[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

A Comparison of the Action of Several Alpha Amylases upon a Linear Fraction from Corn Starch[1]

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Evidence is presented that alpha amylases from different sources differ markedly in their action on the same linear substrate. These differences are discussed.

Introduction

When examined in the very early stages of the hydrolysis of starch, alpha amylases from several different sources were found to give the same ratio of saccharogenic to dextrinogenic activities and the conclusion was drawn that the action of these alpha amylases on the same substrate was the same.[2,3] On the other hand, much evidence[4-8] leads to the conclusion that alpha amylases from different sources differ markedly in their action. Such differences are especially evident in the pioneer spectrophotometric measurements of amylase action reported by Hanes and Cattle[4] and are confirmed by the work reported here. In this investigation, a comparison has been made of the action of several alpha amylases on portions of the same well characterized substrate, a linear fraction from corn starch.[9,10] Strictly comparable measurements were made of the relationship between the disappearance of the substrate, as judged by blue values,[11-14] and the increase in the reducing values[15] of the hydrolyzates during the course of the hydrolyses. This relationship is extremely sensitive and is independent of the concentration of the amylase being studied. Quantitative information of this kind increases our understanding of the action of these important enzymes and justifies interpretation of the results in terms of the architecture of their substrates.

(1) This investigation was supported, in part, by research grants from the National Institutes of Health, Public Health Service, and in part by a grant from the Corn Industries Research Foundation.

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(14) Blue value is defined as follows: B.V. = $(D_t/D_0) \times 100$ where D_0 and D_t are the optical densities of the hydrolyzates at initial, or zero, time and at t minutes of hydrolysis, respectively. The optical density values were measured with a Beckman spectrophotometer and with a Cary recording spectrophotometer at the wave lengths of maximum absorption and also at 610 mμ, with 2-ml. portions of the hydrolyzates diluted to 500 ml. and to a total concentration of 0.002% iodine and 0.02% potassium iodide.

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Experimental

Amylases.—The amylases studied included: three times crystallized swine pancreatic amylase[16]; recrystallized taka amylase;[17,18a,b] crystalline bacterial amylase, *subtilis*[19,20]; human salivary amylase; human pancreatic amylase[21]; swine salivary amylase[22,23] and amylase solutions obtained by pooling fat-free extracts of homogenates of the pancreas, the liver and the spleen of well nourished rats[24-26] as well as fat-free extracts of their blood serum.

The crystalline amylases were free from traces of maltase activity.[16,18a,b,19] The crystalline pancreatic amylase and the crystalline taka amylase had been found to be homogeneous by electrophoresis and by sedimentation measurements.[16,18a,b] In addition, evidence that each of the crystalline proteins, pancreatic amylase and taka amylase, is enzymically homogeneous has been obtained by selective inactivation studies and by comparisons of the solubilities of the protein and of the active amylase in each case.[16,18a,b] Therefore, any differences in the action of these two amylases, at least, appear to be true properties of the amylases themselves. The solutions of human and of swine salivary amylases and of human pancreatic amylase also were free from significant traces of maltase activity. The solutions of the uncrystallized amylases were held in the frozen state until used. No loss of amylase activity was encountered

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(19) K. H. Meyer, M. Fuld and P. Bernfeld, *Experientia*, **3**, 411 (1947).

(20) The authors wish to thank Dr. Ed. H. Fischer who kindly supplied this crystalline amylase.

(21) (a) The authors wish to thank Dr. Henry Doubilet and Dr. Louis Fishman of the New York University College of Medicine who kindly supplied the human pancreatic juice from two male patients. The juice was obtained through tubes placed in the pancreatic ducts.[21b] The juice was frozen immediately after collection. It was thawed, centrifuged, diluted and used without further purification. (b) Henry Doubilet and John H. Mulholland, *Proc. Soc. Exp. Biol. Med.*, **76**, 113 (1951).

(22) The authors wish to thank Mr. Edward M. Caldwell and Dr. K. K. Burriss for their coöperation in this part of the work. Two 250-pound sows were induced to drool by injections of pilocarpine.[23] The saliva was frozen immediately and shipped to the Laboratory in the frozen state. It was thawed, centrifuged, diluted and measured for amylase and for maltase activities and used without further purification and also after partial purification by alcohol fractionation.

(23) The injections of pilocarpine were suggested by Dr. Carl F. Cori.

(24) Young adult rats raised[25] on Sherman diet 13[26] were stunned by a sharp blow at the base of the skull. After severing the jugular vein, the blood pumped by the heart was collected in potassium oxalate in centrifuge tubes. After centrifuging at 0°, the serum was shaken repeatedly with toluene and again centrifuged. The organs were dissected out, rinsed with distilled water and pressed between filter paper. Two-gram portions were ground with 10 ml. of buffer at pH 7.2, 0.01 M phosphate and 0.02 M potassium chloride in an all-glass homogenizer immersed in an ice-bath. The homogenates were centrifuged at 40,000 r.p.m. at 0° for one hour. The upper layer containing lipids was pipetted off, the middle layer containing the amylase, collected and the residue discarded. The amylase extract was shaken repeatedly with toluene to remove the remaining lipids.

(25) Kindness of Mrs. Constance Pearson.

(26) H. C. Sherman, M. E. Rouse, Bernice Allen and Ella Woods, *J. Biol. Chem.*, **46**, 503 (1921),

upon repeated freezing and thawing of any of these solutions.

Substrate.—The substrate was the linear fraction from corn starch, prepared essentially according to Schoch.[9,10] It was dissolved in molar potassium hydroxide, neutralized with hydrochloric acid and diluted with appropriate buffers and salts to 0.25%.

Measurements of Amylase Activities.—Portions of the same composite sample of a linear fraction from corn starch[9,10] were used for all of the measurements reported here. Each substrate was adjusted to the conditions that had been found previously to favor the action of the amylase being investigated.[16-19,27-29] All hydrolyses were carried out at 40°. Portions of each hydrolyzate were examined initially and at suitable intervals for total reducing values[15] and for blue values.[11-14] In this way, it was possible to correlate the disappearance of the substrate and of its products capable of giving blue values[30a,b,31a,b] with the increase of the reducing values of the hydrolyzates. The determinations of the blue values depend upon the fact that the linear components of starches and certain of their hydrolysis products absorb iodine to form complexes that can be determined quantitatively by spectrophotometric methods.[4,11-14,30-34]

Every comparison of amylase action included measurements with three-times crystallized maltase-free swine pancreatic amylase. In addition, repeated experiments were staggered to give direct comparisons of the action of several different amylases upon portions of the same original dispersion of the linear fraction, adjusted to favor the action of each amylase. In this way it was hoped to minimize any influence that might be exerted by differences from day to day in the state of the dispersion of the substrate. Such differences might influence the blue values.[11-14,30-34]

Results and Discussion

Relation between Blue Values and Reducing Values Independent of Concentration of Amylase.—The data given in Fig. 1 for swine pancreatic amylase and for taka amylase illustrate the fact that the relationship between the disappearance of the substrate, as indicated by decrease in blue values, and the increase in the reducing values of a hydrolyzate is independent of the concentration of a given amylase. With the amylases studied here, the data for different concentrations of a given amylase always fell on the same curve when blue values were plotted against reducing values. Similar conclusions were reached by Hanes and Cattle in their work.[4]

Alpha Amylases of Animal Origin.—The data given in Fig. 2 illustrate the fact, repeatedly observed, that human pancreatic amylase and human salivary amylase from the same or from different people hydrolyze the same linear substrate in the same manner, at least during the early stages of the hydrolyses, when not more than 15% of the glucosidic linkages of the substrate have been broken. The data illustrate the results of large numbers of measurements and include pancreatic amylase from two male patients[21] and salivary amylase from one of these patients[21] as well as salivary amylase from the saliva of other persons of different races and of different nutritional habits.

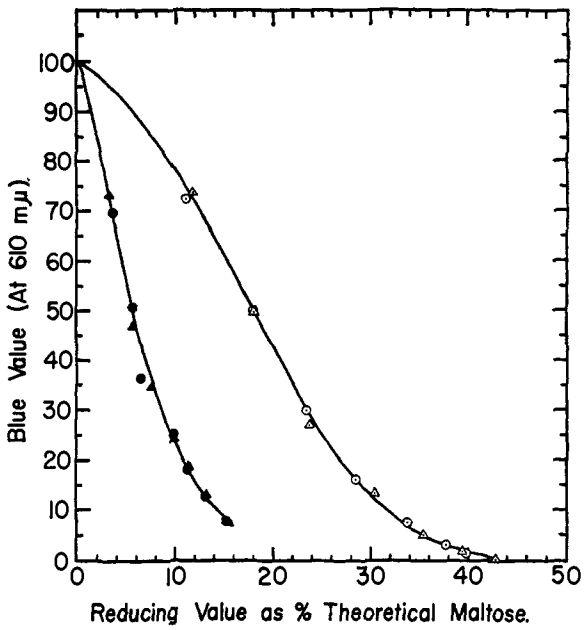

Fig. 1.—Hydrolyses of linear fraction from corn starch by different concentrations of pancreatic amylase from swine and by different concentrations of taka amylase. Relationship between disappearance of substrate, as indicated by blue values, and increase in reducing values of hydrolyzates. Hydrolyses at 40° for different time intervals. Linear fraction from corn starch, 0.25%, adjusted for pancreatic amylase to, 0.01 *M* phosphate, 0.02 *M* potassium chloride, *p*H 7.2; for taka amylase to, 0.02 *M* acetate, 0.02 *M* potassium chloride, *p*H 5.0; ⊙, 0.014 mg. and ▲, 0.056 mg. of recrystallized maltase-free pancreatic amylase per gram linear fraction; ●, 0.02456 mg. and ▲, 0.09824 mg. of recrystallized maltase-free taka amylase per gram linear fraction.

It is interesting to note that while the concentrations of amylase in the saliva of the same person at different times and of different persons often differed widely, the action of the salivary amylase in the different samples of saliva was remarkably uniform in this type of measurement. In the same way, the concentration of amylase in pancreatic juice differs from time to time for any given patient and also for different patients but the action of the different samples of human pancreatic amylase was the same in these studies and identical with that of human salivary amylase from the same and from different persons.

Amylase solutions obtained from different organs of the rat all exerted the same action and this action was very similar to if not identical with that of human pancreatic and human salivary amylase. These findings are illustrated by the data given in Table I and in Fig. 3 and afford additional justification for the use of the rat as a deputy for man in nutritional experiments.

On the other hand, the data given in Table I and in Fig. 3 show that swine pancreatic amylase differs markedly in its action from that of swine salivary amylase and that both of these amylases, in turn, differ in their action from that of human pancreatic and human salivary amylase. These differences in action appear to be true properties

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(30) M. A. Swanson and Carl F. Cori, *J. Biol. Chem.*, **172**, (a) 797; (b) 815 (1948).

(31) M. A. Swanson, *ibid.*, **172**, (a) 805; (b) 825 (1948).

(32) F. L. Bates, D. French and R. E. Rundle, THIS JOURNAL, **65**, 142 (1943).

(33) R. R. Baldwin, R. S. Bear and R. E. Rundle, *ibid.*, **66**, 111 (1944).

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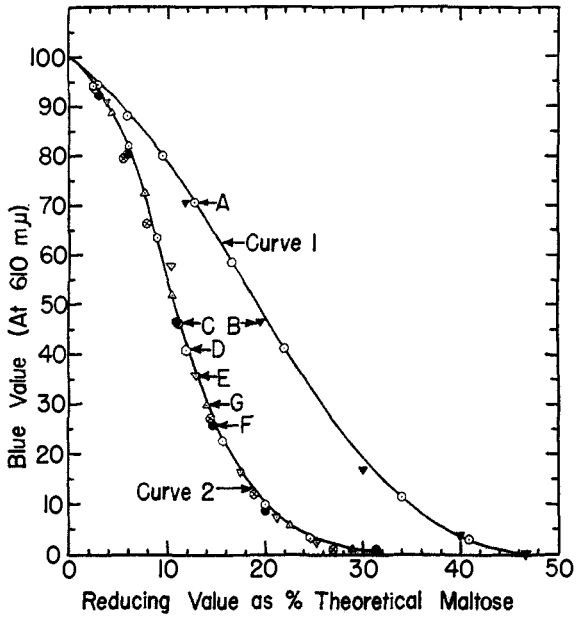

Fig. 2.—Comparison of the action of human salivary amylase, of human pancreatic amylase, and of swine pancreatic amylase on a linear fraction from corn starch. Hydrolyses at 40° for different intervals of time. Linear fraction from corn starch, 0.25%; 0.01 *M* phosphate; 0.02 *M* potassium chloride; *p*H 7.2. Curve 1, pancreatic amylase from swine: A, ⊙, recrystallized, maltase-free swine pancreatic amylase; B, ▼, partially purified swine pancreatic amylase; curve 2, human pancreatic and human salivary amylase, C, ⊗, human pancreatic amylase, male patient #1, D, ⟨·⟩, human pancreatic amylase, male patient 2; E, ▽, human salivary amylase, male patient 1; F, ●, human salivary amylase, female adult, white; G, △, human salivary amylase, female adult, Chinese.

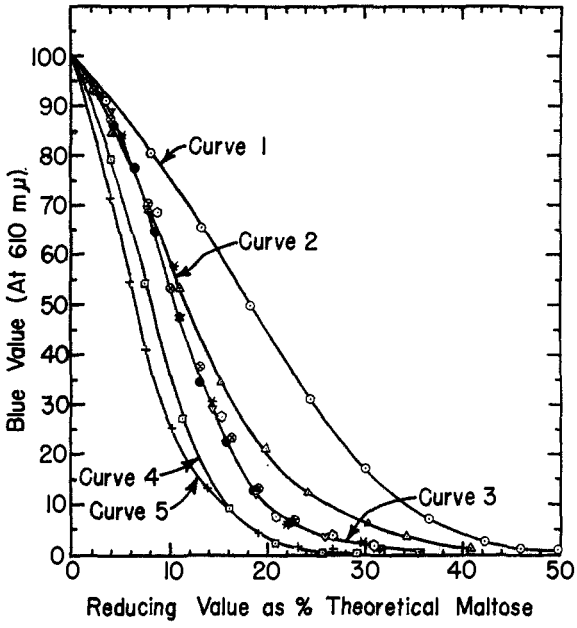

Fig. 3.—Comparison of the action of alpha amylases from different sources upon linear fraction from corn starch. Hydrolyses at 40° for different intervals of time. Linear fraction from corn starch: 0.25%; adjusted for animal amylases to 0.01 *M* phosphate; 0.02 *M* potassium chloride; *p*H 7.2; for bacterial, *subtilis*, amylase, to 0.01 *M* phosphate, 0.02 *M* potassium chloride; *p*H 6.4; for taka amylase to, 0.02 *M* acetate, 0.02 *M* potassium chloride; *p*H 5.0. Curve 1, ⊙, recrystallized maltase-free pancreatic amylase from swine; curve 2, △, swine salivary amylase; curve 3, human salivary amylase and rat amylases; ▽, human salivary amylase; ⟨·⟩, rat pancreatic amylase; ⊗, rat liver amylase; *, rat spleen amylase; ●, rat serum amylase; curve 4, ⊡, recrystallized maltase-free bacterial *subtilis* amylase: curve 5, ×, recrystallized maltase-free taka amylase.

of the amylases involved. Each curve in Figs. 2 and 3 is typical of the results obtained in many comparisons with different solutions and with different concentrations of the amylases as well as for different lengths of time of hydrolysis of the substrate.

Because crystalline maltase-free pancreatic amylase from swine was used as the control in all comparisons of this type, the data for this amylase represent an unusually large number of determinations. In addition, the crystalline amylase was obtained from commercial pancreatin[16] and undoubtedly represented the pancreas glands of a large number of hogs. Moreover, as shown in Fig. 2, the data for partially purified swine pancreatic amylase agree well with those given by the crystalline maltase-free amylase. Therefore, the extensive purification and crystallization procedures[16] did not cause any significant change in the action of the amylase. Moreover, although contaminated by traces of maltase activity,[16] the partially purified pancreatic amylase from swine showed the same amylase action in these studies as the recrystallized maltase-free pancreatic amylase, Fig. 2. Therefore, the differences observed in the action of crystalline maltase-free swine pancreatic amylase and of swine salivary amylase do not appear to be due to possible contamination of the swine salivary amylase by traces of maltase or other glucosidases. Evidently, sufficiently large concentrations of maltose and of other sugars are not available at these early stages in the hydrolyses of the substrate to influence the results in this type of experiment even if traces of maltase and other glucosidases were present. In any case, contamination by maltase or by other glucosidases, except *trans*-glucosidases, would be expected to increase the reducing values of the hydrolyzates at any given stage in the hydrolyses and, thus, would tend to minimize rather than to exaggerate the differences observed in the action of the other amylases as compared with that of the crystalline maltase-free swine pancreatic amylase, Fig. 3, Table I. Incidentally, the best evidence available at present indicates that the alpha amylases studied here are not mixed in nature with other amylases.

Taka Amylase and Bacterial Amylase, *subtilis*.—The data given in Table I and in Fig. 3 also show that maltase-free crystalline taka amylase[18a,b] and maltase-free crystalline bacterial amylase, *subtilis*,[19] differ in their action from each other and from the other amylases in the comparison. Although taka amylase and the bacterial amylase both bring their hydrolyzates to the achroic point very early in the hydrolyses, when only 14% of the

TABLE I

COMPARISON OF THE ACTION OF DIFFERENT ALPHA AMYLASES ON PORTIONS OF THE SAME LINEAR SUBSTRATE[a]

Total reducing val. as theor. maltose[c], %	Glucosidic linkages broken,[c] %	Blue value[b] color remaining, %									
		Pancreatic amylase swine[d]	Salivary amylase swine[d]	Pancreatic amylase human[d]	Salivary amylase human[d]	Pancreatic amylase rat[d]	Blood serum amylase rat[d]	Spleen amylase rat[d]	Liver amylase rat[d]	Bacterial amylase *subtilis*[e]	Taka amylase[f]
2	1	96	92.6	95.6	95.0	94.6	94.6	94.6	94.2	90.6	87.4
4	2	91.2	84.4	89.2	88.0	88.0	88.0	88.0	87.0	79.0	71.4
6	3	86.2	76.2	80.6	80.0	80.2	80.2	80.2	79.8	65.0	54.2
8	4	80.8	68.0	69.0	68.6	71.0	71.0	71.0	69.6	50.6	38.0
10	5	75.0	59.2	54.0	55.0	57.6	57.6	57.6	56.4	36.2	26.2
12	6	69.2	50.2	41.0	41.4	44.6	44.6	44.6	44.6	23.4	18.0
14	7	63.0	41.8	30.0	30.8	33.2	33.2	33.2	33.6	15.0	12.6
16	8	56.8	34.0	21.6	21.8	23.6	23.6	23.6	24.6	9.0	8.8
18	9	50.6	27.0	15.0	14.6	16.0	16.0	16.0	17.4	5.6	5.6
20	10	44.4	21.0	10.0	8.6	9.6	9.6	9.6	12.0	3.0	3.0
22	11	38.4	16.4	6.6	6.3	6.2	6.2	6.2	8.0	1.8	1.8
24	12	32.2	12.6	4.2	4.5	4.7	4.7	4.7	5.0	1.0	1.0
26	13	26.8	9.4	2.8	3.2	3.6	3.6	3.6		0.4	0.4
28	14	21.8	7.2	1.6	2.2	2.8	2.8	2.8		0	0
30	15	17.2	5.8	0.8	1.4	2.4	2.4	2.4			
32	16	13.2	4.6								
34	17	10.0	3.6								
36	18	7.2	2.9								
38	19	5.2	2.0								
40	20	4.0	1.6								
42	21	3.0									

[a] Portions of same linear fraction from corn starch[9,10] adjusted to conditions most favorable to the action of each amylase; hydrolyses at 40°. [b] Blue values[14] read from large graph of data given in Fig. 3; human pancreatic amylase from data in Fig. 2. [c] Iodometric method[15] gives stoichiometric measure of glucosidic linkages broken in substrate. [d] 0.25% substrate; 0.01 *M* phosphate; 0.02 *M* potassium chloride; *p*H 7.2.[16,19,28] [e] 0.25% substrate; 0.01 *M* phosphate; 0.02 *M* potassium chloride; *p*H 6.4.[27] [f] 0.25% substrate; 0.02 *M* acetate; 0.02 *M* potassium chloride; *p*H 5.0.[17,18a,b]

glucosidic linkages of the substrate have been broken, it is evident that the action of these two amylases on the same substrate is very different in the earlier stages of the hydrolyses. Thus 62% of the substrate had disappeared when 4% of the glucosidic linkages had been broken by taka amylase, whereas 50% of the substrate remained at the same stage in the hydrolysis by the bacterial amylase, Table I.

Additional Comparisons of the Action of Alpha Amylases from Different Sources.—A comparison was made of the changes in the absorption spectra of the iodine complexes formed, first with portions of the unhydrolyzed substrate and then with portions of the products present at different stages in its hydrolyses by the different amylases. These data were obtained with a Cary recording spectrophotometer and all of the hydrolyses in the comparison were carried out not only with portions of the same composite sample of the same linear substrate but, except for human pancreatic amylase, with portions of the same original dispersion of that substrate, adjusted to favor the action of each amylase. Therefore, the data for the different amylases are strictly comparable. The data obtained with crystalline maltase-free, enzymically homogeneous pancreatic amylase and with crystalline maltase-free enzymically homogenous taka amylase represent the two extremes observed and are given in Figs. 4 and 5. Similar data for the other amylases are omitted for the sake of brevity.

A study of the data given in Figs. 4 and 5 shows that the peaks of the absorption spectra of the iodine complexes shift toward shorter wave lengths as the hydrolyses proceed. A similar shift was reported by Hanes and Cattle in their work[4] and was observed with the other amylases studied here. This shift is related to differences in the average chain length of the products present in the hydrolyzates.[12,30b,35,36] Furthermore, if the data given in Figs. 4 and 5 are compared at equivalent stages in the hydrolyses, as judged by the same total reducing values, it is evident that the shift in the absorption peaks of the iodine complexes formed with the hydrolysis products is much more rapid when the substrate is hydrolyzed by taka amylase than when it is hydrolyzed by pancreatic amylase. This comparison gives additional evidence that these two highly purified maltase-free, enzymically homogeneous amylases[16,18a,b] hydrolyze the same substrate into fragments of different chain lengths.

Additional information about the comparison is given in Tables II to VI and in Fig. 6. The average wave lengths of the peaks of the absorption spectra of the iodine complexes, reported in Tables II to VI and in Fig. 6, were read directly from the large graphs of the original data. The average chain lengths of the dextrins present in the hydrolyzates at different stages in the hydrolyses, reported in Tables II to VI were estimated by comparison of the average wave lengths of the absorption peaks of the iodine complexes formed by the products present at each of the different stages in the hydrolyses with the wave lengths reported by

(35) R. W. Kerr, "Chemistry and Industry of Starch," 2nd Ed., Academic Press, Inc., New York, N. Y., 1950, p. 188.

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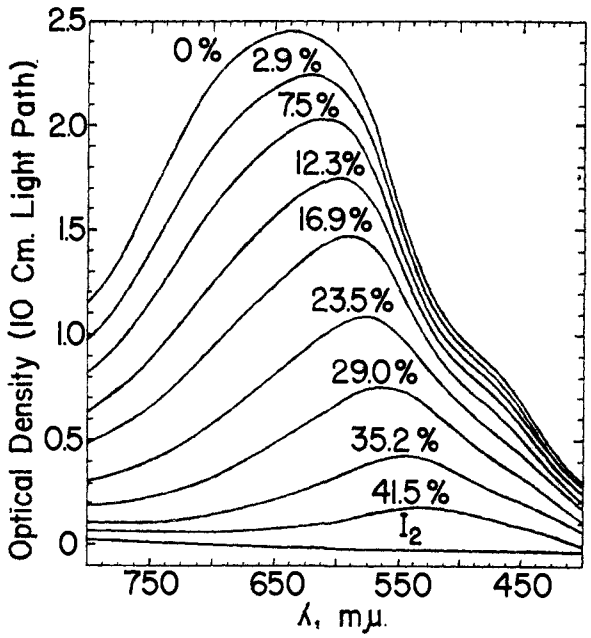

Fig. 4.—Changes in the absorption spectra of iodine complexes during hydrolysis of linear fraction from corn starch by recrystallized maltase-free enzymically homogeneous pancreatic amylase from swine. Hydrolyses at 40°; the percentage of hydrolysis is indicated on each curve as per cent. theoretical maltose. Linear fraction from corn starch; 0.25%; 0.01 *M* phosphate; 0.02 *M* potassium chloride; *p*H 7.2.

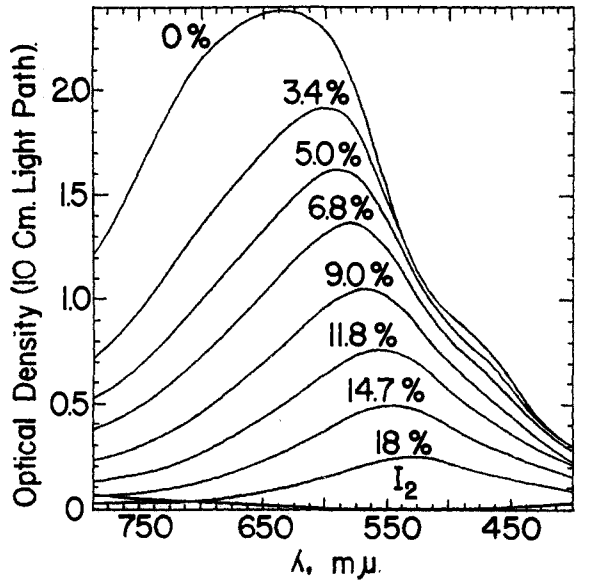

Fig. 5.—Same as for Fig. 4, but for recrystallized maltase-free enzymically homogeneous taka amylase. Linear fraction from corn starch: 0.25%; 0.02 *M* acetate; 0.02 *M* potassium chloride; *p*H 5.0.

Swanson[31b] for the absorption peaks of the iodine complexes given by synthetic linear polysaccharides of known chain lengths.

Taken all together, the data reported here show that all of the amylases in this investigation exhibit an important characteristic of alpha amylases. They all cause the relatively rapid breakdown of the linear substrate to products that no longer give colored complexes with iodine, to products that average 7–13 glucose residues.[31b] On the other hand, the number of glucosidic linkages of the substrate broken to reach this achroic stage and the course of the earlier stages of the hydrolyses of the substrate differ markedly from amylase to amylase. Thus, the data in Tables II to VI give the following

TABLE II

ACTION OF CRYSTALLINE MALTASE-FREE PANCREATIC AMYLASE, SWINE, ON LINEAR FRACTION FROM CORN STARCH[a]

Total reducing val. as theor. maltose,[b] %	Glucosidic linkages broken,[b] %	Blue value[c] remaining, % At max. absorp.[d]	Blue value[c] remaining, % At 610 mμ[d]	Absorp. peak,[d] mμ	Av. chain lengths[e] remaining in hydrolyzate, glucose units
0	0	100	100	623–635	>56
2.9	1.5	92.0	92.5	610–628	>56
7.5	3.8	83.0	84.5	611–617	>56
12.3	6.2	71.8	72.2	591–605	56
16.9	8.5	60.5	59.2	587–595	35–56
23.5	11.8	45.2	41.8	569–580	20–29
29.0	14.5	31.7	26.8	559–565	20
35.2	17.6	18.1	13.1	540–550	18–20
41.5	20.8	8.1	5.3	518–540	13–18
46.0	23.0		1.0	505–522	7–13

[a] Linear fraction: 0.25%; 0.01 *M* phosphate; 0.02 *M* potassium chloride; *p*H 7.2; 40°.[16,28] [b] Reducing values by iodometric method.[15] [c] Percentage of blue value[14] remaining was calculated by taking optical density of unhydrolyzed substrate as 100. [d] Taken from graphs of original data. [e] The average chain lengths of dextrins remaining were estimated by comparing the wave lengths of the absorption peaks of the iodine complexes with those shown by Swanson.[31b]

TABLE III

ACTION OF SWINE SALIVARY AMYLASE ON LINEAR FRACTION FROM CORN STARCH[a]

Total reducing val. as theor. maltose,[b] %	Glucosidic linkages broken,[b] %	Blue value[c] remaining, % At max. absorp.[d]	Blue value[c] remaining, % At 610 mμ[d]	Absorp. peak,[d] mμ	Av. chain lengths[e] remaining in hydrolyzate, glucose units
0	0	100	100	620–634	>56
6.2	3.1	76.5	76.3	597	56
12.9	6.5	53.5	49.6	575–580	29–35
17.6	8.8	36.6	29.6	555–566	20
24.1	12.1	20.2	13.3	540	18
31.0	15.5	9.5	5.4	520–535	13–18
41.0	20.5		1.0	505–522	7–13

TABLE IV

ACTION OF HUMAN SALIVARY AMYLASE OR HUMAN PANCREATIC AMYLASE ON LINEAR FRACTION FROM CORN STARCH[a]

Total reducing val. as theor. maltose,[b] %	Glucosidic linkages broken,[b] %	Blue value[c] remaining, % At max. absorp.[d]	Blue value[c] remaining, % At 610 mμ[d]	Absorp. peak,[d] mμ	Av. chain lengths[e] remaining in hydrolyzate, glucose units
0	0	100	100	625–635	>56
4.2	2.1	88.1	89.0	604–612	>56
7.5	3.8	73.9	72.5	588–596	35–56
10.3	5.2	56.8	51.9	570–580	20–29
13.9	7.0	39.2	30.0	546–556	18–20
17.7	8.9	25.7	16.5	533–543	13–20
22.5	11.3	12.2	5.8	514–526	7–13
28.9	14.5	3.9	1.2	500–514	7–13

TABLE V

ACTION OF CRYSTALLINE MALTASE-FREE BACTERIAL AMYLASE, *subtilis*, ON LINEAR FRACTION FROM CORN STARCH[a]

Total reducing val. as theor. maltose,[b] %	Glucosidic linkages broken,[b] %	Blue value remaining, % At max. absorp.[d]	Blue value remaining, % At 610 mμ[d]	Absorp. peak,[d] mμ	Av. chain lengths[c] remaining in hydrolyzate, glucose units
0	0	100	100	627–635	>56
1.6	0.8	88.5	88.7	600–610	>56
6.0	3.0	74.2	72.9	592–595	35–56
9.1	4.6	53.0	48.6	573–580	20–29
13.6	6.8	29.6	23.0	550–562	18–20
18.0	9.0	16.3	10.5	533–545	13–20
22.6	11.3	6.3	3.3	516–536	13–18
24.0	12.0		1.0	505–522	7–13

[a] Linear fraction: 0.25%; 0.01 *M* phosphate; 0.02 *M* potassium chloride; *p*H 6.4[27]; 40°. [b,c,e] Same as those in Tables II–IV. [d] Taken from graphs of original data.

TABLE VI

ACTION OF CRYSTALLINE MALTASE-FREE TAKA AMYLASE ON A LINEAR FRACTION FROM CORN STARCH

Linear substrate: 0.25%; 0.01 *M* acetate; 0.02 *M* potassium chloride; *p*H 5.0.[17,18a,b,29] 40°.

Total reducing val. as theor. maltose,[b] %	Glucosidic linkages broken,[b] %	Blue value[c] remaining, % At max. absorp.[d]	Blue value[c] remaining, % At 610 mμ[d]	Absorp. peak,[d] mμ	Av. chain lengths[c] remaining in hydrolyzate, glucose units
0	0	100	100	625–635	>56
3.4	1.7	81.4	81.5	594–603	56
5.0	2.5	69.1	67.6	585–590	35–56
6.8	3.4	58.1	54.7	575–584	29–35
9.0	4.5	44.5	39.2	565–577	20–29
11.8	5.9	32.2	25.8	548–561	18–20
14.7	7.4	20.8	14.2	542–547	18–20
18.0	9.0	10.6	5.2	525–539	13–18
24.0	12.0		1.0	505–522	7–13

values for the percentages of glucosidic linkages of the substrate broken by the different amylases at approximately the achroic stage of the hydrolyses, when products capable of giving approximately 1% of the blue value remained: swine pancreatic amylase, 23%; swine salivary amylase, 20.5%; human pancreatic amylase or human salivary amylase, 14.5%; bacterial, *subtilis*, amylase, 12% and taka amylase, 12%. Even greater differences in the action of these amylases are seen when comparisons are made at earlier stages of the hydrolyses. For example, it is evident that swine pancreatic amylase at one extreme causes a much more gradual breakdown of the substrate than that caused by taka amylase at the other extreme. It also is evident that the action of each of these amylases follows its own pattern in the hydrolysis of a given substrate. Therefore, these alpha amylases do not cause a random hydrolysis of the glucosidic linkages of the substrates. The products present at different stages in these hydrolyses are being investigated.

It should be emphasized that the differences noted in this investigation occur in the relatively early stages of the hydrolyses of the substrate, when not more than 25% of the glucosidic linkages of the substrate have been broken and when not more than approximately 50% of the so-called

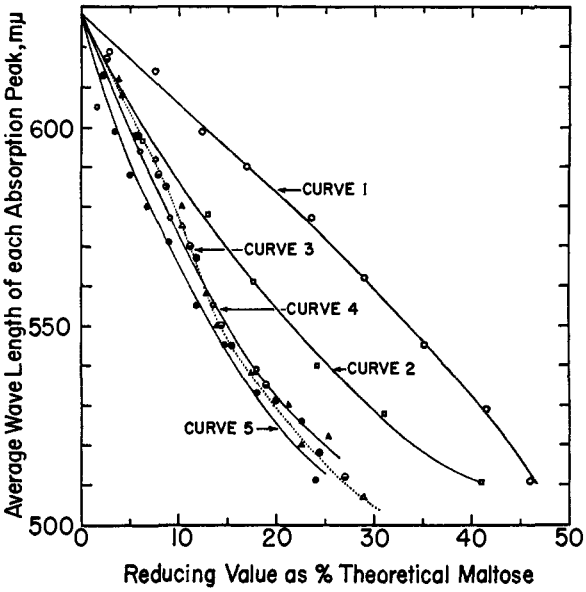

Fig. 6.—A comparison of the changes in the average wave length of the absorption peaks of the iodine complexes obtained with the products formed from a linear fraction from corn starch at different stages in its hydrolyses by alpha amylases from different sources. Curve 1, O, recrystallized, maltase-free enzymically homogeneous swine pancreatic amylase; curve 2, □, swine salivary amylase; curve 3, △, human salivary amylase, female, adult, Chinese; ▲, human salivary amylase, adult, male patient 1; ◒, human pancreatic amylase, adult, male patient 1; ⊗, human pancreatic amylase, adult, male patient 2; curve 4, O, recrystallized maltase-free bacterial, *subtilis*, amylase; curve 5, ●, recrystallized maltase-free enzymically homogeneous taka amylase.

theoretical maltose has been formed. The final products of the action of these amylases on a given substrate or on similar substrates may or may not differ. Thus, Meyer and Gonon[37] found that crystalline maltase-free swine pancreatic amylase[38] produced only glucose and maltose as the final products of the hydrolysis of linear substrates from corn or potato starches. Similarly, maltose and glucose were the only products present in the final hydrolyzates when a linear fraction from corn starch was hydrolyzed by crystalline maltase-free taka amylase.[18a,b] On the other hand, Roberts and Whelan[39] have given beautifully convincing evidence that the final hydrolyzates from a linear fraction from potato starch contain malto-triose and maltose but no glucose when hydrolyzed by maltase-free highly purified salivary amylase.[27] However, they report that crystalline salivary amylase was found by Meyer to hydrolyze malto-triose.[39]

In conclusion, when the early stages of the hydrolyses are considered, it is evident from the work reported here, that alpha amylases from a number of different sources show marked differences in their action on a given linear substrate. The rapid

(37) K. H. Meyer and W. F. Gonon, *Helv. Chim. Acta*, **34**, 294 (1951).

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

(39) P. J. P. Roberts and W. J. Whelan, *Biochem. J.*, **49**, 1VI (1951); *J. Chem. Soc.*, 1293, 1298 (1953).

breakdown of the substrate to small dextrins observed on one extreme with taka amylase and the relatively slow disappearance of long chain saccharides observed with swine pancreatic amylase on the other extreme appear to be definite properties of these amylases. It is challenging to speculate about and to seek the cause for these differences in the action of two amylases, both of which appear to be so-called simple proteins.[3,16-18,38]

NOTE ADDED OCTOBER 20, 1953.—Since this paper was submitted for publication, two additional series of experiments have been completed with results that confirm and strengthen the conclusions.

When all of the amylases in the comparison reacted with the substrate at the same hydrogen ion activity, at *p*H 7.2, the results were identical with those reported in Fig. 3 and in Table I for comparisons made when each amylase reacted with the substrate at the *p*H most favorable to its action. These results show that the differences observed in the action of the amylases are true differences and are not due to the influence of differences in the hydrogen ion activities of the substrate. An unfavorable hydrogen ion activity may decrease materially the concentration of active amylase but does not influence its unique action. It must be remembered that the comparisons given here for amylase action are independent of amylase concentration, Fig. 1.

Similarly, results identical with those reported in Fig. 3, curve 1, were obtained when suitable volumes of swine saliva that had been held at 100° for 5 minutes to inactivate the amylase were added to recrystallized swine pancreatic amylase. Thus, the substances that accompanied the unpurified swine salivary amylase had no influence on the action of recrystallized swine pancreatic amylase and presumably had no influence on the action of the swine salivary amylase they accompanied. These results give additional evidence that the differences observed above in the action of swine pancreatic amylase and of swine salivary amylase represent true differences in the specific mode of action of these two amylases.

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Conversion of Acetate and Pyruvate to Tyrosine in Yeast[1]

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$CH_3C^{14}OCOOH$ and $CH_3C^{14}OOH$ have been compared as carbon sources for the formation of tyrosine in bakers' yeast. The intramolecular distribution of radioactivity in the isolated tyrosine indicated that the aromatic ring was formed from pyruvate *via* oxalacetate or a similar unsymmetrical C_4-acid as intermediate. The side chain appeared to arise from pyruvate as an intact C_3-unit.

Considerable interest has been focused recently upon the biosynthesis of tyrosine in microörganisms. Davis's studies[3] with mutant strains of *Escherichia coli* point to shikimic acid as a normal precursor of tyrosine and phenylalanine, as well as other aromatic compounds in that organism. Baddiley, *et al.*,[4] have suggested that when acetate is used as the sole carbon source for adapted Torula yeast, the carboxyl group gives rise to the carboxyl of tyrosine, as well as to carbon 4 of the ring; the other seven carbon atoms were considered derived chiefly from the methyl group of acetate. Finally, the side chain of tyrosine may arise from pyruvate as an intact unit in *E. coli* (Cutinelli, *et al.*[5]).

In this Laboratory, C^{14}-labeled pyruvate and acetate have been compared as carbon sources for yeast growth. The radioactivity distribution patterns in the isolated tyrosine suggest that oxalacetate or other unsymmetrical C_4 acid may function as an intermediate in the conversion of pyruvate to this amino acid.

Experimental

Use was made of the yeast samples obtained previously,[6] in which 20 mmoles each of $CH_3C^{14}OCOOH$ or $CH_3C^{14}OOH$ with a specific activity of 1.85×10^6 c.p.m. per mmole, were administered as the sole carbon source to Fleischmann's bakers' yeast that had been previously grown on glucose. In the pyruvate experiments, all of the labeled substrate was utilized in 4 hours aerobically, and 5 hours anaerobically. Acetate was employed only under aerobic conditions, 39% of the labeled substrate being used in 4 hours. Details of these fermentations have been presented elsewhere.[6]

Tyrosine was isolated from the yeast hydrolysate[7] by concentration and crystallization at the isolectric point, following a fivefold dilution with non-isotopic L-tyrosine. The specific activities were thus one-fifth of the values given in Table I. Yields of the (diluted) tyrosine, obtained from three grams of dry yeast in each sample, were: from acetate, 81.0 mg.; from pyruvate (aerobic), 91.2 mg.; and from pyruvate (anaerobic), 66.1 mg. Purity of the isolated samples was established by paper chromatography.

The tyrosine obtained was degraded according to the method of Baddiley, *et al.*,[4] on the same scale in the following manner: (1) combustion to CO_2 for the specific activity of the whole molecule; (2) decarboxylation with ninhydrin for the specific activity of the carboxyl carbon; (3) fusion with KOH and NaOH to give *p*-hydroxybenzoic acid. Combustion and radioactivity assay gave the specific activity of this compound directly and the specific activity of the amino carbon of tyrosine by difference; (4) nitration of *p*-hydroxybenzoic acid to 3,5-dinitro-4-hydroxybenzoic acid. The latter was oxidized with $Ca(OBr)_2$ to bromopicrin which was in turn converted to CO_2. This represented carbon atoms 3 and 5 of the tyrosine ring; (5) nitration of *p*-hydroxybenzoic acid to picric acid. A small amount of the picric acid was burned to CO_2 to obtain the specific activity of the benzene ring as well as the specific activity of the methylene carbon atom of the side chain by difference. The remainder of the picric acid was oxidized with $Ca(OBr)_2$ to bromopicrin, which was oxidized to CO_2. This represented carbon atoms 1, 3 and 5 in the tyrosine ring. The average specific activity of carbon atoms 2, 4 and 6 could be thus obtained by difference.

Carbon atom 4 of the ring was not differentiated from

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(2) National Science Foundation Predoctoral Fellow.

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