REFERENCES

- Abou Akkada, A. R. & Howard, B. H. (1960). *Biochem. J.* **76**, 445.
- Abou Akkada, A. R. & Howard, B. H. (1961). *Biochem. J.* **78**, 512.
- Abou Akkada, A. R. & Howard, B. H. (1962). *Biochem. J.* **82**, 313.
- Archibald, A. R. & Manners, D. J. (1959). *Biochem. J.* **73**, 292.
- Bailey, R. W. (1958). *N.Z. J. agric. Res.* **1**, 825.
- Bailey, R. W., Clarke, R. T. J. & Wright, D. E. (1962). *Biochem. J.* **83**, 517.
- Bailey, R. W. & Howard, B. H. (1962). *Arch. Biochem. Biophys.* **99**, 299.
- Bailey, R. W. & Robertson, A. M. (1962). *Biochem. J.* **82**, 272.
- Dahlqvist, A. (1961). *Biochem. J.* **80**, 547.
- Duncan, W. A. M. & Manners, D. J. (1958). *Biochem. J.* **69**, 343.
- Hobson, P. N., Pirt, S. J. & Whelan, W. J. (1951). *J. chem. Soc.* p. 801.
- Howard, B. H. (1957). *Biochem. J.* **67**, 643.
- Howard, B. H. (1959). *Biochem. J.* **71**, 675.
- Huggett, A. St G. & Nixon, D. A. (1957). *Lancet*, ii, 368.
- McIlvaine, T. C. (1921). *J. biol. Chem.* **49**, 183.
- Mould, D. L. & Thomas, G. J. (1958). *Biochem. J.* **69**, 327.
- Nigam, V. N. & Giri, K. V. (1960). *J. biol. Chem.* **235**, 947.
- Taylor, P. M. & Whelan, W. J. (1962). *Chem. & Ind.* p. 44.
- Whelan, W. J., Bailey, J. M. & Roberts, R. J. P. (1953). *J. chem. Soc.* p. 1293.
- Wilson, C. M. (1959). *Analyt. Chem.* **31**, 1199.

Biochem. J. (1963) **86**, 452

The Action of some α -Amylases on Starch Granules

By GWEN J. WALKER AND PAMELA M. HOPE

Department of Biochemistry, University of Sydney, Sydney, Australia

(Received 24 May 1962)

Many enzymes that can convert starch solution into sugars are known, but it is not known whether all the enzymes that degrade dissolved starch can also attack starch granules. The researches of early workers, which have been reviewed by Reichert (1913), indicated that certain crude α -amylases could slowly hydrolyse raw starch. The view is still held that α -amylase is responsible for the breakdown of intact starch granules (Kneen, Beckord & Sandstedt, 1941), but it remains to be explained why the speed of the hydrolysis of raw starch observed *in vitro* is so much slower than *in vivo*. In the present paper the action is described of both crude and crystalline α -amylase from various sources on cereal, tuber and protozoal starch. Some factors that are of importance for the rapid digestion of starch granules are discussed.

MATERIALS AND METHODS

Tuber starch. Starch was prepared from potatoes as described by Schoch (1957). Soluble starch (Lintner) was supplied by Hopkin and Williams Ltd.

Cereal starch. Waxy-maize starch was prepared by the method of Schoch (1957) from hand-sorted single-cross Tapicorn seed kindly given by the Bear Hybrid Corn Co., Ill., U.S.A. Maize starch was prepared similarly. Rice starch was prepared as described by Whelan (1955).

Protozoal starch. Starch was prepared from the rumen ciliates *Holotricha* spp. as described by Mould & Thomas (1958), the deproteinization with organic solvent being

omitted. Maltose hydrate was supplied by Thomas Kerfoot Ltd., Vale of Bardsley, Lancs. Sodium deoxycholate was Laboratory Reagent grade (British Drug Houses Ltd.).

Amylases. Saliva was collected from several people and centrifuged after 1 hr. to remove mucins. Salivary α -amylase was purified by the method of Fischer & Stein (1961). Human salivary α -amylase (crystalline) was a gift from Dr E. A. Stein. A crude α -amylase from pig pancreas was prepared by grinding the minced pancreas (148 g.) with borate buffer (60 ml.) at pH 8.5. The mixture was allowed to stand at 0° for 1 hr. with occasional stirring, and then was filtered through gauze. The extract was clarified by centrifuging at 80 000g and an acetone-dried powder was prepared from the supernatant. A portion (489 mg.) of this powder was extracted with a solution (5 ml.) containing 0.1M-sodium chloride, 0.01M-calcium acetate and 0.02M-tris-maleate buffer, pH 6.9. The insoluble material was removed by centrifuging and the clear extract (7500 units/ml.) was used as the source of crude α -amylase. Pig pancreatic α -amylase (crystalline) was purchased from the Worthington Biochemical Corp., Freehold, N.J., U.S.A. A crude *Bacillus subtilis* α -amylase was prepared from the extracellular α -amylase produced in a broth culture of *B. subtilis*. The broth was clarified by centrifuging and dialysed against distilled water containing 1 mM-Ca²⁺ ion to remove reducing substances. The dialysed broth (400 units/ml.) was used as the source of crude α -amylase. *Bacillus subtilis* α -amylase (crystalline) was a gift from Daiwa Biochemical Products Co. Ltd., Osaka, Japan. A crude *Aspergillus oryzae* α -amylase was prepared from Clarase 900 supplied by the Takamine Laboratory Inc., N.J., U.S.A. Clarase 900 (6 g.) was stirred slowly with water (37.5 ml.) at 2° overnight. The undissolved residue

Table 1. *Comparison of the amylase activity of saliva taken from different subjects*

For conditions see text. Incubation of the starch digests was for 30 min. at 35°. In all the Tables 'hydrolysis' means 'apparent conversion into maltose'.

Saliva	Hydrolysis of maltose (%)	Hydrolysis of dissolved maize starch (%)	Hydrolysis of maize-starch granules (%)	D/R
1	0	3.3	2.1	15.9
2	0	2.4	1.7	14.2
3	0	2.3	1.7	13.6
4	1	17.2	9.2	18.6
5	2	2.5	1.7	15.1
6	7.2	10.6	6.3	16.9
7	9.8	8.9	5.9	15.1
8	27.7	10.3	6.6	15.6

was removed by centrifuging and the clear supernatant (3840 units/ml.) was used as the source of crude α -amylase. *Aspergillus oryzae* α -amylase (crystalline) (Fischer & Stein, 1954) was a gift from Dr E. A. Stein. Sweet-potato β -amylase (crystalline) was purchased from the Worthington Biochemical Corp.

Amylase activity. (i) Dissolved starch. The digests (5 ml.) contained 1% of starch solution, 10 mM-tris-maleate buffer, 1 mM-calcium acetate and enzyme (1 unit). The digests were buffered to the optimum pH of the amylase being assayed (pH 6.9 for salivary and pancreatic α -amylase, pH 6.4 for *B. subtilis* α -amylase and pH 5.7 for *A. oryzae* α -amylase). Sodium chloride, when present, was 10 mM. The copper reagent of Shaffer & Hartmann (1921) was used to obtain a reducing value for the digest, from which an apparent maltose value was calculated. Provided that the reducing power liberated by the enzyme did not exceed the equivalent of 7.5 mg. of maltose in 30 min. the hydrolysis was proportional to enzyme concentration. One unit of activity was defined as the amount of amylase that produced 5 mg. of apparent maltose in 30 min. at 35° under the above conditions. Unless stated otherwise, saliva from one person (G.W.) was used throughout. The activity of this saliva was about 300 units/ml.

(ii) Starch granules. Raw starch (50 mg.) replaced dissolved starch in the digest described above and the flasks were shaken to maintain the starch in suspension. At least 10 units of amylase activity were added. It was confirmed that no adsorption of maltose occurred on the starch granules and that maltose could be correctly determined in the activity digests.

(iii) Ratio of amylase activity on dissolved and raw starch. The results for amylase activity were expressed in terms of the ratio

$$\frac{D}{R} = \frac{\text{Wt. of maltose from dissolved starch}}{\text{Wt. of maltose from raw starch}}$$

Low values of D/R were given by amylases that could hydrolyse raw starch. Greatly increased values were given when the amylase had negligible action on raw-starch granules. No allowance for loss of α -amylase activity by adsorption of the enzyme on starch granules was made in the calculation of D/R .

Maltase activity. Maltose hydrate (30 mg.; 3 ml.) was incubated at 35° with saliva (1.5 ml.) and 0.1M-tris-maleate buffer, pH 6.4 (0.5 ml.). The conversion into glucose which occurred during 20 hr. was determined with the Shaffer & Hartmann (1921) reagent.

Table 2. *Activity of saliva taken from one person at intervals during a day*

Saliva (0.2 ml.) was diluted to 25 ml. Portions (0.2 ml.) were added to α -amylase-activity digests containing dissolved starch. The starch-granule digests contained 2.0 ml. of enzyme soln. Incubation was at 35° for 30 min. Meals were taken at 07.15, 13.05, 16.30 and 21.00 hr.

Time (hr.)	Hydrolysis of dissolved maize starch (%)	Hydrolysis of maize-starch granules (%)	D/R
06.30	7.0	5.5	12.8
07.00	12.6	7.6	16.6
08.30	14.4	8.8	16.4
10.45	16.1	10.0	16.1
13.00	17.5	10.2	17.3
14.10	13.1	9.7	13.5
17.15	17.5	10.4	16.9
20.15	9.7	7.2	13.4
21.45	15.9	10.9	14.6

RESULTS

Action of saliva and crystalline salivary α -amylase on maltose, dissolved maize starch and maize-starch granules

Saliva was collected from eight people and portions (0.2 ml.) were diluted to 25 ml. with 1 mM-calcium acetate. The diluted saliva (0.2 ml.) was incubated with dissolved maize starch in activity digests containing 10 mM-sodium chloride and buffered to pH 6.9. Similar digests containing maize-starch granules were incubated with 2 ml. of diluted saliva. The results are shown in Table 1. Saliva taken from one person at different times during 1 day was then tested for its activity on the two starch substrates. Variation in activity towards starch solution was again reflected in a similar variation in activity towards starch granules, leading to a fairly constant value for D/R (Table 2).

The action of saliva on dissolved and raw maize starch was then compared with that of purified salivary α -amylase and with crystalline salivary

α -amylase (Table 3). Purification of saliva did not lead to a loss of activity of the amylase towards starch granules, the value obtained for D/R being of the same order at each stage.

The hydrolysis of both maize-starch granules and dissolved maize starch by crystalline α -amylase ceased when the apparent conversion into maltose was 97–98% (Fig. 3).

Action of heat-inactivated saliva on maize starch. Dialysed saliva was heated at 55° and at intervals between 0 and 93 min. portions were withdrawn and tested for amylase activity. The samples were then diluted so that they all contained 4.0 amylase units/ml. When the samples were incubated with maize-starch granules the hydrolysis was the same in each case, showing that heat had destroyed the activity of α -amylase towards both starch substrates to the same extent (Table 4).

Effect of chloride ions on the α -amylolysis of dissolved starch and raw-starch granules. The action of dialysed saliva on dissolved maize starch and on maize-starch granules was studied in varying concentrations of Cl^- ions. The degree of α -amylolysis of the two substrates was affected in a similar manner by Cl^- ions and a constant value was obtained for D/R (Table 5).

Table 3. *Comparison of the actions of saliva, purified salivary α -amylase and crystalline α -amylase on maize starch*

The dissolved-starch digests contained 1.45 units of α -amylase. Starch granules were shaken with 14.5 units. Incubation was for 30 min. at 35°. All digests contained sodium chloride and were buffered to pH 6.9.

	Hydrolysis of maize starch (%)		D/R
	Dissolved starch	Starch granules	
Saliva	14.5	8.4	17.2
Purified salivary α -amylase	14.7	8.1	18.2
Crystalline salivary α -amylase	15.3	8.3	18.4

Optimum pH of salivary α -amylase action. Dissolved maize starch and maize-starch granules were incubated with saliva at various H^+ -ion concentrations. Citrate buffer was used to obtain values at pH 5.8 and lower. The pH optimum for dissolved maize starch was between 6.8 and 7.1. For the starch granules the optimum was more widely spread and the activity did not vary greatly between pH 5.4 and 6.7. Values of D/R obtained from these results were not constant (Fig. 1), and gradually increased from 6 at pH 4.8 to 18 at pH 7.9.

Effect of temperature on salivary α -amylase action. The action on dissolved maize starch and maize-starch granules of both saliva and crystalline α -amylase was studied at a range of temperatures between 25° and 50°. At temperatures higher than 50° there was a possibility that gelatinization of some of the starch granules would occur; for this reason no estimations of α -amylolysis were made above 50°. In a control experiment in which starch granules in digests without α -amylase were kept for 1 hr. at the same range of temperature, then cooled to 25° and incubated with enzyme, the degree of hydrolysis was the same in all cases. This showed that the granules had not been ruptured and that no irreversible swelling of the granules had occurred. The results, shown in Fig. 2, suggest that, as temperature is increased, an effect in addition to the expected activation of α -amylase influences the hydrolysis of raw-starch granules. Further experiments were conducted with both saliva and crystalline salivary α -amylase at lower temperatures. These showed that the ratio D/R continued to increase with decrease in temperature. At 6° the value obtained was 250, whereas at 3° no hydrolysis of maize-starch granules could be detected in the presence of five times the amount of amylase present in a corresponding digest containing dissolved starch. The hydrolysis of dissolved starch was 14% in 30 min. at 3°. When the starch granules were removed from the digest at 3° and the supernatant was tested for its activity on dissolved

Table 4. *Effect of heat on the action of saliva on starch*

Dissolved-starch digests contained 1.6 units of α -amylase and the starch-granule digests contained 12 units. Incubation was at 35° for 30 min.

Time of heating (min.)	Percentage of original α -amylase activity	Hydrolysis of maize starch (%)		D/R
		Dissolved starch	Starch granules	
0	100	15.8	7.5	15.8
7.5	80	15.0	7.9	14.2
15	63	16.0	7.5	16.0
22.5	48	16.0	7.5	16.0
30	37	17.2	7.3	17.7
60	18	12.0	5.9	15.4
93	6.5	13.9	5.8	18.1

starch, the amylase was found to have only 5% of the activity of a control solution in which the enzyme had not been in contact with starch granules.

Adsorption of salivary α -amylase on starch granules

Rate of adsorption on maize-starch granules. Saliva was shaken with maize-starch granules at three different temperatures. Controls without starch granules were treated similarly. At intervals samples were withdrawn and the granules removed by centrifuging for 2 min. The amylase remaining in the supernatant was determined by testing the activity on dissolved starch. By comparing this result with the amylase activity in the controls the

extent of adsorption could be determined. It was found that within 30 min. the enzyme was adsorbed to the fullest extent possible under the conditions of the experiment (Table 6).

Effect of temperature on the degree of adsorption. The results shown in Fig. 2 suggested that the value for D/R might increase with decrease in temperature because of the increased adsorption of α -amylase on starch granules at lower temperatures. This was found to be the case when saliva was shaken with maize-starch granules at four different temperatures for 30 min. The amylase activity still remaining in solution in each digest was expressed as a percentage of the activity in controls incubated without starch. Adsorption was greatest at the lowest temperature (Table 7a). When adsorption experiments were conducted at higher

Table 5. *Activation of salivary α -amylase by chloride ions*

Saliva was dialysed against running tap water for 1 day and against distilled water for 2 days. The dissolved-starch digests contained 3 units of enzyme and were incubated for 20 min. The starch granules were incubated for 30 min. with 30 units of enzyme. Incubation was at 39° and pH 6.9.

Concn. of chloride (mm)	Hydrolysis of maize starch (%)		D/R
	Dissolved starch	Starch granules	
0	1.40	1.87	11.3
1	15.4	17.3	13.4
5	—	19.5	—
10	20.2	20.7	14.6
50	—	19.0	—
100	19.7	18.1	16.3

Fig. 1. Effect of H^+ -ion concentration on the α -amylolysis of maize starch by saliva. Dissolved-starch digests (●) contained 2.5 units of α -amylase and the maize-starch granules (○) were shaken with 18.75 units. Incubation was at 35° for 30 min. The ratio of rate of hydrolysis of dissolved starch and starch granules (D/R) was plotted at each pH (▲).

Fig. 2. Effect of temperature on the α -amylolysis of maize starch. Dissolved-starch digests (●) contained 1.8 units of crystalline salivary α -amylase, and the maize-starch granules (○) were shaken with 18 units. Incubation was at pH 6.9 for 30 min. The ratio of the rate of hydrolysis of dissolved starch and starch granules (D/R) was plotted at each temperature (▲).

Table 6. *Rate of adsorption of salivary α -amylase on maize-starch granules*

Starch-granule digests (5 ml.) contained maize starch (50 mg.) and saliva (110 units). At intervals portions (0.5 ml.) of the enzyme in solution in the digests and in the controls incubated without starch were tested for α -amylase.

Time of shaking with starch granules (min.)	Adsorption of α -amylase (%)		
	Temp. 5°	Temp. 10°	Temp. 20°
5	60.4	59.8	55.4
30	82.6	80.4	70.1
60	84.0	81.5	70.0

temperatures appreciable hydrolysis of starch granules occurred, and a proportion of the maltose liberated was introduced into the dissolved-starch-activity digests with the amylase being tested. It was confirmed that the weight of maltose transferred in this way was never sufficient to inhibit amylase activity.

Effect of pH on the degree of adsorption. Since the variation in D/R with temperature was accounted for by increased adsorption of α -amylase with decreasing temperature, tests were made to find whether the variation in D/R with pH could similarly be explained. Saliva was shaken with maize-starch granules for 30 min. at 35° in digests of different pH. The results (Table 7b) showed that adsorption was dependent on pH, the extent of adsorption increasing as pH was raised.

The conditions that yielded low values of D/R were high temperature and low pH (Figs. 1, 2). Under these same conditions the adsorption of α -amylase on starch granules was decreased (Table 7a and b). It followed therefore that, when the correct combination of temperature and pH was found for reducing adsorption to a minimum, these

conditions would lead to the lowest possible value of D/R . Accordingly, the adsorption of salivary α -amylase was measured at three different temperatures. At each temperature digests were buffered to three different pH values. Adsorption continued to decrease as the temperature was raised to 45°, and at this temperature the influence of pH was not so marked as at 35°. Thus by changing the conditions of incubation of α -amylase with starch granules from 35° and pH 7 to 45° and pH 7 the amylase activity remaining in solution was increased 3.5-fold (Table 7c). At the same time hydrolysis of the granules was also increased. It appeared that α -amylase was not adsorbed in a manner that facilitated hydrolytic degradation of the granule.

Release of α -amylase from starch granules. The reversibility of the process by which α -amylase is removed from solution by starch granules could be demonstrated by allowing α -amylase to adsorb on starch granules at 0° and by then raising the temperature to release part of the α -amylase into solution. In one experiment, saliva (10 units) was shaken with maize starch (50 mg.) for 30 min. at 0°. The starch was removed by centrifuging at 0°, and the supernatant was found to contain no amylase activity. After the starch granules were shaken with 10 mM-tris-maleate buffer, pH 6.9, at 40° for 30 min. it was shown that 25 % of the α -amylase was present in solution. This experiment could not prove that α -amylase was fully active when adsorbed on starch at 0°, since during the incubation at 40° the enzyme again distributed itself between the granules and the suspending medium. Also, some of the α -amylase released at 40° was unavoidably adsorbed again during the short period of centrifuging at room temperature. A second experiment was designed to give complete release of α -amylase from starch granules. As described above, saliva (10 units) was adsorbed on starch granules at 0° and then the granules were suspended in dissolved-starch solution (50 mg.) for 30 min. at 40°. After removal of the starch granules the supernatant solution was found to contain 9 units of amylase activity. Dissolved starch, having a higher affinity for α -amylase than starch granules, released most of the enzyme from the granules, a phenomenon previously described by Ambard (1921).

In another experiment, starch granules (50 mg.) that had adsorbed crystalline salivary α -amylase (10 units) at 0°, as described in the preceding experiments, were suspended at 40° in a solution (5 ml.) containing 10 mM-sodium chloride and 10 mM-tris-maleate buffer. The rate of hydrolysis of the starch granules in this digest was compared with that of a control in which the α -amylase was added to the granules at 40°. The results (Table 8) showed that the α -amylolysis proceeded at the

Table 7. *Effect of temperature and pH on the adsorption of salivary α -amylase on maize-starch granules*

(a) Saliva (8.7 units) was incubated with maize-starch granules for 30 min. at pH 6.9. Portions (0.75 ml.) of the enzyme remaining in solution were then tested for α -amylase activity.

Temperature	Adsorption (%)
20°	86.2
25	84.3
30	79.3
35	67.9

(b) Saliva (27.5 units) was incubated with maize-starch granules for 30 min. at 35°. Portions (0.5 ml.) of the enzyme remaining in solution were then tested for α -amylase activity.

pH	Adsorption (%)
7.8	77.0
7.1	68.5
6.0	63.6
5.2	57.4
4.7	41.6

(c) Saliva (20 units) was incubated with maize-starch granules for 30 min. at various temperatures and pH values. Portions (0.5 ml.) of the enzyme remaining in solution were then tested for α -amylase activity.

pH	Adsorption (%)		
	Temp. 35°	Temp. 40°	Temp. 45°
4.7	43.9	38.7	17.8
5.9	71.4	41.9	23.4
7.1	77.5	40.9	20.9

Table 8. *Release of crystalline salivary α -amylase from maize-starch granules*

For conditions see text. The digest contained α -amylase that had been completely adsorbed on the starch granules at 0° before incubation at 40°. In the control α -amylase was added to buffered substrate at 40°. Results are also given for amylase activity in a digest incubated without substrate.

Time (hr.)	Hydrolysis (%)		α -Amylase in soln. (units)		
	Digest	Control	Digest	Control	Enzyme
0	—	—	0	10	10
0.5	9.9	10.6	4.4	5.0	9.6
1.0	20.5	22.7	4.8	5.6	9.1
2.0	34.3	36.9	5.5	6.0	8.5

same rate in each case. During the incubation, tests for α -amylase activity revealed that both digests contained similar amounts of enzyme in solution (Table 8). Degradation of the starch granules allowed a slow release of α -amylase into solution.

Effect of α -amylase concentration on the degree of adsorption on maize-starch granules. In previous experiments the amount of α -amylase included in digests containing starch granules was variable. Since this is a factor that could influence the extent of adsorption, the effect of enzyme concentration on percentage adsorption was determined. The results (Table 9) were fitted to Freundlich's equation, used in the form $m = k_1 c^{k_2}$, where m is amount adsorbed and c is equilibrium concentration of α -amylase. A linear relation was found between $\log m$ and $\log c$.

Effect of chloride ions on adsorption. Salt may influence adsorption in two ways. Hockenhull & Herbert (1945) found that the adsorption of the amylase of *Clostridium acetobutylicum* on starch was inhibited by salts. In other cases salts enhance adsorption; this has been termed 'salting-out' adsorption by Tiselius (1950). When dialysed saliva was shaken with starch granules in the presence and absence of Cl^- ions (Table 10), adsorption of α -amylase on the granules was not influenced by the presence of 100 mM-chloride. The difference that occurred at 30° could be accounted for by the tenfold amount of amylase present in the digests incubated without Cl^- ions. Effects due to amylase concentration were expected to be less marked at 0°.

Adsorption of salivary α -amylase on starch granules of different size from different sources. Schwimmer & Balls (1949) found that the efficiency of adsorption of malt α -amylase on starch at 0° in the presence of 40% ethanol was inversely proportional to the granule size. As shown in Table 11, the adsorption of salivary α -amylase on various starches at 35° also showed a marked variation. Adsorption was greatest on protozoal starch, which, having granules of very small size, presented a large surface area for the adsorption of α -amylase.

Table 9. *Relationship between amount adsorbed and equilibrium concentration of α -amylase*

Digests containing different amounts of saliva were shaken with maize-starch granules for 30 min. at 35°. The percentage of enzyme adsorbed was calculated from assays of the enzyme remaining in solution. m , Amount adsorbed; c , equilibrium concentration of α -amylase.

α -Amylase (units)	Adsorption (%)	$\log m$	$\log c$
2.3	84.1	0.924	0.204
4.6	79.0	1.20	0.623
23	60.5	1.78	1.60

Table 10. *Adsorption of salivary α -amylase on starch granules in the presence of chloride ions*

Dialysed saliva was incubated for 30 min. with maize-starch granules at three different temperatures. Digests incubated with chloride (100 mM) contained 15 units of enzyme. When chloride was absent, the digests contained 150 units of α -amylase. Hydrolysis of the granules was in this way kept constant.

Temperature	Adsorption (%)	
	Chloride present	Chloride absent
45°	32.5	31.0
30	82.4	61.2
0	100.0	97.0

Potato-starch granules were the largest in size but showed the lowest adsorption of all starches investigated. Rice starch was hydrolysed very rapidly so that the adsorption of α -amylase on the granules was apparently less than would be predicted from the size of the starch granules. As the starches became degraded amylase was released into solution. Potato starch was not hydrolysed by the enzyme, and the adsorption of α -amylase increased with time. Acid-treated granules of potato starch (Lintner) were degraded during the first few minutes, but since the amylase was rapidly inactivated the extent of its adsorption could not be calculated. The rate of hydrolysis of the starches studied was not directly related to the size of the granules.

Action of the α -amylases of pig pancreas, Bacillus subtilis and Aspergillus oryzae on starch

Both crude and crystalline α -amylases from each source were tested for their action on dissolved maize starch and maize-starch granules. The mean values of D/R obtained from the results of the hydrolyses are shown in Table 12. In each case the crystalline enzyme had the same effect on starch granules as the crude α -amylase. Each amylase had a different rate of action on starch granules and no prediction of the degrading activity of an amylase on starch granules could be made from a knowledge of its activity on starch solution. Although pancreatic α -amylase gave results closely similar to those obtained with salivary α -amylase,

the action of *B. subtilis* α -amylase on maize-starch granules was at least one-tenth of the rate. *Aspergillus oryzae* α -amylase did not degrade raw starch.

Adsorption of amylases from different sources on maize-starch granules

When crystalline amylases from different sources were incubated with maize-starch granules at 35° (Table 13), it was found that three of the amylases were completely unadsorbed. Since adsorption may be increased at lower temperatures, the experiment was repeated at 3°. Two of the amylases, those of *Aspergillus oryzae* and sweet potato, remained unadsorbed, but *Bacillus subtilis* α -amylase was adsorbed to the extent of 59 % at the

Table 11. *Action of salivary α -amylase on starch granules from different sources*

The starch was dried to constant weight *in vacuo* over P_2O_5 at 60°. Saliva (23 units) was incubated with starch granules (50 mg.) at 35°. Portions (0.5 ml.) were removed after 30 min. and the percentage of α -amylase remaining in solution was determined.

Source of starch	Granule size (μ)		Hydrolysis (%)		Adsorption (%)	
	Range	Average	0.5 hr.	2 hr.	0.5 hr.	24 hr.
Holotrich protozoa	1	1	7.9	25.0	92	74
Rice	3-8	5	30.5	—	52	24
Waxy maize	5-25	15	11.5	28.3	77	31
Maize	5-25	15	21.1	41.8	61	29
Potato	15-75	35	0	0.5	20	30
Lintner potato	—	—	8.1	10.1	—	—

Table 12. *Hydrolysis of maize starch by α -amylases from three different sources*

(a) Dissolved-starch digests contained 1.5 units of crude pancreatic (pig) α -amylase or 0.5 unit of crystalline α -amylase. Starch-granule digests contained 15 units of crude α -amylase or 4.7 units of crystalline α -amylase. Incubation was at 35° and pH 6.9. (b) Dissolved-starch digests contained 1 unit of *Bacillus subtilis* crude amylase or 1 unit of crystalline α -amylase. The starch-granule digests contained 112 units of crude α -amylase or 75 units of crystalline α -amylase. Incubation was at 35° and pH 6.4. (c) Dissolved-starch digests contained 1 unit of *Aspergillus oryzae* crude α -amylase or 1 unit of crystalline α -amylase. The raw-starch digests contained 1000 units of crude α -amylase or 140 units of crystalline α -amylase. Incubation was at 35° and pH 5.7.

Time (hr.)	Hydrolysis (%)				<i>D/R</i>	
	Dissolved starch		Starch granules			
	Crude α -amylase	Cryst. α -amylase	Crude α -amylase	Cryst. α -amylase	Crude α -amylase	Cryst. α -amylase
	(a) Pig pancreatic α -amylase					
0.25	7.7	—	—	—	23	22
0.5	15.1	5.0	7.0	2.2		
1.0	—	9.8	12.6	4.2		
(b) <i>Bacillus subtilis</i> α -amylase						
0.25	5.2	5.3	—	—	348	323
0.5	9.2	10.1	3.3	—		
1.0	—	—	6.0	4.8		
3.0	—	—	—	14.5		
(c) <i>Aspergillus oryzae</i> α -amylase						
0.25	4.6	4.9	—	—	16 000	120 000
0.5	9.6	10.0	—	—		
1.0	—	—	1.2	0		
3.0	—	—	3.3	0		
24.0	—	—	—	0		

lower temperature. After incubation at 35° for 24 hr. the β -amylase digest contained no maltose, *Bacillus subtilis* α -amylase had produced 10% hydrolysis and salivary and pancreatic α -amylases had converted 45% of the starch granules into maltose.

Table 13. *Adsorption of various amylases on maize-starch granules*

Digests contained 4 units of amylase activity and were buffered to pH 6.1. β -Amylase was stabilized with serum albumin (0.05%) and glutathione (0.5 mM). After incubation for 30 min. the starch granules were removed and the amylase activity remaining in solution in the digests and controls was estimated.

Source of crystalline amylase	Adsorption (%)	
	Temp. 35°	Temp. 3°
Saliva	89	93
Pig pancreas	88	98
<i>Bacillus subtilis</i>	0	59
<i>Aspergillus oryzae</i>	0	0
Sweet-potato β -amylase	0	0

Table 14. *Effect of bile and deoxycholate on the degradation of maize-starch granules by salivary α -amylase*

(a) Increasing concentration of bile. Digests contained 60 mM-phosphate buffer and were incubated for 1 hr. at 35° with 10 units of saliva.

Bile (%)	Hydrolysis (%)
0	10.4
5	13.2
10	14.1
15	14.9
25	14.8
50	14.3

(b) Increasing concentration of phosphate. Digests contained 10% of bile and were incubated for 1 hr. at 35° with 10 units of saliva.

Phosphate buffer (mM)	Hydrolysis (%)
0	10.8
30	13.8
60	14.3
120	14.5
240	14.8

(c) Increasing concentration of deoxycholate. Digests contained 60 mM-phosphate buffer, and were incubated for 1 hr. at 35° with 10 units of saliva.

Sodium deoxycholate (%)	Hydrolysis (%)
0	9.9
0.01	11.5
0.05	12.2
0.10	13.0

Action of salivary α -amylase and Bacillus subtilis α -amylase on potato-starch granules

Salivary α -amylase did not degrade potato-starch granules under conditions that led to a rapid attack on maize-starch granules (Table 11). A slight hydrolysis was obtained when 500 times the amount of enzyme was used. In this way it was calculated that purified salivary α -amylase was 1000 times as active on maize-starch as on potato-starch granules. Crystalline *Bacillus subtilis* α -amylase was similarly 1150 times as active on maize-starch as on potato-starch granules.

Effect of detergents on the hydrolysis of starch granules

Bile. Pig bile was stored at 0° for 3 days. At the end of this period the bile contained no amylase activity and did not inactivate salivary α -amylase. The presence of bile in standard starch-granule digests resulted in an increased hydrolysis of maize-starch granules by salivary α -amylase. A concentration of 10–15% of bile was the most effective and phosphate buffer enhanced the activating effect of bile (Table 14a, b). In the absence of bile, phosphate buffer did not stimulate increased hydrolysis of starch granules.

Sodium deoxycholate. The rate of hydrolysis of maize-starch granules was increased by 30% when sodium deoxycholate (0.1%) was present (Table 14c). No such activating effect was observed in digests containing dissolved starch.

DISCUSSION

Badenhuizen (1959) stated that amylases do not act on native-starch granules unless some swelling has occurred. In consequence starch granules are incubated with amylases at 37–40°, a temperature which is high enough to cause sufficient swelling for the granules to become accessible. The results described in the present paper suggest another reason why amylases should be incubated with starch granules at 40–45°. Although the adsorption of α -amylases on granular starch in the presence of cold ethanol has long been known (Holmberg, 1933; Hockenhull & Herbert, 1945; Schwimmer & Balls, 1949), few workers (Ambard, 1921; Boekestein, 1932) have studied the efficiency of adsorption at higher temperatures. The relationship between adsorption of amylase and the amylolytic degradation of the starch granule has not been considered.

The results of the present experiments suggest that the increased rate of hydrolysis of starch granules at higher temperatures is due more to decreased adsorption of the enzyme on the granules than to increased swelling of the granules. Potato-

starch granules have higher swelling power and lower gelatinization temperature than maize-starch granules. On this basis one would expect potato-starch granules to be more easily digested than the more rigid maize-starch granules, yet the reverse is well known to be true. None of the amylases in the present study could degrade potato-starch granules. Balls & Schwimmer (1944) also found potato-starch granules much more resistant than maize-starch granules to their enzyme mixture of mould bran and pancreatin, and Hattori & Takenchi (1961) reported that rice-, corn- and wheat-starch granules were digested more easily than potato-starch granules by a purified amylase from *Endomycopsis fibuliger*. Turvey & Hughes (1958) have suggested that, in the potato, starch granules are degraded by phosphorylase and not by α -amylase.

Additional evidence of a relationship between the degree of adsorption of salivary α -amylase and the rate of amylolytic degradation of maize-starch granules was given in experiments conducted at different pH values. Adsorption of amylase was greater at pH 7.8 than at 4.7 (Table 7*b* and *c*), and values of D/R increased with pH (Fig. 1). Thus when adsorption of amylase on the starch granules was decreased the rate of digestion of the starch was increased. Schwimmer & Balls (1949) have concluded that the site for adsorption of malt α -amylase on starch granules at 0° is not the same as the site of union of enzyme with substrate to form a hydrolysable complex. The same conclusion has been reached in the present work. When salivary α -amylase was incubated with starch granules, a large proportion of the enzyme could be adsorbed on starch by choosing (*a*) a low temperature, (*b*) a high pH, and (*c*) a small-sized starch granule, which would present a large surface area for adsorption of the enzyme. These three conditions all led to a decreased rate of hydrolysis. Values of D/R were high at low temperature and high pH (Figs. 1, 2), and holotrich-starch granules (1 μ diameter) were hydrolysed more slowly than maize-starch granules (25 μ diameter). The greater ease of hydrolysis of rice-starch granules compared with maize-starch granules (Table 11) could be due to a solubilizing effect on the granule of the alkaline treatment of the rice grains, which occurred during the preparation of this starch. Sandstedt (1955) noted that the action of a malt extract from wheat was two to three times as rapid on rye-starch granules (40–50 μ diameter) as on wheat-starch granules (2–8 μ and 25–35 μ diameter).

Further evidence that adsorption does not take place in a manner that is favourable for hydrolysis was given in experiments with potato-starch granules, which adsorbed salivary α -amylase without being hydrolysed by this enzyme.

Although adsorption does not lead to hydrolysis, and must in fact be avoided if rapid degradation of starch granules is to occur, the present experiments have indicated that the ability of a particular α -amylase to digest maize-starch granules may be associated with its ability to adsorb on the granules. β -Amylase from sweet potatoes and α -amylase from *Aspergillus oryzae* were not adsorbed at either 35° or 0° and these amylases did not degrade starch granules. *Bacillus subtilis* α -amylase was not adsorbed at 35° but was adsorbed at 0°; its hydrolytic activity towards starch granules was low. Both salivary α -amylase and pancreatic α -amylase were strongly adsorbed on the granules, the adsorption being appreciable even at 35°; these two amylases attacked maize-starch granules extremely rapidly. At 50° salivary α -amylase hydrolysed granular starch at one-eighth of the rate of action on dissolved starch. Further work with a wide variety of α -amylases would be necessary before a confident prediction could be made that amylases that can be adsorbed on cereal-starch granules are consequently capable of degrading the granules.

Care was taken with the preparation of the starch granules to avoid mechanical damage. Damaged granules are reported to be much more susceptible to amylolytic degradation than normal granules (Jones, 1940), and when a granule is crushed or otherwise physically injured it swells in cold water in the same manner as a normal granule does in hot water (Sandstedt, 1955). In spite of the mild conditions used in the present work many of the waxy-maize granules appeared broken. The results in Table 11 showed that granules of waxy-maize starch, though less physically perfect than maize starch, were more slowly hydrolysed. When the amylolytic degradation of two commercial maize starches was compared with the laboratory preparation, little difference in rate of hydrolysis was seen. It might have been expected that the commercial preparations of starch would have undergone a more rigorous treatment during extraction, resulting in more damage, with concomitant ease of hydrolysis. The experiments reported in this paper suggest that starch granules can be extracted from the plant material without damage and that a broken or cracked granule is an indication of either enzymic activity or adverse environmental conditions during the growth of the parent plant. The fact that a specimen of native maize starch can be completely digested by one type of amylase, for example salivary α -amylase (Fig. 3), and yet be completely resistant to another type of α -amylase, for example *Aspergillus oryzae* α -amylase (Table 12*c*), rules out any possibility that enzymic degradation of raw starch is dependent on prior mechanical damage.

Sandstedt & Gates (1954) found a wide variation in the ability of amylases from various sources to digest raw starch. Their enzymes were from 165 to 7000 times as active on dissolved starch as on raw starch. This variation was considered to be due either to fundamental differences in the properties of the α -amylases or to the possibility that the enzymes responsible for the digestion of raw starch were not α -amylases, so that a raw-starch enzyme different from α -amylase would be necessary for degrading starch granules. The experiments with salivary α -amylase reported in the present paper have demonstrated conclusively that no special raw-starch enzyme exists in saliva, and that it is the α -amylase in saliva which is responsible for the digestion of maize-starch granules. The results were as follows. (i) Eight subjects had the same amount of starch-granule-degrading activity in their saliva relative to α -amylase. This would be unlikely if the two activities were those of different enzymes. The amount of maltase in the saliva samples varied considerably relative to α -amylase (Table 1). (ii) No change in starch-granule-degrading activity relative to α -amylase activity occurred when the α -amylase was partially inactivated by heat. (iii) Hydrolysis of starch granules by saliva was activated by Cl^- ions to the same extent as the well-known activation of salivary α -amylase by sodium chloride (Table 5). (iv) Crystalline salivary α -amylase and crude saliva had the same ratio of hydrolytic activities with both dissolved starch and with starch granules.

The crystalline enzyme was as active as the crude α -amylase in the hydrolysis of starch granules for all the α -amylases studied. If the crude-

amylase systems contained an enzyme or factor other than α -amylase, which was required for the digestion of granular starch, then the crystalline α -amylases prepared from the crude sources would have had little or no action on starch granules.

Schwimmer (1945) found that the addition of maltase from *Aspergillus oryzae* considerably accelerated the degradation of starch granules by pancreatic α -amylase. In the present experiments salivary maltase did not influence the degradation of maize-starch granules by saliva.

Sandstedt & Gates (1954) have shown that there is an increased rate of digestion of raw starch in the presence of the cationic detergent Roccal. When the bile salt sodium deoxycholate was tested in this respect (Table 14) the maximum increase in rate of hydrolysis was 30%. The activating effect occurred only in the presence of phosphate buffer, an observation previously noted with Roccal.

SUMMARY

1. α -Amylases from human saliva, pig pancreas, *Bacillus subtilis* and *Aspergillus oryzae*, and β -amylase from sweet potatoes, were examined for their action on starch granules.

2. Salivary α -amylase rapidly degraded maize-starch granules. The starch-granule-degrading activity relative to the hydrolysis of dissolved starch varied according to the pH and temperature of the reaction.

3. Adsorption of salivary α -amylase occurred on the surface of starch granules. The extent of adsorption increased at low temperature and high pH; if conditions of temperature and pH were chosen whereby adsorption of the enzyme was decreased, the rate of hydrolysis of starch granules was increased.

4. The efficiency of adsorption was proportional to the surface area of the starch granules and the adsorption obeyed the Freundlich adsorption equation.

5. Pig pancreatic α -amylase behaved in a similar manner to salivary α -amylase in its hydrolytic action on raw starch and in its adsorption on starch granules. *Bacillus subtilis* α -amylase hydrolysed maize-starch granules slowly; this amylase was adsorbed on the granules at 0° but not at 35°. *Aspergillus oryzae* α -amylase and sweet-potato β -amylase did not degrade maize-starch granules; these amylases were not adsorbed on starch granules under any of the conditions tested.

6. Each crystalline α -amylase had the same ability to hydrolyse starch granules as the crude source of α -amylase from which it was prepared. It was concluded that certain α -amylases could degrade raw-maize starch and that no special raw-starch enzyme was necessary.

Fig. 3. Hydrolysis of dissolved maize starch (●) and maize-starch granules (○) by crystalline salivary α -amylase. Dissolved-starch digest contained 0.5 amylase unit/ml. and the starch-granule digest contained 7 amylase units/ml. At 45 hr. and at 75 hr. (↓) more enzyme (7 amylase units/ml.) was added to each digest. Incubation was at 40°.

7. None of the amylases studied could degrade potato-starch granules.

The authors are grateful to the University of Sydney for a grant towards the costs of this work and for a Fellowship to G. J. W.

REFERENCES

- Ambard, L. (1921). *Bull. Soc. Chim. biol., Paris*, **3**, 51.
 Badenhuisen, N. P. (1959). *Protoplasmatologia*, **2B**, sect. 2b, 8.
 Balls, A. K. & Schwimmer, S. (1944). *J. biol. Chem.* **156**, 203.
 Boekestein, P. T. (1932). *Acta brev. neerl. Physiol.* **2**, 132.
 Fischer, E. H. & Stein, E. A. (1954). *Arch. Sci., Genève*, **7**, 131.
 Fischer, E. H. & Stein, E. A. (1961). *Biochem. Prep.* **8**, 27.
 Hattori, Y. & Takenchi, I. (1961). *Rika Gaku Kenkyūsho Hōkoku*, **37**, 37.
 Hockenhull, D. J. D. & Herbert, D. (1945). *Biochem. J.* **39**, 102.
 Holmberg, O. (1933). *Biochem. Z.* **258**, 134.
 Jones, C. R. (1940). *Cereal Chem.* **17**, 133.
 Kneen, E., Beckord, O. C. & Sandstedt, R. M. (1941). *Cereal Chem.* **18**, 741.
 Mould, D. L. & Thomas, G. J. (1958). *Biochem. J.* **69**, 327.
 Reichert, E. T. (1913). *Publ. Carneg. Instn.*, no. 173, part 1.
 Sandstedt, R. M. (1955). *Cereal Chem.* **32** (Suppl.), 17.
 Sandstedt, R. M. & Gates, R. L. (1954). *Food Res.* **19**, 190.
 Schoch, T. J. (1957). In *Methods in Enzymology*, vol. 3, p. 5. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Schwimmer, S. (1945). *J. biol. Chem.* **161**, 219.
 Schwimmer, S. & Balls, A. K. (1949). *J. biol. Chem.* **180**, 883.
 Shaffer, P. A. & Hartmann, A. F. (1921). *J. biol. Chem.* **45**, 365.
 Tiselius, A. (1950). *Naturwissenschaften*, **37**, 25.
 Turvey, J. R. & Hughes, R. C. (1958). *Biochem. J.* **69**, 4P.
 Whelan, W. J. (1955). In *Modern Methods of Plant Analysis*, vol. 2, p. 160. Ed. by Paech, K. & Tracey, M. V. Heidelberg: Springer Verlag.

Biochem. J. (1963) **86**, 462

The Metabolism of C₂-Compounds in Micro-organisms

9. ROLE OF THE GLYOXYLATE CYCLE IN PROTOZOAL GLYCOGENOGENESIS*

BY J. F. HOGG† AND H. L. KORNBERG‡

Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford

(Received 19 July 1962)

Although evidence has previously been presented (Hogg, 1959) for the presence of the key enzymes of the glyoxylate cycle (Kornberg & Krebs, 1957; Kornberg & Madsen, 1958) in cells of *Tetrahymena pyriformis*, strain E, which can convert fats into glycogen (Wagner, 1956; Hogg & Wagner, 1956), the presence of isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) in cells lacking this ability has brought into question the intracellular role of these enzymes (Hogg, 1959; Reeves, Seaman, Papa & Aji, 1961).

It is the main purpose of this paper to present results obtained from analysis of *Tetrahymena* cells grown either in synthetic or in proteose-peptone media. Such results show that the intracellular activities of isocitrate lyase and malate synthase are profoundly affected by the composition of the growth media. Moreover, in cells capable of glyconeogenesis from fats, these enzymes were found to be confined to one of the two 'large-granule'

fractions obtained after disruption of the cells in a mannitol solution; in cells not capable of glyconeogenesis from fats, the enzymes (though present) were not concentrated at one intracellular locale. These findings indicate that the conversion of fats into glycogen by *Tetrahymena* necessitates both the presence of large amounts of glyoxylate-cycle enzymes and their incorporation into an organized intracellular structure. This work has been presented in part to The Biochemical Society (Hogg & Kornberg, 1961).

MATERIALS AND METHODS

Growth of cells. The routine procedures for maintenance, growth and harvesting of *T. pyriformis* have been described by Elliott & Hogg (1952) and Wu & Hogg (1952). However, the stock culture medium, which contains (g./l.) proteose-peptone (Difco) 10, sodium acetate 1.0, glucose 1.0 and K₂HPO₄ 1.0, was varied by omitting acetate or glucose or both. The minimal synthetic medium (Elliott & Hayes, 1953) was altered by reducing the concentrations of the essential ketogenic amino acids phenylalanine, leucine, isoleucine and lysine to 20, 20, 15 and 25 mg./l. respectively. In each case the altered concentration corresponded to 2.5 times the concentration required to obtain half-

* Part 8: Kornberg & Sadler (1961).

† Present address: Department of Biological Chemistry, University of Michigan, Ann Arbor, Mich., U.S.A.

‡ Present address: Department of Biochemistry, University of Leicester.