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Further Studies of the Action of Pancreatic Amylase: Extent of Hydrolysis of Starch[1]

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Although pancreatic amylase has been known and studied for many years, remarkably little quantitative information is available concerning its action. A survey of the literature shows contradictory statements about the extent of the hydrolysis of starches by this amylase[3-10] and uncertainty about the products formed or the order of their appearance.[3,4,10-16]

The present report is a summary of part of a detailed study of the action of highly purified pancreatic amylase[17] carried out under conditions which have been found to favor its activity and to protect it from inactivation.[18] Information of this kind is important to a better understanding both of the amylase and of the chemical nature of starch.

Experimental

Preparations of highly purified pancreatic amylase were obtained from pancreatin[19] by a modification of the method of Sherman, Caldwell and Adams.[17] They showed no evidence of maltase activity in the concentrations used in this work.

The hydrolyses of starch were carried out at 40° and the reaction mixtures were adjusted to *p*H 7.2 in the presence of 0.01 *M* phosphate (sodium) and 0.02 *M* chloride (sodium), conditions which had been shown to protect this amylase from inactivation and to favor its activity.[16] The amylase preparations, also, were dissolved in 0.01 *M* phosphate, 0.02 *M* chloride at *p*H 7.2 at 0° and used as promptly as possible.[18]

The substrates included whole potato starch, a straight chain component of corn starch[20] and Lintner's soluble potato starch. The latter was included for comparative purposes as it has been the most widely used substrate for the laboratory study of amylase action. The starches were washed repeatedly with distilled and with redistilled water and air dried.

In experiments designed to study the extent of the hydrolysis of starch, concentrations of the amylase were chosen so that the reactions would proceed rapidly and be practically complete before contamination by yeasts and bacteria might be expected appreciably to influence the results. Phemerol[23] and toluene were added to hydrolysis mixtures which were allowed to react for more than five hours. Neither of these reagents was found to influence the activity of the amylase nor the reducing values of the reaction mixtures in the concentrations used.

The reducing values of the reaction mixtures were converted to their equivalents of maltose and usually are given in terms of the percentage yield of the maltose which could be obtained theoretically from the substrate. Usually, the reducing values were determined by iodometric titration[24] although a ferricyanide ceric sulfate method[25] also was used.

Results

Extent of Hydrolysis of Starch.—The average data given in Fig. 1 are typical of the results obtained when different concentrations of pancreatic amylase reacted with soluble potato starch, whole potato starch or with the linear fraction from corn starch.[26,27] They show that the extent of the hydrolysis of these substrates by pancreatic amylase depends in each case within wide limits upon the concentration of amylase used. Similar results have since been obtained with corn starch[28] and with waxy maize starch.[29] With each substrate the hydrolysis curves show a change from a rapid to a slow phase of the reaction, typical of many other enzyme reactions but, here, the reaction curves tend to flatten at higher values as the concentration of amylase is increased. With different concentrations of pancreatic amylase, there was no evidence of a common limit in the extent of the hydrolysis of any of these substrates such as has been reported for this amylase[6,7,8,10] or as is observed when different concentrations of beta amylase act on starch or on its branched-chain components.[30] These results illustrate an important difference between the action of pancreatic amylase and that of beta-amylase. They also cast doubt upon the rather common practice[6,7,8] of assuming a limit in the hydrolysis of starch by

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

(3) Kuhn, *Ann.*, **443**, 1 (1925).

(4) Freeman and Hopkins, *Biochem. J.*, **30**, 442 (1936).

(5) Freeman and Hopkins, *ibid.*, **30**, 451 (1936).

(6) Willstätter, Waldschmidt-Leitz and Hesse, *Z. physiol. Chem.*, **126**, 143 (1923).

(7) Blom, Bak and Braae, *ibid.*, **250**, 104 (1937).

(8) von Euler and Svanberg, *Ber.*, **56**, 1749 (1923).

(9) Pringsheim and Liebowitz, *ibid.*, **59**, 991 (1926).

(10) Hopkins, *Adv. in Enz.*, **6**, 389 (1946).

(11) Freeman and Hopkins, *Biochem. J.*, **30**, 446 (1936).

(12) Myrbäck, Örtenblad and Ahlborg, *Biochem. Z.*, **307**, 49 (1940).

(13) Örtenblad and Myrbäck, *ibid.*, **307**, 123 (1941).

(14) Myrbäck, *ibid.*, **307**, 132 (1941).

(15) Myrbäck, *ibid.*, **307**, 140 (1941).

(16) Sherman and Punnett, THIS JOURNAL, **38**, 1877 (1916).

(17) Sherman, Caldwell and Adams, *J. Biol. Chem.*, **88**, 295 (1930).

(18) Sherman, Caldwell and Adams, THIS JOURNAL, **50**, 2529, 2535, 2538 (1928).

(19) Parke Davis and Company, pancreatin from swine.

(20) The linear fraction from corn starch was kindly furnished by Dr. T. J. Schoch. It was hydrolyzed completely to fermentable sugar by beta amylase and corresponded to 94% crystalline "amylose"[21] by potentiometric titration.[22]

(21) Kerr and Severson, THIS JOURNAL, **65**, 193 (1943).

(22) Bates, French and Rundle, *ibid.*, **65**, 142 (1943).

(23) Phemerol is *p-t*-octyl-phenoxy-ethoxy-ethyl-dimethyl-benzyl-ammonium chloride.

(24) Caldwell, Doebbeling and Manian, *Ind. Eng. Chem., Anal. Ed.*, **8**, 181 (1936).

(25) Hassid, *ibid.*, **8**, 138 (1936).

(26) Meyer, Brentano and Bernfeld, *Helv. Chim. Acta*, **23**, 845 (1940).

(27) Schoch, THIS JOURNAL, **64**, 2957 (1942).

(28) Caldwell and Daly, unpublished.

(29) Caldwell and Mindell, unpublished.

(30) Caldwell and Adams, "Am. Assoc. Cer. Chem., Monograph Series," Vol. I, Chapter II (1946).

pancreatic amylase at 75% theoretical maltose when solutions of unknown amylase concentrations are being compared and evaluated.

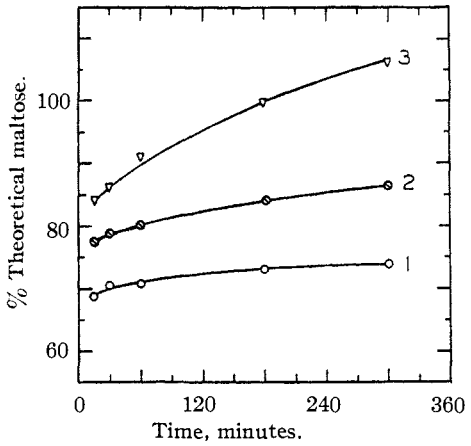

Fig. 1.—Influence of amylase concentration upon the extent of the hydrolysis of Lintner soluble potato starch by purified maltase-free pancreatic amylase: amylase preparation, mg. per 1000 mg. starch: Curve 1, 1 mg.; Curve 2, 8 mg.; Curve 3, 32 mg.; optimal conditions.[18]

Study of Hydrolysis Mixtures at Stages of Very Slow Action.—It was found repeatedly that the introduction of additional amylase into reaction mixtures which had reached stages of very slow rates of change resulted in further increases in their reducing values and, as would be expected, that larger increases in reducing value followed the use of larger additions of amylase. It is evident that products capable of further hydrolysis by the enzyme remained in reaction mixtures which had reached stages of very slow rates of change.

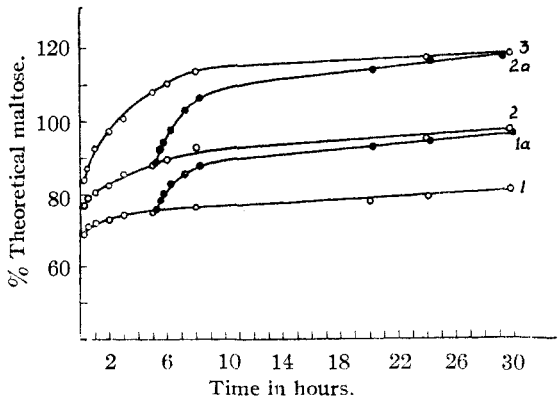

Fig. 2.—Extent of hydrolysis by additional pancreatic amylase of hydrolysis mixtures which had reached stages of very slow rates of change: Curve 1, 1 mg. amylase preparation per 1000 mg. starch; Curve 2, 8 mg.; Curve 3, 32 mg.; Curve 1a, addition of amylase to reaction 1 to bring amylase concentration equal to that in reaction 2; Curve 2a, addition of amylase to reaction 2 to bring amylase concentration equal to that in reaction mixture, 3; optimal conditions.[18]

Moreover, the data summarized in Fig. 2 show that the extent of hydrolysis attained was practically the same, as judged by the theoretical maltose calculated from the reducing values, whether a given concentration of amylase was added at the start of the reaction or in part after the reaction had reached the stage of slow action. The data given in Fig. 2 are strictly comparable as the reaction mixtures which had reached stages of very slow action were divided and one portion was treated with additional amylase while the other was continued at 40° as before.

Similarly, the data summarized in Fig. 3 show that the amylase had not been inactivated irreversibly to any appreciable extent in reaction mixtures which had reached stages of very slow rates of change. Additional substrate, introduced into such reaction mixtures, was hydrolyzed to an extent which was very similar to that attained in comparable reaction mixtures which had contained initially an equivalent concentration of amylase and ratio of amylase to substrate. It should be pointed out that the conditions of these experiments were chosen to protect the amylase from inactivation.[18]

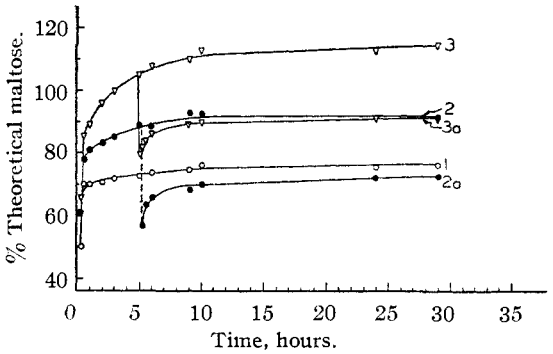

Fig. 3.—Hydrolysis of additional substrate by pancreatic amylase in starch hydrolyzates which had reached stages of very slow rates of change: Curve 1, 1 mg. amylase preparation per 1000 mg. starch; Curve 2, 8 mg.; Curve 3, 32 mg.; Curve 2a, part of reaction mixture of Curve 2 diluted with substrate to give amylase concentration equal to that in reaction mixture of Curve 1; Curve 3a, part of reaction mixture of Curve 3 diluted with substrate to bring amylase concentration equal to that in reaction mixture of Curve 2; optimal conditions.[18]

These findings, that the per cent. of theoretical maltose, calculated from the reducing values of the reaction mixtures, is practically the same under a number of different conditions, does not mean necessarily that the same products are formed from the starch in these different reaction mixtures. This point will be considered more fully elsewhere.[31]

Removal of Products by Dialysis.—The results so far reported suggested the possibility that under favorable conditions the hydrolysis of starch by pancreatic amylase continues until

(31) Alfin and Caldwell, unpublished.

equilibrium is established and that this equilibrium is upset by the addition either of more amylase or of more substrate. The slowing down of enzyme reactions has often been attributed to reaction with or equilibrium between the enzyme and its substrate or between the enzyme and the products of its action.

These possibilities were studied by the use of efficient dialysis to remove the readily dialyzable products during the hydrolyses. Starch–amylase mixtures were prepared and divided into two portions. One of these was allowed to react as usual in a flask held at 40° and was examined at intervals for reducing value. The other portion was placed in small dialyzing bags[32] and dialyzed at 40° against a buffer solution of the same electrolyte concentration and *p*H value as the substrate. The bags held approximately 5-ml. portions. They were kept in rapid motion during the dialysis and the outside solution, which was used in portions equal to four times the total volume of the hydrolysis mixture being dialyzed, was replaced ten to twenty times during a five-hour dialysis. At intervals during the reaction, one or two of the dialysis bags were removed and the contents measured for volume and examined for reducing value and for total solids. At intervals, the outside solution, also, was concentrated and examined for reducing value. Typical data for comparable hydrolyses conducted with and without dialysis are summarized in Tables I and II.

TABLE I

INFLUENCE OF DIALYSIS DURING HYDROLYSIS UPON THE EXTENT OF THE HYDROLYSIS OF LINTNER'S SOLUBLE POTATO STARCH BY PANCREATIC AMYLASE

Reaction[a] time, min.		Theoretical maltose: Hydrolysis accompanied by dialysis,[b] % Inside	Outside	Total	Hydrolysis without dialysis, %
A	300	12.4	56.3	68.7	68.8
		11.0	57.8	68.8	
B	300	Not measurable	70.8	70.8	71.7
C	300	Not measurable	66.4	66.4	69.7
D	360	Not measurable	74.3	74.3	72.6
E	360	6.5	75.8	82.3	82.9
F	600	2.8	82.2	85.0	83.5

[a] Lintner's Soluble Potato Starch: 1%; 0.01 *M* phosphate; 0.02 *M* chloride; *p*H 7.2. Hydrolyses at 40°; amylase preparation, 1 mg. per 1000 mg. starch. [b] Dialyzed during the hydrolysis against 0.01 *M* phosphate; 0.02 *M* chloride at *p*H 7.2.

The data summarized in Table I show remarkably good agreement between the per cent. of theoretical maltose calculated from the reducing values of comparable dialyzed and undialyzed hydrolysis mixtures of soluble potato starch and pancreatic amylase. These data lead to the conclusion that the slowing down of the reaction in the hydrolysis of starch by pancreatic amylase cannot be explained by assuming reaction with or equilibrium between the amylase and maltose or glucose or other readily dialyzable products of the reaction. If this were the case, the removal of such products would be expected to increase the extent of the hydrolysis of the dialyzed reaction mixtures.

(32) Cellophane tubing purchased from the Visking Corporation was used to make the bags.

TABLE II

INFLUENCE OF DIALYSIS DURING HYDROLYSIS UPON THE EXTENT OF THE HYDROLYSIS OF POTATO STARCH BY PURIFIED PANCREATIC AMYLASE

Reaction[a] time, minutes		Theoretical maltose: Hydrolysis accompanied by dialysis,[b] % Inside	Outside	Total	Hydrolysis without dialysis, %	Diff., %
A	60	31.1	37.4	68.5	70.9	2.4
B	180	6.9	65.2	72.1	75.5	3.4
C	360	Not measurable	66.7	66.7	73.7	7.0
D	360	1.24	70.7	71.9	78.5	6.6
E	360	1.04	70.7	71.7	79.8	8.1
F	360	1.08	75.1	76.2	85.8	9.6
G	600	0.4	69.1	69.5	79.9	10.4
H	600	0.4	72.5	72.9	86.4	13.5
I	600	0.4	74.5	74.9	88.1	13.2

[a] Potato starch, 1%; 0.01 *M* phosphate; 0.02 *M* chloride; *p*H 7.2; hydrolyses at 40°. Amylase preparation, 1 mg. per 1000 mg. of starch. [b] Dialyzed during hydrolysis against 0.01 *M* phosphate; 0.02 *M* chloride at *p*H 7.2.

When whole potato starch was used as the substrate (Table II) instead of Lintner soluble starch (Table I), there was not such good agreement in the per cent. of theoretical maltose calculated from the reducing values of comparable dialyzed and undialyzed hydrolysis mixtures. However, as the values for the undialyzed hydrolysis mixtures were consistently higher than those for the dialyzed hydrolysis mixtures, these data, also, lead to the conclusion that inter-reaction with or equilibrium between readily dialyzable products and amylase does not explain the slowing down of the reaction between pancreatic amylase and starch.

The reducing products formed from Lintner soluble potato starch hydrolyzed with and without dialysis were differentiated into fermentable sugars (maltose and glucose) and non-fermentable reducing products by a modification of the method of Somogyi.[33] The data summarized in Table III show that when the hydrolysis was accompanied by dialysis, reducing products capable of further hydrolysis by the amylase escaped hydrolysis, presumably by being dialyzed away. In this comparison again, the total reducing values of dialyzed and of undialyzed hydrolysis mixtures were very similar even though the products responsible for that total reducing action were quite different.

When the initial concentrations of amylase were not too large, the addition of amylase to dialyzed hydrolysis mixtures which had reached stages of very slow rates of change resulted in small but measurable increases in their reducing values. These results showed the presence in the dialyzed

(33) Somogyi, *J. Biol. Chem.*, **119**, 741 (1937).

TABLE III

INFLUENCE OF DIALYSIS DURING THE HYDROLYSIS UPON THE PRODUCTS FORMED FROM LINTNER'S SOLUBLE POTATO STARCH BY PURIFIED MALTASE-FREE PANCREATIC AMYLASE

		Reducing values calculated as per cent. theoretical maltose								
		Total			reducing dextrins[b]			maltose and glucose[b]		
Hydrolyses[a]	Time, hours	Inside	Outside	Total	Inside	Outside	Total	Inside	Outside	Total
Dialyzed	6	1.4	80.0	81.4	1.4	32.8	34.2	0	47.2	47.2
Undialyzed	6			82.9			24.3			58.6

[a] Lintner Soluble Potato Starch, 1%; 0.01 *M* phosphate, 0.02 *M* chloride, *p*H 7.2; amylase preparation, 1 mg. per 1000 mg. starch. One-half of reaction mixture was dialyzed during the hydrolysis against 0.01 *M* phosphate, 0.02 *M* chloride, *p*H 7.2. Hydrolysis and dialysis at 40°. [b] Determined by selective fermentation with washed baker's yeast; dextrins by difference.

hydrolysis mixtures of products capable of further hydrolysis by the amylase.

Similarly, the addition of substrate to dialyzed hydrolysis mixtures which had reached stages of very slow action resulted in extensive hydrolysis of the added substrate. The typical data given in Table IV show that a second portion of substrate was hydrolyzed in five hours to a similar extent (66.2% theoretical maltose) as the original portion of the substrate (69.7% theoretical maltose). It is evident that there had been no significant irreversible loss or inactivation of pancreatic amylase in the dialyzing hydrolysis mixtures under the conditions of these experiments.

TABLE IV

EVIDENCE FOR THE PRESENCE OF PANCREATIC AMYLASE AFTER HYDROLYSIS OF LINTNER'S SOLUBLE STARCH ACCOMPANIED BY DIALYSIS

Reaction[a] time, minutes	Theoretical maltose, %
A. Original Hydrolysis with Dialysis	
300	69.7
B. Hydrolysis of Fresh Substrate by Amylase Remaining in an Equal Volume of A (above)	
15	49.8
30	56.8
60	60.8
120	65.6
300	66.2
1200	68.6

[a] Lintner Soluble Potato Starch: 1%; 0.01 *M* phosphate; 0.02 *M* chloride; *p*H 7.2; 40°. Amylase preparation, 1 mg. per 1000 mg. starch.

On the other hand, marked inactivation of the amylase (86 to 87%) occurred when portions of the same solutions of purified pancreatic amylase were dialyzed for five hours at 40° under the same conditions, but in the absence of substrate, against a buffer solution of the same electrolyte concentration and *p*H value. These results give experimental evidence for the suggestion often advanced that the amylase unites with its substrate, in this case with the larger less readily dialyzable products of the hydrolysis of starch, and thus is protected from appreciable irreversible inactivation or from appreciable loss due to dialysis.

The loss of pancreatic amylase activity which occurs during dialysis of its aqueous solutions has not yet been reversed[17,34] by uniting the dialyzed solution with its dialyzate as has been possible under suitable conditions with certain enzymes which contain dialyzable prosthetic groups.

The data reported here indicate that the slowing down of the reaction in the hydrolysis of starch by pancreatic amylase is due to the replacement of the original substrate by products for which the amylase has less affinity. These dextrins are hydrolyzed only slowly by pancreatic amylase and are present in relatively low concentrations. Under the conditions of these experiments, the slowing down of the reactions is not due to irreversible inactivation of the amylase nor, as is often reported,[10] to action with or equilibrium between the more readily dialyzable products of the hydrolysis and the amylase. However, there is evidence of union between the amylase and the less readily dialyzable products of the hydrolysis of starch. These products are being investigated.

Examination of Preparations of Purified Pancreatic Amylase for Traces of Maltase, Phosphorylase and Phosphatase Activities.—No evidence of *maltase* activity was found in composite samples of the preparations of purified pancreatic amylase even when the highest concentrations used in this work were held for twenty-four hours with 1% maltose under the conditions used for the hydrolysis of starch. This failure to find maltase activity furnishes conclusive evidence that the results reported here were not influenced to any significant extent by traces of maltase activity.

Similarly, no evidence of *phosphorylase*[35,36] or of *phosphatase*[37] activity was found in relatively high concentrations of the preparations of purified pancreatic amylase.

Examination of Preparations of Purified Pancreatic Amylase for Traces of Other Carbohydrases.—The preparations of purified pancreatic amylase were also examined for traces of carbohydrases other than amylase by attempts to cause the selective inactivation of amylase activity, on the one hand, and of other carbohydrase (glucosidase, or dextrinase) activities on the other. Amylase activity refers here to the increase in the reducing value (mg. maltose) per unit weight of amylase preparation in the early stages of hydrolysis of 1% starch or to the

(34) Meyer, Fischer and Bernfeld, *Helv. Chim. Acta*, **30**, 64 (1947); *Arch. Biochem.*, **14**, 149 (1947).

(35) Green and Stumpf, *J. Biol. Chem.*, **142**, 355 (1942).

(36) Allen, *Biochem. J.*, **34**, 858 (1940).

(37) Prebluda and McCollum, *J. Biol. Chem.* **127**, 495 (1939).

disappearance of starch[38] per unit weight of amylase preparation when these measurements were made in thirty minutes at 40° under specified conditions.[18] "Glucosidase" activity refers to the increase in the reducing value (calculated as mg. maltose) per unit weight of amylase preparation in two hours when the substrates were hydrolysis mixtures of starch and pancreatic amylase which had reached stages of very slow change but which still contained products capable of further hydrolysis by additional amylase preparation.

TABLE V

A SUMMARY OF ATTEMPTS TO CAUSE THE SELECTIVE INACTIVATION OF AMYLASE AND OF "GLUCOSIDASE" ACTIVITIES OF PURIFIED PANCREATIC AMYLASE PREPARATIONS

Treatment of amylase solution	Amylase activities: Saccharogenic[a]	Amyloclastic[b]	"Glucosidase" activity[c]
A. Amylase Solution Held at 50° for Five Minutes			
Unheated[d]	100	100	100
Heated[e]	17	17	10
Unheated[f]	100	100	100
Heated[g]	54	50	50
B. Amylase Solution Held at Unfavorably High Hydrogen-Ion Activities			
Untreated[d]	100	100	100
Acid-treated[h]	61	59	67
C. Influence of calcium ions at 50°			
Heated with Ca^{++}[i]	100	100	100
Heated without Ca^{++}[j]	36	40	29
D. Influence of calcium ions at unfavorable hydrogen-ion activities			
Held with Ca^{++}[k]	100	100	100
Held without Ca^{++}[l]	69	71	57
E. Influence of HNO_2			
Held at *p*H 4.8 and 0°[m]	100	100	100
The same plus nitrite[n]	55	59	47

[a] Saccharogenic activity: mg. of "maltose" formed in thirty minutes at 40° per mg. amylase preparation acting on 1% soluble potato starch under specified conditions.[18] [b] Amyloclastic activity: mg. of soluble potato starch hydrolyzed per mg. amylase preparation in thirty minutes at 40° under specified conditions to products which give a clear red color with iodine. [c] "Glucosidase" activity: increase in mg. "maltose" per mg. added amylase preparation when amylase is added at stage of very slow rate of action to a potato starch-amylase reaction mixture and allowed to react for two hours. [d] Amylase solution: 25 mg. of amylase preparation in 100 ml. solution (0.02 *M* sodium chloride, 0.01 *M* phosphate, at *p*H 7.2). Held at 0° until examined for activity. [e] A portion of amylase solution of *d* held at 50° for five minutes before being examined for activity. [f] and *g* Same as *d* and *e* but the dry purified amylase preparation had been stored in the refrigerator for several weeks. [h] Same as *d* but held at *p*H 4.5 or at *p*H 4.6 or at *p*H 5.0 at 0° for five, ten or fifteen minutes before being adjusted to *p*H 7.2 and measured for amylase activity (average values). [i] Amylase solution: 25 mg. of amylase preparation in 100 ml. solution (0.02 *M* sodium chloride, 0.02 *M* calcium chloride, at *p*H 7.2) held at 50° for five minutes. [j] Same as *i* but without calcium chloride. [k] Amylase solution: 25 mg. of amylase preparation in 100 ml. solution (0.02 *M* sodium chloride, 0.02 *M* calcium chloride), held at 0° at *p*H 4.0, *p*H 4.5, or at *p*H 5.0 for five or ten minutes before being adjusted to *p*H 7.2 and measured for amylase activity. [l] The same as *k* but containing no calcium chloride. [m] Amylase preparation dissolved in 0.25 *M* sodium acetate, 0.01 *M* sodium phosphate and 0.02 *M* sodium chloride at *p*H 4.8 held at 0° for fifteen minutes before being adjusted to *p*H 7.2 and measured for amylase activity. [n] Same as *m* but with addition of 1.0 *M* sodium nitrite. All data are averages of several determinations.

(38) Hydrolysis of starch to products which give a clear red color with iodine.

All determinations of amylase and of "glucosidase" activities in any given series of measurements were kept strictly comparable. Amylase solutions were prepared and divided into two portions. After one had been treated to cause partial inactivation of the amylase, both portions were examined side by side, with portions of each of the two substrates, for amylase and for "glucosidase" activities.

The conditions chosen for the partial inactivation of the amylase were based upon the results of much previous work in this laboratory with similar purified preparations[17,39,40] and on preliminary experiments.

The data summarized in Table V (A and B) show that there was appreciable loss of "glucosidase" as well as of amylase activity when aqueous solutions of purified pancreatic amylase were partially inactivated at 50° or at unfavorably high hydrogen-ion activities.

Kneen[41] has shown that calcium ions protect malt α-amylase from inactivation when its aqueous solutions are held at unfavorable temperatures or at unfavorably high hydrogen-ion activities. The data summarized in Table V (C and D) show that both the amylase and the "glucosidase" activities of purified pancreatic amylase were protected by calcium ions from inactivation in aqueous solutions held at 50° or at unfavorably high hydrogen-ion activities.

In 1942, Little and Caldwell[39,40] showed that the amylase activity of pancreatic amylase depends upon the presence of free primary amino groups in the protein molecule. Any treatment which caused the loss of primary amino nitrogen caused a corresponding loss of amylase activity. The data summarized in Table V (E) show that "glucosidase" as well as amylase activity was decreased when solutions of purified pancreatic amylase were treated with nitrous acid under conditions which caused the rapid loss of primary amino groups. In these experiments the influence of the unfavorably high hydrogen-ion activities was taken into consideration. Aliquots of the amylase solution adjusted to *p*H 4.8, and 0.25 *M* acetate, 0.01 *M* phosphate and 0.02 *M* chloride were held at 0° for the specified length of time in the absence of and in the presence of 1.0 *M* nitrite. The solutions were then adjusted to *p*H 7.2 with phosphate and compared with an untreated control solution for amylase and for "glucosidase" ac-

(39) Little and Caldwell, *J. Biol. Chem.*, **142**, 585 (1942).

(40) Little and Caldwell, *ibid.*, **147**, 229 (1943).

(41) Keen, Sandstedt and Hollenbeck, *Cereal Chem.*, **20**, 399 (1943).

tivities. The excess nitrite was removed by treatment with sulfamic acid before reducing values were determined.

The data summarized in Table V fail to give any evidence by selective inactivation for the presence of a second carbohydrase in addition to amylase in the preparations of purified pancreatic amylase used here. The differences obtained in the inactivation studies of "glucosidase" activities were small and undue emphasis cannot be placed on comparisons of inactivation percentages when different activities, obtained by different types of measurements, are involved. However, the results as a whole give the same trend for the inactivation of "glucosidase" and of amylase activities and appear to justify the conclusion that the properties of pancreatic amylase observed in this investigation are not influenced to any important extent by the presence of contaminating carbohydrases.

Summary and Conclusions

A study of the action of highly purified pancreatic amylase shows that the extent of the hydrolysis of unfractionated potato starch, Lintner soluble starch and of the linear fraction from corn starch depends in each case upon the concentration of amylase. Relatively very high concentrations of the amylase gave no evidence of a limit in the hydrolysis of starch by pancreatic amylase such as is observed with β-amylase.

The reaction curves showed a rapid phase of reaction followed by a phase of very slow rate of change but the extent of the hydrolysis attained was dependent within wide limits upon the concentration of amylase. Under the conditions of these experiments the slowing down of the reactions was not due to any appreciable inactivation of the amylase nor to inter-reaction with or equilibrium between amylase and maltose, glucose or other readily dialyzable products of hydrolysis.

Evidence is presented which shows that the slowing down of the hydrolysis is due to relatively low concentrations of products which the amylase hydrolyzes slowly, for which it has low affinity.

Data are presented which show that the results obtained here are not influenced to any appreciable extent by the presence of maltase or of other carbohydrases.

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(42) Original manuscript received May 7, 1947.

[CONTRIBUTION FROM PHILLIPS PETROLEUM COMPANY, RESEARCH DEPARTMENT]

Butylation of Benzene during Propylation in the Presence of Isobutane. Ratio of Reactivities of Benzene and Isobutane

BY FRANCIS E. CONDON AND MARYAN P. MATUSZAK

In an experiment originally designed to determine the ratio of the rates of alkylation of benzene and isobutane, a mixture of these two hydrocarbons was subjected to alkylation with propylene in the presence of hydrofluoric acid as catalyst. It was found that, besides isopropylation of benzene, considerable *t*-butylation occurred. The formation of *t*-butylbenzene may be taken as an indication of the intermediate formation of *t*-butyl carbonium ions, which must have been derived from isobutane in accordance with the following considerations.

According to the ionic mechanism of catalytic alkylation of a hydrocarbon, the initial step is formation of a carbonium ion[1]; for example

$$H^+ + C_3H_6 \longrightarrow i\text{-}C_3H_7^+ \qquad (1)$$

When the alkylatable hydrocarbon is an isoparaffin like isobutane, the various subsequent steps may be generalized as reactions in which a carbonium ion, however formed, produces another carbonium ion, either by acquiring hydrogen with its bonding electrons from isobutane, thereby producing also a paraffin

$$i\text{-}C_3H_7^+ + i\text{-}C_4H_{10} \xrightarrow{k} t\text{-}C_4H_9^+ + C_3H_8 \qquad (2)$$

(1) Price and Ciskowski, THIS JOURNAL, **60**, 2499 (1938).

or by uniting with an olefin, which may have been introduced as such, or may have been formed by the reverse of a reaction like 1

$$i\text{-}C_3H_7^+ + i\text{-}C_4H_8 \longrightarrow C_7H_{15}^+ \qquad (3)$$

The new carbonium ions formed by such reactions undergo similar reactions; in addition, some of them undergo preliminary rearrangement, thereby accounting for the multiplicity of products from isoparaffin alkylation.[2]

In the aforementioned experiment, substantially no alkylation of isobutane occurred, in spite of a 50-fold molecular excess of isobutane over benzene. Consequently, it was not possible to deduce, from the composition of the alkylation product, a numerical value for the ratio of the rate of alkylation of benzene to the rate of alkylation of isobutane. There was deduced, however, an approximate numerical value for the ratio of the reactivity of benzene with isopropyl carbonium ion to the reactivity of isobutane with this ion. This deduction was possible because the isopropylation of benzene

$$i\text{-}C_3H_7^+ + C_6H_6 \xrightarrow{k'} i\text{-}C_3H_7C_6H_5 + H^+ \qquad (4)$$

(2) (a) Bartlett, Condon and Schneider, *ibid.*, **66**, 1531 (1944); (b) see also Schmerling, *ibid.*, **68**, 275 (1946); Ciapetta, *Ind. Eng. Chem.*, **37**, 1210 (1945).