Isolation of Large and Small Granules of Barley Starch and a Study of Factors Influencing the Adsorption of Barley Malt α-Amylase by These Granules[1]

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ABSTRACT

An elutriation column was used to separate barley starch granules into a prime starch fraction and a secondary starch fraction with mean particle diameters of 16.7 and 1.87 μm, respectively. By microscopic examination, each fraction was relatively homogeneous. Secondary starch, presumably because of its greater available surface area, was more efficient than prime starch in adsorbing malt α-amylase from solution. Adsorption was greatest in the pH range 5–6 and was seven times higher at 5° C than at 28° C.

Starches and starch derivatives are able to absorb α-amylases isolated from a variety of sources. These have included hepatic (Starkenstein 1910), salivary (Ambard 1921, Walker and Hope 1963), pancreatic (Boekestein 1932), fungal (Tokuoka 1936), bacterial (Hockenhull and Herbert 1945), and cereal (Sandstedt and Ueda 1969, Schwimmer and Balls 1949b) amylases. This selective adsorption by starch has been used as a means for purifying α-amylases (Schwimmer and Balls 1949a, Tokuoka 1936).

For cereal α-amylases, however, adsorption by starch may pose practical problems. Because these amylases exist in close contact with starch in vivo, adsorption by starch granules could interfere with extraction and isolation of the enzymes. Therefore, extraction of amylases under conditions of minimum adsorption and minimum denaturation would be advantageous. However, very little information is available on the adsorption of cereal α-amylases by untreated starch isolated from the same cereal grain. The present research was conducted to investigate the dependence of malt α-amylase adsorption on granule size of barley starch, concentration of barley starch, time of contact with starch, pH, and temperature.

MATERIALS AND METHODS

Preparation of Starch

Conquest barley was washed thoroughly with distilled water and steeped for 30 hr in acetate buffer (0.02*M*, pH 6.5, 0.01*M* $HgCl_2$) that was changed frequently. The softened kernels were ground to a fine grist in a blender, and the starch was passed through two sieves (150 μm and 75 μm mesh). Particulate material was reground to ensure complete disruption of barley endosperms.

The crude starch preparation was suspended in distilled water and separated roughly into two starch fractions by decanting the lighter particles (containing secondary starch) from the heavy, fast-sedimenting material (containing prime starch).

To remove the bulk of the proteinaceous material, each fraction was further purified by a process of resuspension, partial sedimentation, and centrifugation (5,000 × *g*, 10 min). Final purification was achieved by multiple extractions of starch slurries with toluene (Greenwood and Robertson 1954). The purified starch fractions contained less than 0.2% protein.

Fractionation of Starch Granules

The two purified but crudely separated starch fractions were individually fractionated using an elutriation column (Scott 1968). Starch slurry (30% by weight) was added to the top of the column at a rate of 2 ml/min. At the same time, distilled water was pumped in at the base of the column at 6–8 ml/min. Large granules sedimented inside the column and small granules were washed out at the top. Starch was regained from the large volumes of starch suspensions obtained by centrifugation (5,000 × *g*, 10 min). Two passes of both starch fractions through the column were required to remove small granules from the prime starch and large granules from the secondary starch. Starch fractions were partially dried on a Buchner funnel, dehydrated with ethanol, dried at 65° C under vacuum, and stored in a desiccator.

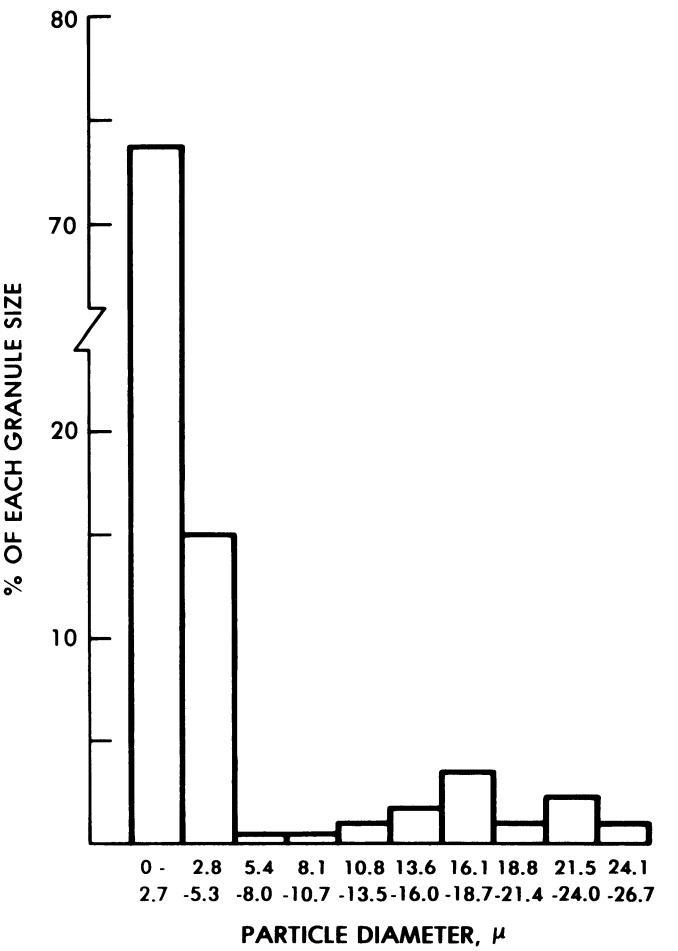

Fig. 1. Size distribution of starch granules from mature Conquest barley.

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Size Distribution of Starch Granules

Small samples of homogeneous starch suspensions were placed on microscope slides with a drop of 0.2% iodine in 2% potassium iodide. The diameter of at least 400 granules for each sample was determined by using a microscope with a calibrated ocular eyepiece and a hemocytometer chamber (May and Buttrose 1959).

Scanning Electron Microscopy

Starch samples were attached to stubs with double-sided tape and coated with gold. The mounted samples were examined in a JEOL 35C scanning electron microscope at an accelerating potential of 15 kV. Photographs were taken on Polaroid type 55 P/N film.

Preparation of α-Amylase

α-Amylase II was prepared from malted Conquest barley using the extraction, heat treatment, and glycogen precipitation conditions previously described (MacGregor 1977). The enzyme was further purified by ion-exchange chromatography on carboxymethyl cellulose (MacGregor et al 1971 b), precipitated in saturated ammonium sulfate solution, and stored at 5°C. Fresh enzyme solutions were prepared daily from this stock preparation by centrifuging a portion of the ammonium sulfate precipitate and dissolving the precipitate in a suitable volume of acetate buffer (0.2M, pH 5.5, $10^{-3}M$ $CaCl_2$) to give a final α-amylase activity of 150 IDC units per milliliter.

α-Amylase Activity

This was determined using the β-limit dextrin of waxy maize starch as substrate as described (MacGregor et al 1971a).

Adsorption of α-Amylase

A 25 or 50 mg starch sample was weighed into a small vial and, at zero time, 10 or 20 ml of enzyme solution was added and the vial was mixed gently on a Labline rotary shaker. After the appropriate elapsed time, a portion of the suspension was removed and centrifuged (10 min, 4,000 × g). Contact time between starch and enzyme was assumed to terminate when the centrifuge started. The α-amylase content of the supernatant solution was determined and compared with the activity of a blank α-amylase solution treated in the same way but containing no starch.

RESULTS AND DISCUSSION

Previous studies (MacGregor et al 1971a, May and Buttrose 1959) showed that starch from mature barley contained two populations of starch granules. There were large numbers of small granules of particle size < 2.7 μm and a small number of much larger granules in the size range 13–20 μm. The results of the present study agree with those findings (Fig. 1). Almost 90% of the granules were less than 5.3 μm in diameter, but there was another discrete group in the 13.6–18.8 μm size range. Very few granules were in the range of 5.3–13.6 μm.

The bimodal distribution of starch granules in barley kernels has been thoroughly discussed by May and Buttrose (1959). Their results and those of Bathgate and Palmer (1972) suggested that, although small granules represented about 90% of the total number of granules at maturity, they occupied only 10% of the total volume or weight of the starch. More recent results on wheat starch (Evers and Lindley 1977, Hughes and Briarty 1976) suggested that small starch granules may represent a much higher proportion of the weight of starch in the mature wheat endosperm than had been thought previously. In the current study, however, no attempt was made to determine the relative amounts of the two granule sizes.

Crude separation of large and small starch granules was achieved by repeated suspension and sedimentation, but the two fractions obtained were not uniform (Figs. 2A and 3A). They both had a bimodal distribution of starch granules showing a natural division between the large and small granules. The object of the fractionation was to prepare pure samples both of small granules (<5.3 μm) and of large granules (>5.3 μm), and so these preparations were not acceptable. An elutriation system, used for fractionating ion-exchange resins (Scott 1968), offered the possibility of achieving such a fractionation with relatively large

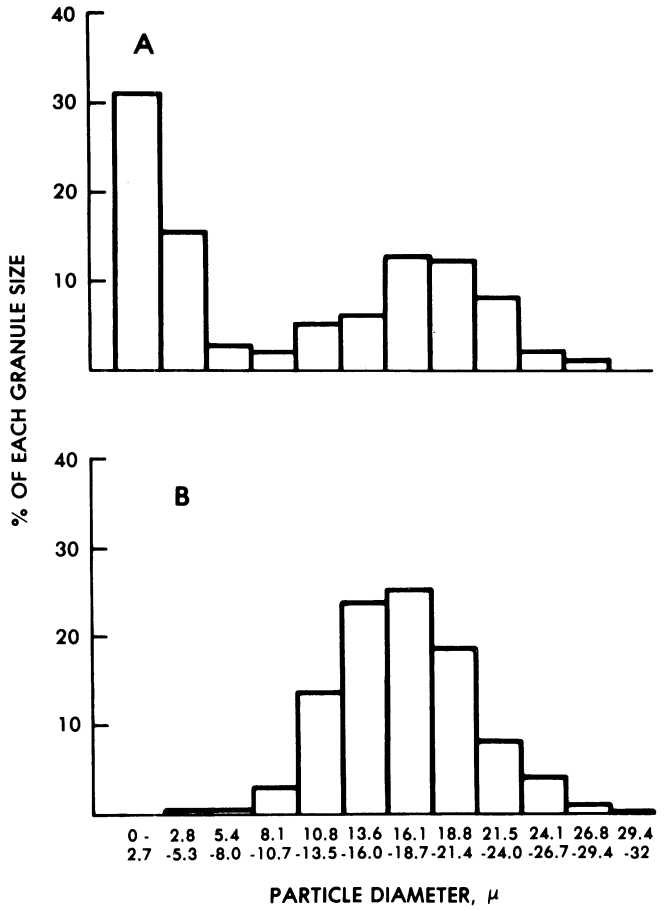

Fig. 2. Size distribution of prime starch. **A**, unfractionated sample. **B**, Fractionated sample.

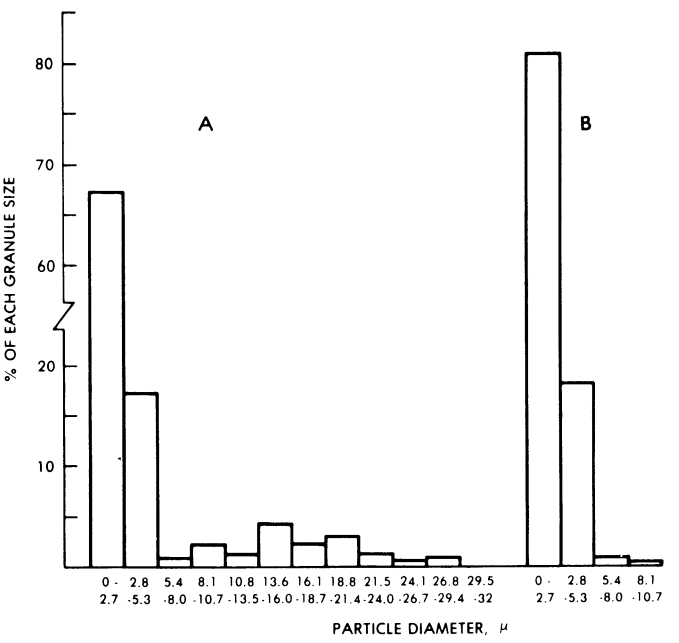

Fig. 3. Size distribution of secondary starch. **A**, unfractionated sample. **B**, fractionated sample.

amounts of starch. Each of the two original fractions was passed through the elutriation column twice. The size distributions of the resultant fractions are shown in Figs. 2B and 3B.

Essentially all of the starch granules smaller than 5.3 μm were removed from the prime starch fraction after two passes through the column. Although these small granules constituted 47% by number of the prime starch fraction, they represented only about 1% of the weight of the starch. During fractionation, the mean particle diameter of the prime starch rose from 10.3 to 16.7 μm. The purified sample was somewhat heterogeneous, but 80% of the granules fell into a size range of 10.8–21.4 μm.

Most (84%) of the particles in the unseparated secondary starch fraction (small granules) had a diameter less than 5.3 μm; the mean particle diameter of this fraction was 4.1 μm. After two fractionations, however, essentially all granules larger than 5.3 μm had been removed from the sample, and the mean particle size had fallen to 1.87 μm. Only about 25% of the small granules in the original crude fraction were recovered in a pure form from the elutriation column. The remaining small granules sedimented with the large granules, and repeated attempts to effect a separation were unsuccessful. This suggested that either the small granules were clumping and were, therefore, sedimenting like large granules or that the specific gravity of these particular small granules was similar to that of the large granules. Microscopic examination showed some clumping, but at least 50% of the small granules existed in suspension as discrete particles. Perhaps there are two kinds of small starch granules, each having the same size but different specific gravities. This possibility will be investigated further. Scanning electron photomicrographs of the two purified fractions are shown in Figs. 4 and 5. Small granules were not present in the large granule sample and very few large granules were detected in the sample of small granules. These pictures confirmed that the elutriation column method could be successfully used to separate the two populations of starch granules that occur

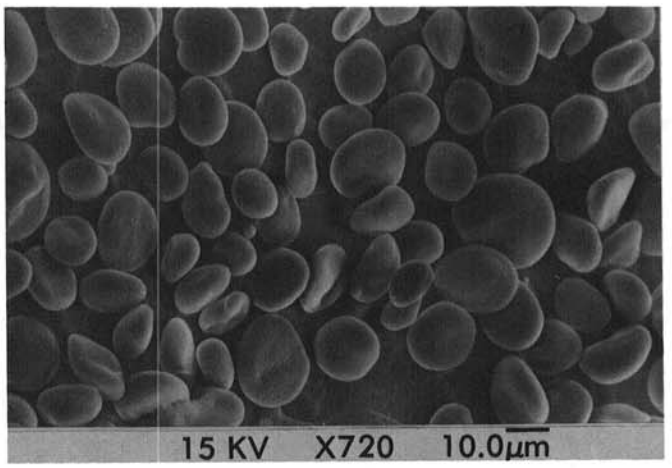

Fig. 4. Scanning electron photomicrograph of large starch granules.

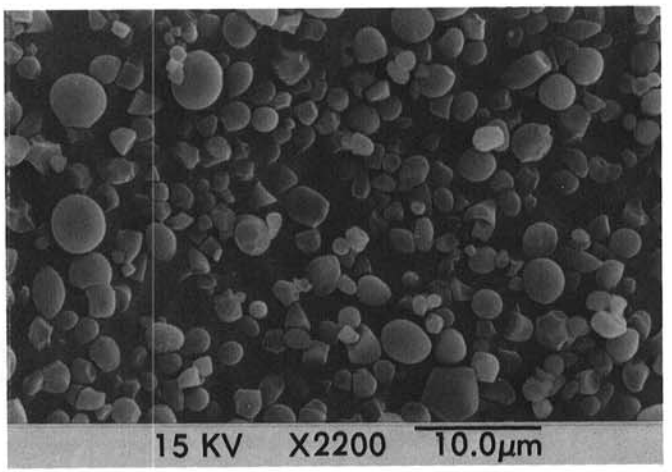

Fig. 5. Scanning electron photomicrograph of small starch granules.

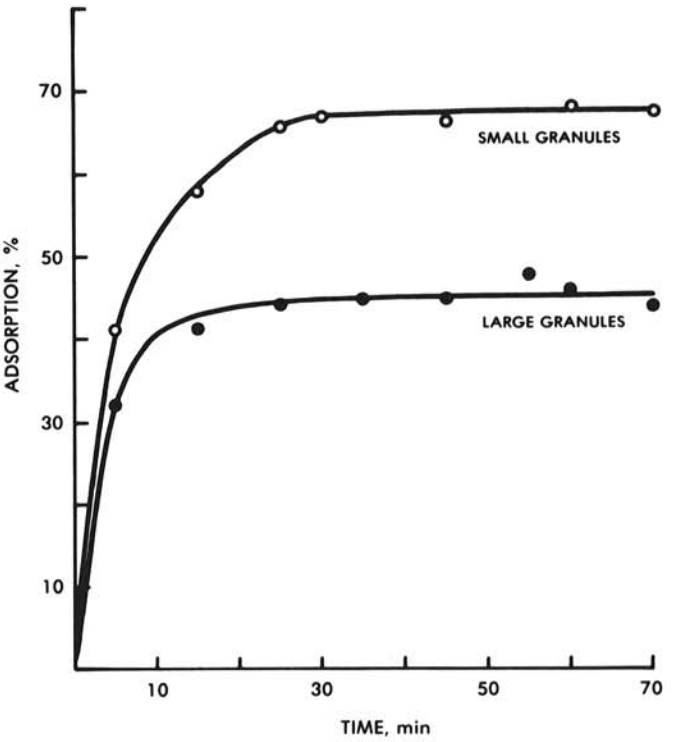

Fig. 6. Effect of contact time on the adsorption of malted barley α-amylase by large and small granules at 5° C and pH 5.5.

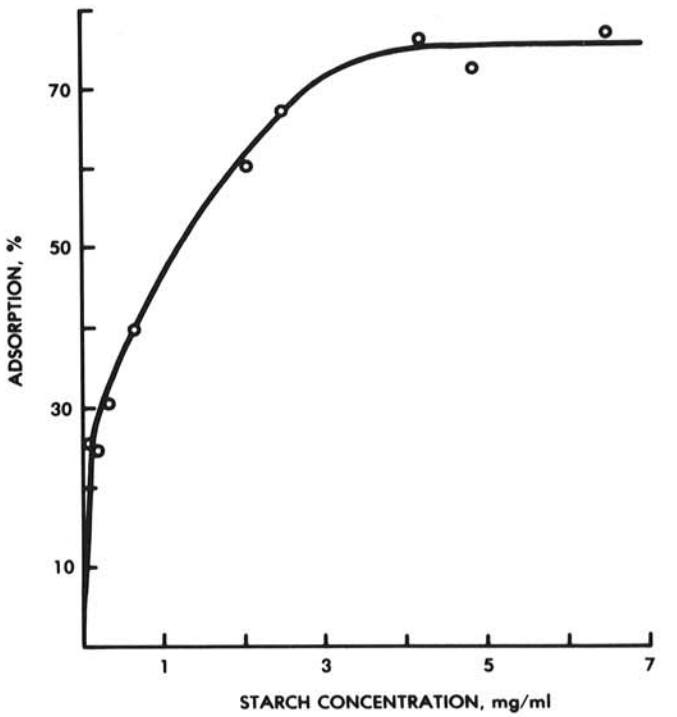

Fig. 7. Effect of the concentration of small starch granules on α-amylase adsorption at 5° C and pH 5.5.

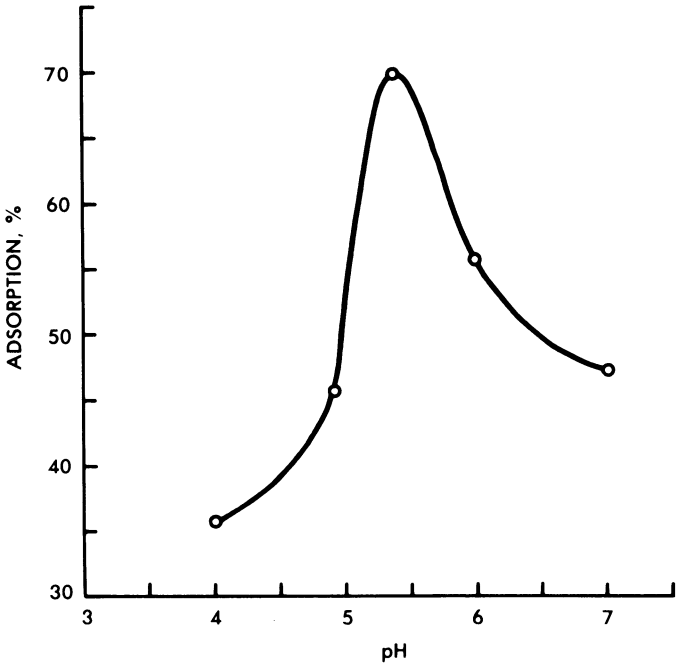

Fig. 8. Effect of pH on α-amylase adsorption by small starch granules at 5° C.

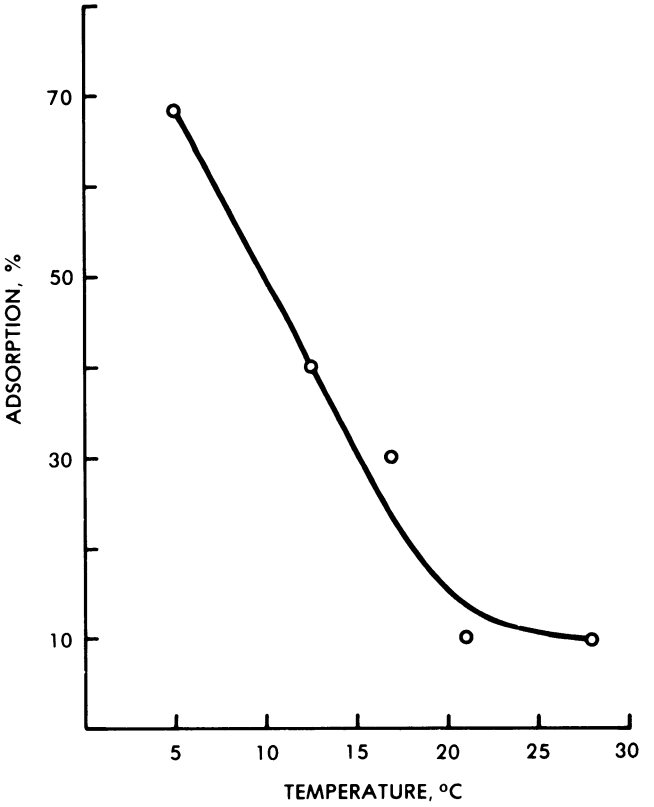

Fig. 9. Effect of temperature on α-amylase adsorption by small starch granules at pH 5.5.

naturally in mature barley. These starch granule fractions were used for α-amylase adsorption studies.

Adsorption of malt α-amylase by both large and small granules was very rapid at pH 5.5 and low temperature (Fig. 6). For both samples, maximum adsorption was achieved after a contact time of 30 min and there was very little change during the next 40 min. The large granules adsorbed a maximum of 45% of the enzyme activity, and the small granules adsorbed 67%. These results are in general agreement with previous studies (Sandstedt and Ueda 1969, Schwimmer and Balls 1949b) in which the extent of adsorption of α-amylases from various sources was inversely related to the size of the starch granules. Because adsorption phenomena depend on the surface area of the adsorbing material, this is not surprising.

The variation in α-amylase adsorption with different concentrations of small starch granules is shown in Fig. 7. Contact time between starch and enzyme was 50 min. Adsorption was significant at very low starch concentrations—more than 30% of the enzyme was adsorbed at a starch concentration of 0.3 mg/ml. Thereafter, increase in adsorption with increased starch concentration was much less, and a starch concentration of 4.5–5 mg/ml was required to achieve the maximum adsorption of 75%. Adsorption is an equilibrium phenomenon, so some unadsorbed α-amylase will always remain.

The effect of pH on the adsorption of α-amylase by secondary starch granules at 5° C is shown in Fig. 8. The general shape of the curve is similar to that reported for changes in α-amylase activity with pH (MacGregor 1977). More data in the pH 5–6 range would be required to determine precisely the pH of maximum adsorption, but this value was very close to pH 5.5, the pH of maximum activity. The concurrence of maximum activity and maximum adsorption of malt α-amylase at almost the same pH may be fortuitous and not necessarily because activity sites and adsorption sites on the enzyme are identical. Results were similar with an α-amylase from *Aspergillus oryzae* (Sandstedt and Ueda 1969). The pH of the two maxima were quite different, however, for α-amylases from saliva (Walker and Hope 1963), pancreas, and *Bacillus subtilis* (Sandstedt and Ueda 1969). Therefore, the evidence accumulated thus far suggests that adsorption and enzymatic activity occur at two different sites on the enzyme molecule.

Enzyme adsorption decreased very rapidly as the temperature of the enzyme-starch suspension was increased from 5 to 28° C (Fig. 9). This confirms Holmbergh's findings (1933), that adsorption of malt α-amylase by rice starch granules was "greatly promoted" at low temperatures.

The phenomenon of decreased adsorption at higher temperatures would clearly be useful in the isolation of α-amylase from solutions containing suspended starch granules, such as extracts of barley and malted barley. Because barley malt α-amylase is fairly thermostable, it could easily withstand extraction at 25–30° C, and the amount of adsorption would be very low. In addition, adsorption could be minimized by extracting with high pH (7–8) buffers. Adsorption was low, also, at lower values of pH (5–4) but cereal α-amylases are not stable at these pH values (Greenwood and Milne 1968), so extensive denaturation of the enzyme would occur.

Adsorption of α-amylase by starch granules probably is not a serious problem in the extraction and determination of α-amylases in germinated cereals. Because large amounts of α-amylase are present in such samples, only a relatively small proportion would be adsorbed. In mature or lightly germinated samples, however, the situation could be much more serious; a significant proportion of the α-amylase could adsorb to the starch granules during extraction and so remain undetected. To minimize this potential problem, α-amylases should be extracted from cereals at 20° C and with buffers at pH 7–8.

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