ic activity, and (ii) the occurrence of shock following electromyographic activity. It seems reasonable to assume that at least the former type of reinforcement could occur in completely curarized subjects. If this is so, then the completely curarized preparation would not avoid the difficulties described above. Before we can decide on the adequacy of the completely curarized preparation unequivocally, however, we must determine how much electromyographic activity, if any, is necessary for operant conditioning and transfer to occur. It may be that no electromyographic activity is necessary, and that the transfer could be mediated by the reinforcement of central nervous system events associated with movement (*14*). If this is the case, then the operant reinforcement of motoneuron electrical activity in completely curarized dogs should show the same type of transfer as was shown by the dogs in this experiment.

A. H. BLACK

Department of Psychology, McMaster University, Hamilton, Ontario, Canada

References and Notes

1. A. H. Black, N. J. Carlson, R. L. Solomon, *Psychol. Monog.* **76**, 29 (1962).
2. A. H. Black and W. H. Lang, *Psychol. Rev.* **71**, 80 (1964).
3. A. H. Black, in *Classical Conditioning*, W. Prokasy, Ed. (Appleton-Century-Crofts, New York, 1965), p. 20.
4. R. M. Church V. LoLordo, J. B. Overmier, R. L. Solomon, L. H. Turner, *J. Comp. Physiol Psychol.* **62**, 1 (1966).
5. In this report classical conditioning refers to conditioning that depends on the pairing of two stimuli—the conditioned stimulus and unconditioned stimulus. Operant conditioning refers to conditioning that depends on the pairing of a response and a reinforcing stimulus.
6. J. A. Trowill, paper presented at the meetings of the Eastern Psychological Association, New York, 1966.
7. L. V. Di Cara, paper presented at the meetings of the Eastern Psychological Association, New York, 1966.
8. A. H. Black, *J. Comp. Physiol. Psychol.* **51**, 519 (1958).
9. R. L. Solomon and L. H. Turner, *Psychol. Rev.* **69**, 202 (1962).
10. R. C. Leaf, *J. Comp. Physiol. Psychol.* **58**, 446 (1964).
11. J. B. Overmier, *ibid.* **62**, 15 (1966).
12. R. F. Hefferline, B. Keenan, and R. A. Harford [*Science* **130**, 1338 (1959)] have demonstrated that human subjects could be operantly trained to make extremely small electromyographic responses. It should be pointed out also that training a subject to refrain from electromyographic activity is not identical with training a subject to "hold still" in the normal state. The latter could involve a cessation of electromyographic activity as in relaxation, or a high level of electromyographic activity as in isometric contractions where movement does not occur because one set of muscles is pitted against another.
13. The *d*-tubocurarine chloride was provided by E. R. Squibb and Sons of Canada Ltd.
14. While the central nervous system events that might be involved cannot be specified, it does seem that feedback from the pedal-press is not a crucial factor, since Gorska and Jankowska [*Acta. Biol. Exp. Polish Acad. Sci.* **21**, 219 (1961)] and Taub, Bacon, and Berman [*J. Comp. Physiol. Psychol.* **59**, 275 (1965)] have demonstrated operant conditioning after deafferentation. The present results are consistent with theirs in that operant conditioning in curarized dogs had a clear-cut effect on subsequent pedal-pressing even though the afferent feedback was very different under curare from that occurring during pedal-pressing.
15. Supported by research grant MY-2741 from the National Institute of Mental Health, USPHS, by research grant No. 81 from the Ontario Mental Health Foundation, and by research grant APY-42 from the National Research Council of Canada. I thank C. Batenchuk and A. Dalton for their assistance in carrying out the experiment.

25 October 1966

Reversible Aggregation of α-Gliadin to Fibrils

Abstract. *Acetic-acid (0.01 molar) extracts of wheat flour contain fibrils of α-gliadin which are about 80 angstroms thick and up to several thousand angstroms long. These fibrils dissociate to globular protein subunits at very low ionic strength and low* p*H. The fibrils can be reformed by increasing the* p*H to 5.1 and the ionic strength to about 0.005.*

Bernardin, Kasarda, and Mecham (*1*) observed that the wheat protein α-gliadin aggregated specifically in aqueous solutions under certain conditions of *p*H and ionic strength, and they devised a procedure, based on the aggregation reaction, to separate it from other wheat proteins. We have studied the form of these aggregates by electron microscopy, and we find they are long threads with a fairly uniform thickness of about 80 Å (Fig. 1, A and B). These aggregates are formed from subunits that have a molecular weight of 49,000 or less (*1*). Their appearance in the electron micrographs, combined with the observation that such aggregates can be dispersed and reformed reversibly by varying conditions of *p*H and ionic strength, indicate their similarity to such proteins as actin (*2*) and insulin (*3*), which are known to undergo a reversible transformation from globular to fibrous form (G-F transformation). The formation of threads or fibrils does not necessarily mean that the globular form of the protein subunit involved is unfolded. Electron micrographs of actin (*2*) show fibrils of approximately spherical subunits linked into chains. Until recently (*4*), no plant protein was known to undergo a similar reversible transformation. The phenomenon may be more general, however, and simply may have been overlooked, as gliadin mixtures have been studied by analytical ultracentrifugation since 1935 (*5*) without recognition of the ability of α-gliadin to form specific aggregates.

A 0.01*M*-acetic-acid extract of wheat flour contains in solution gliadins, glutenin, albumins, globulins, carbohydrates, and lipids in addition to salts and low-molecular-weight organic molecules. For electron microscopy, specimens of such a crude extract were sprayed onto grids and shadow-cast with uranium. Micrographs showed the presence of fine fibrils, some with lengths up to 5000 Å, imbedded in a film of low-molecular-weight material. The apparent thickness of these fibrils was about 50 Å, but this estimate is undoubtedly low since the film partially obscured their contours.

Centrifugation of the crude extract at 133,000*g* (average) for 2 hours sedimented a clear, gelatinous pellet. Electron micrographs of the supernatant solution showed a film of low-molecular-weight material, but almost no fibrils, with the exception of a relatively few short segments. The pellet was dissolved in 0.017*M* aluminum lactate, *p*H 3.1, a solvent known to dissociate gliadin proteins to aggregates with particle weights less than 50,000 (*6*); the solution was then analyzed by electrophoresis on polyacrylamide gel. The pellet consisted almost entirely of α-gliadin.

We tried to purify the pellet by repeated sedimentation. When the material was resuspended to the original volume either in the 0.01*M* acetic acid used for the initial extract (but now buffered only by α-gliadin as opposed to the many other substances contained in the flour extract) or in 0.001*M* HCl, it could not be sedimented by the usual centrifugation. The absence of fibrils in the solutions was confirmed by electron microscopy. By contrast, about 50 percent of the redissolved α-gliadin could be sedimented when the solvent was an ammonium-acetate buffer of 0.006 ionic strength, *p*H 5.1.

Polyacrylamide-gel electrophoresis of twice-sedimented α-gliadin aggregates subsequently dissolved in aluminum lactate buffer showed the α-gliadin to be free of contaminating low-molecu-

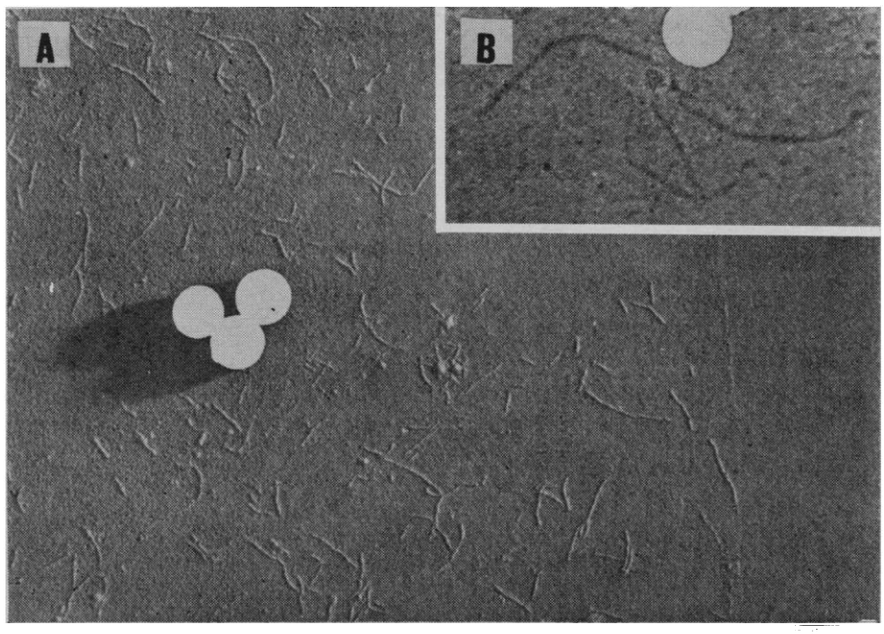

Fig. 1. Electron micrographs of purified α-gliadin aggregates fixed with gluteraldehyde. (A) Shadowed spray-droplet preparation. The edge of the droplet is seen at the lower right; 0.264-μ polystyrene spheres serve to indicate the magnification and shadow angle (reverse contrast print $\times$ 28,000). (B) Preparation negatively stained with uranyl acetate. A 0.088-μ polystyrene sphere on the back side of the grid serves to indicate the magnification (reverse contrast print, $\times$ 100,000).

lar-weight proteins. Evidence for slight contamination by a material of higher molecular weight, probably glutenin, was obtained by gel filtration of the protein in the aluminum-lactate buffer on a dextran column that excluded from the gel molecules with molecular weights greater than 100,000. The contaminant was readily separated by this procedure. Calibration of this column (*1*) yielded a molecular weight of 49,000 for α-gliadin in this solvent. This may not be the monomeric weight of α-gliadin, but it does set an upper limit.

The purified, low-molecular-weight α-gliadin obtained on the gel-filtration column was reaggregated in the ammonium-acetate buffer, and a specimen was prepared for electron microscopy as before. Micrographs showed fibrils similar to those in the original flour extract, but again a continuous film of material partially obscured the threads. This film was evidently formed from low-molecular-weight α-gliadin in equilibrium with the aggregated form.

To obtain preparations free from low-molecular-weight material we dialyzed a solution of α-gliadin, which had already been aggregated by dialysis against 0.005*M* KCl, against a solution containing 2.5 percent glutaraldehyde in 0.005*M* KCl and 0.0005*M* NH_4Cl, *p*H 5.1. By this treatment the aggregates were fixed, presumably by intersubunit crosslinking (*7*). The aggregated protein was dialyzed against distilled water and centrifuged to separate it from unfixed and disaggregated protein. Before centrifugation, the *p*H of the water solution was lowered to 3 with HCl so that aggregated, but unfixed, protein was dissociated. The pellet was extracted with distilled water, and the resulting solution was used to prepare the specimens seen in the electron micrographs (Fig. 1).

Figure 1A shows part of a dried spray-droplet of the material. The solution of aggregates in distilled water was mixed with 0.264-μ polystyrene latex spheres for comparison, sprayed on a carbon-surfaced collodion membrane, and shadowed with uranium. The edge of the droplet is seen at the lower right; comparison of the background inside and outside the edge indicates the nearly complete absence of low-molecular-weight material. The fibrils have a fairly uniform thickness of about 80 Å and are a few hundred to 3000 or 4000 Å long. The individual filaments show little tendency toward lateral aggregation and when clearly separated from their neighbors appear unbranched. Except for the absence of low-molecular-weight material occluding the threads, this preparation looks similar to spray-droplets of the original, crude flour-extract.

Figure 1B shows a negatively stained preparation. The carbon-collodion-filmed grid was sprayed on the back side with 0.088-μ polystyrene spheres that served as aids in calibration and focusing. A solution of the fixed aggregates in 0.01*M* ammonium-acetate buffer containing 0.5 percent uranyl acetate was sprayed onto the front side and dried in air. The fibrils again show a uniform thickness of about 80 Å. No convincing evidence of substructure can be seen in the threads although the background grain should permit resolution to about 30 Å. We do not know whether the absence of visible substructure represents a failure in the preparation of the specimen or an inherent property of the fibrils.

It seems unlikely that extensive change in conformation of the subunits occurs when α-gliadin undergoes the G-F transformation, since the transformation occurs under relatively mild conditions and the conformation of gliadins seems relatively stable (*5*, *8*). About 20 percent of the amino-acid residues in the gliadins exist in the α-helix form in aqueous solution, as estimated from optical rotatory dispersion measurements (*8*), so other structures must play an important role in stabilizing them. Since about one in three amino-acid residues in α-gliadin is glutamine and about one in three residues has a nonpolar side chain (*1*), hydrogen bonds and hydrophobic forces are probably involved. The small number of side chains capable of assuming the charged form in solution results in a low charge-density for α-gliadin. Aggregation occurs when the ionic strength is increased to only about 0.005 at *p*H 5, whereas higher ionic strengths precipitate the protein at this *p*H. Increasing the ionic strength should weaken general ionic interactions. However, it is possible that decreasing the repulsive effect of the net positive charge on the molecules at *p*H 5 permits interaction of local concentrations of positive and negative charges on the surface of the molecules. It is also possible that such interactions could act in concert with hydrogen bonding and hydrophobic forces.

Gel electrophoresis of purified α-gliadin results in three bands (*1*). Reaggregation and collection by centrifugation of α-gliadin, although effective in separating it from at least 20 other proteins in the flour extract, does not change the relative intensi-

ties of these bands. All α-gliadin components appear to participate in the aggregation and, accordingly, may interact randomly, or cooperate in some specific way, or even form separate fibrils.

These aggregates may reflect native structure in the wheat kernel, and the property of forming ordered aggregates most likely reflects the functional role of this protein in the seed.

DONALD D. KASARDA
JOHN E. BERNARDIN
RICHARD S. THOMAS
Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Albany, California 94710

References and Notes

1. J. E. Bernardin, D. D. Kasarda, D. K. Mecham, *J. Biol. Chem.*, in press.
2. A. Szent-Gyorgyi, *General Physiology of Contraction in Body and Heart Muscle* (Academic Press, New York, 1953), pp. 21–2; J. Hanson and J. Lowy, *J. Mol. Biol.* **6**, 46 (1963).
3. D. F. Waugh, *J. Amer. Chem. Soc.* **68**, 247 (1946); J. L. Farrant and E. H. Mercer, *Biochim. Biophys. Acta* **8**, 355 (1952); T.-H. Kung and T.-C. Tsao, *Sci. Sinica (Shanghai)* **13**, 395 (1964).
4. B. G. Audley and E. G. Cockbain, *J. Mol. Biol.* **18**, 321 (1966).
5. L. E. Krejci and T. Svedberg, *J. Amer. Chem. Soc.* **57**, 946 (1935).
6. R. W. Jones, G. E. Babcock, N. W. Taylor, F. R. Senti, *Arch. Biochem. Biophys.* **94**, 483 (1961).
7. Our conclusion that we fixed the aggregates was based primarily on an improvement in our specimens for electron microscopy.
8. Y. V. Wu and J. E. Cluskey, *Arch. Biochem. Biophys.* **112**, 32 (1965).
9. We thank Joanne Remy for technical assistance.

31 October 1966

Hexokinase Isoenzymes in Liver and Adipose Tissue of Man and Dog

Abstract. *A hexokinase, with a low Michaelis constant, not previously described, has been found in extracts of human and dog liver but not of rat liver. Earlier reports are contradicted in that glucokinase occurs in extracts of liver from well-nourished humans and dogs; it is absent, or almost so, during states of poor nutrition.*

The discovery of an enzyme in rat liver that phosphorylates glucose at high concentrations (K_m, 0.01 to 0.02*M*), thereby differing from previously described hexokinases (adenosine triphosphate : D-hexose 6-phosphotransferase), was an important advance in knowledge of regulation of glucose utilization by the liver, and thus of glucose tolerance (*1*). The enzyme designated glucokinase, because of its greater substrate specificity, disappears during fasting, a carbohydrate-free diet, or alloxan diabetes: this fact suggests dependence on insulin as well as on substrate for synthesis. After administration of insulin, restoration of enzyme activity in the livers of diabetic rats requires 12 to 24 hours (*2*, *3*); this delay explains the slow response to insulin of utilization of glucose by this tissue.

Subsequently Gonzáles *et al.* (*4*) found four hexokinases in rat-liver extracts, using DEAE-cellulose columns, a finding confirmed by starch-gel electrophoresis (*5*). The types of hexokinase are designated I to IV in order of increasing mobility on starch-gel electrophoresis. Types I to III are low-K_m hexokinases; the fourth, the high-K_m glucokinase, accounts for most of the total activity in the livers of well-fed rats and is the component that varies with the carbohydrate content of the diet. Katzen and Schimke found that the presence of multiple hexokinases is a general phenomenon in the tissues of rats and other species and is not restricted to the liver, although only the liver contains glucokinase.

Each hexokinase retains its unique properties from tissue to tissue. The proportions of hexokinase types are variable in different tissues and with age and nutrition. Type II is the predominant hexokinase in the epididymal fat pad of young adult rats, but decreases with age and fasting. During an earlier study we observed that, in the epididymal fat pad of alloxan-diabetic rats, type-II hexokinase was greatly reduced relative to type I, a change analogous to the changes in hepatic glucokinase (*7*).

The importance of hepatic glucokinase presumably resides in its ability to phosphorylate the tide of glucose reaching the liver by way of the portal circulation, following carbohydrate meals. Since adaptation of glucose-tolerance curves to dietary carbohydrate is a well-established phenomenon in man, it was surprising that Boxer (*8*) and Lauris and Cahill (*9*) could not find glucokinase in normal human liver.

We have determined the total hexokinase and glucokinase activity, and the patterns of isoenzymes, in human liver and in adipose tissue obtained from patients free of liver disease who were undergoing surgery on the intestines or gall bladder. Similar studies were made of tissues from mongrel dogs that had been fasted after feeding. The tissues were either lyophilized immediately for later use, or homogenized in a cold medium (*10*) containing 150 m*M* KCl, 5 m*M* $MgCl_2$, 5 m*M* EDTA, and 10 m*M* mercaptoethanol and adjusted to *p*H 7.4. The homogenates were centrifuged for 45 minutes at 100,000*g*, and the supernatants, after dialysis against the same medium for 1 hour in the cold, were

Table 1. Total hexokinase and glucokinase activities in extracts of liver obtained from well-nourished and poorly nourished humans. Results are given as millimicromoles of glucose-6-phosphate formed per minute, at 25°C, per gram of liver or per milligram of liver protein in the supernatant after centrifugation for 45 minutes at 100,000*g*. The enzymes were estimated from the rate of formation of NADP-hydrogenase at 340 mμ in the presence of excess G6PD and 6-phosphogluconate dehydrogenase; the change in optical density was halved to correct to millicromoles glucose-6-phosphate formed. The method of Sharma, Manjeshwar, and Weinhouse (*3*) was modified in that, in addition to hexokinase activity (0.5 m*M* glucose), a blank omitting only adenosine triphosphate was also subtracted from the activity obtained with 0.1*M* glucose. Protein content was determined by the method of Lowry (*13*). Means and S.E. appear in parentheses.

Protein (mg/g)	Hexokinase		Glucokinase	
	Absolute (unit/g)	To protein (unit/mg)	Absolute (unit/g)	To protein (unit/mg)
		Well-nourished man		
92	130	1.41	283	3.07
109	211	1.94	261	2.39
139	183	1.32	402	2.90
(113 ± 14)	(175 ± 24)	(1.56 ± 0.19)	(315 ± 44)	(2.79 ± 0.20)
		Poorly nourished man		
66	193	2.91	0	0
74	180	2.42	25	0.34
55	171	3.13	0	0
101	220	2.18	0	0
(74 ± 10)	(191 ± 11)	(2.66 ± 0.22)		