	Phosphate, *M*	Chloride, *M*	Chloride, *M*	Ca^{++}, *M*	Acetate, *M*	Phosphate, *M*	Chloride, *M*	Ca^{++}, *M*	
0.04	0	0	0.02	0	0	0.01	0.02	0	100
.04	0	0	0.02	0	.04	0	.02	0	96
.04	0	0	0	0	.04	0	0	0	0
.04	0	0	0	0	0	0	0	0	0
.04	0	0	0	0.02	.04	0	0	0.02	0
.04	0	0	.02	0.02	.04	0	.02	0.02	91
0	0.01	0.02	.02	0	0	0.01	.02008	0	100
0	.01	.02	.02	0	.04	0	.02008	0	93
0	.01	.02	0	0	.04	0	.00008	0	9
0	.01	.02	0	0	0	0	.00008	0	11
0	.01	.02	0	0.02	.04	0	.00008	0.02	11
0	.01	.02	0.02	0.02	.04	0	.02008	0.02	95

[a] Amylase solution; 3 times crystallized pancreatic amylase; 0.038 mg. per ml.; *p*H 7.2; 0°. [b] Mg. maltose equivalents per mg. amylase acting on Lintner's soluble potato starch, 1%; *p*H 7.2; 30 minutes; 40°. The unbuffered hydrolyzates were adjusted to *p*H 7.2 with dilute sodium hydroxide.

Influence of Calcium Ions and of Chloride Ions upon the Stability of Pancreatic Amylase in Aqueous Solutions.—The data given in Table III con-

(13) R. B. Alfin and M. L. Caldwell, THIS JOURNAL, **70**, 2534 (1948).

Calcium ions cannot replace chloride ions in the activation of pancreatic amylase at *p*H 7.2. The data given in Table IV show no activity for pancreatic amylase in the absence of chloride ions even when 0.02 *M* calcium ions were present in the hydrolyzates at *p*H 7.2. The marked influence of slight changes in the *p*H of its hydrolyzates upon the action of pancreatic amylase is emphasized by the lower activity of the amylase in the otherwise comparable but unbuffered or poorly buffered hydrolyzates used for the study of the influence of calcium ions. Phosphate ions[5a,b] and acetate ions[5a] had been found previously to have no influence on the activity of pancreatic amylase.

Protection or Activation of Pancreatic Amylase Distinction between Protection and Activation of Pancreatic Amylase.—The data given in Tables III and IV show that there is a fundamental difference between the influence exerted by chloride ions in activating pancreatic amylase and the influence exerted by calcium ions and by chloride ions in protecting the amylase from inactivation on standing in dilute aqueous solution. It appears probable that chloride ions, and to a less extent certain other anions,[5b] are necessary for the effective union of the amylase protein and its substrate. Attempts to clarify the fundamental differences between the activation of pancreatic amylase by anions such as chloride and the protection of the amylase by ions such as chloride ions and calcium ions are being continued.

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[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

Reactions of Fatty Materials with Oxygen. XIV.[2] Polarographic and Infrared Spectrophotometric Investigation of Peroxides from Autoxidized Methyl Oleate

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Methyl oleate has been autoxidized from 35 to 120° in the presence or absence of ultraviolet radiation. Polarographic and iodometric analysis of the autoxidation mixtures, and peroxide concentrates obtained from them by urea complex precipitation of unoxidized methyl oleate, has shown that, although the bulk of the peroxides formed are hydroperoxides, a significant proportion is not. Evidence is presented which indicates that the non-hydroperoxide portion probably consists of cyclic peroxides. Furthermore, the hydroperoxides have the *trans* configuration, predominantly. It is extremely unlikely that methyl oleate hydroperoxide (*cis* configuration) has ever been isolated since the free-radical nature of the autoxidation reaction precludes its formation to any appreciable extent.

Three major points bearing on the autoxidation of methyl oleate are reported and discussed in this paper. First, although it will be shown that hydroperoxides are the major early products of autoxidation of methyl oleate, a significant proportion (as much as 28%) of the total peroxides is not. Second, evidence will be presented which leads to the conclusion that the non-hydroperoxide portion of the total peroxides isolated from autoxidized methyl oleate is probably cyclic, at least in part. Third, the hydroperoxides from autoxidized methyl oleate have the *trans* configuration predominantly and are preferably referred to as methyl *trans*-octadecenoate hydroperoxides. A consequence of this last statement is that, in view of the isolation procedures presently available, methyl oleate (*cis*) hydroperoxide has undoubtedly never been isolated. Furthermore, the present investigation suggests that it will be extremely difficult to isolate a pure *cis* hydroperoxide from autoxidized methyl oleate, or from any other long-chain *cis* olefinic compound.

All the conclusions drawn in this paper refer to autoxidized methyl oleate in which the peroxide content has not yet reached its peak. After the peak in peroxide content has been reached and the peroxide values are decreasing, the extent of oxidation is so great and the mixture so complicated that there is serious question regarding the reliability of the various analytical methods.

Non-hydroperoxide Content of Autoxidized Methyl Oleate.—Polarographic analysis is a convenient and accurate way to determine hydroperoxides in the presence of other peroxide types.[3] Study of the polarographic properties of methyl oleate autoxidized to different peroxide contents and peroxide concentrates obtained from them clearly demonstrates (Table I) that hydroperoxides are not the sole peroxide substances formed, although they predominate, and that the non-hydroperoxide portion may amount to as much as 28% of the total peroxides (no. 9, Table I). In every case studied so far, in which the peroxide concentrates were not further fractionated by solvent partition methods, the values obtained for total peroxides by chemical (iodometric) analysis[4] exceed those obtained for hydroperoxides by polarographic analysis.[3] Results representative of over fifty autoxidations and concentrations of peroxides are listed in Table I.

It appeared to make little or no difference in the results obtained whether methyl oleate was oxidized in the dark or in the presence of ultraviolet radiation, between 35 and 120°, or in the presence or absence of metal deactivators. A benzoyl peroxide-

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) Presented in part at the Meeting-in-Miniature of the Philadelphia Section of the American Chemical Society, January 29, 1953, and at the meeting of the American Chemical Society held in Los Angeles, California, March 15–20, 1953. The preceding paper in this series is *J. Polymer Sci.*, in press.

(3) C. O. Willits, C. Ricciuti, H. B. Knight and D. Swern, *Anal. Chem.*, **24**, 785 (1952).

(4) D. H. Wheeler, *Oil and Soap*, **9**, 89 (1932); W. O. Lundberg and J. R. Chipault, THIS JOURNAL, **69**, 833 (1947).