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)		

used immediately, or were lyophilized for later use, for starch-gel electrophoresis and for enzyme determinations.

Vertical starch-gel electrophoresis accorded with Katzen and Schimke's (*5*) except for these modifications: veronal buffer, 0.02*M*, *p*H 7.4; EDTA, 4.5 m*M*; mercaptoethanol, 2.3 m*M*. Gels were sliced, and stained in the dark at room temperature for 6 to 8 hours in the following medium: glycylglycine buffer, 50 m*M*, *p*H 7.4; nicotinamide-adenine dinucleotide phosphate (NADP), 2 m*M*; $MgCl_2$, 5 m*M*; adenosine triphosphate, 5 m*M*; KCN, 1 m*M*; nicotinamide, 40 m*M*; with low (0.5 m*M*) or high (0.1*M*) glucose. Just before use, the following were added: poly(vinyl pyrrolidone), 3.5 percent; glucose-6-phosphate dehydrogenase (G6PD), 0.4 unit per milliliter; phenazine methosulfate, 4 mg/100 ml; and nitroblue tetrazolium, 40 mg/100 ml.

Figures 1 and 2 show five isoenzymes of hexokinase in tissues of man and dog in contrast with only four in the rat. The band that migrates in the cathodal direction under these conditions has never been described; found only in liver, it is a low-K_m hexokinase and reacts when glucose is present in a concentration of $10^{-6}M$ in the staining solution; it stains well with fructose as substrate. Thus in all these characteristics it resembles the hexokinases of rat tissues, except for the direction of migration on starch-gel electrophoresis under these conditions. There is no apparent difference in this isoenzyme of hexokinase between man and dog.

The slowest toward-anode-moving hexokinase band found in human and dog liver and adipose tissue corresponds in mobility to type I of the rat. It is an intensely staining and relatively stable hexokinase in contrast with the toward-cathode-moving band that tends to disappear when the liver has been frozen longer than 1 week. The second anodal band migrating with type II hexokinase of the rat occasionally occurs in human liver and is usually seen in the dog, but it occurs consistently in extracts of human and dog adipose tissue. The third hexokinase, corresponding to type III of the rat, occurs consistently in human and dog liver and in extracts of adipose tissue of the dog, but faintly and only occasionally in extracts of human adipose tissue. Since this enzyme is strongly inhibited by high concentrations of glucose, as in the rat, it is difficult to detect when the staining solution contains 0.1*M* glucose (Fig. 1, *adipose*). All the hexokinases stain when fructose is added as substrate to the staining solution, a characteristic similar to that of rat hexokinases.

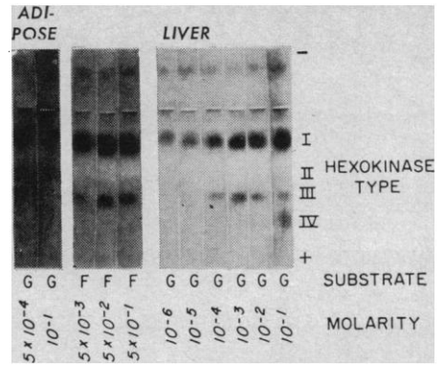

Fig. 1. Starch-gel electrophoresis of isoenzymes of hexokinase in extracts of liver and adipose tissue from a well-nourished human. The substrate included in the staining solution was glucose (*G*) or fructose (*F*). The top band, which migrates toward the cathode under these conditions, is previously undescribed and is not numbered. Glucokinase (type IV) is seen only in liver extracts stained in a solution containing 0.1*M* glucose; not in lower concentrations or in fructose up to 0.1*M*. When fructose was used as substrate, phosphohexose isomerase (0.4 unit/ml) was included in the staining solution.

The most anodal band, migrating with type IV of the rat and characterized as glucokinase, has been found in four samples of human liver and in many dog livers; but, because it depends on good nutrition, it is often absent from specimens of liver obtained at surgery or autopsy. The type IV enzyme does not stain until the gel is bathed in a solution containing 0.01 to 0.1*M* glucose, and does not use fructose as a substrate. Thus, by its mobility, low affinity for glucose, and substrate specificity, it is characteristic of glucokinase as described in the rat.

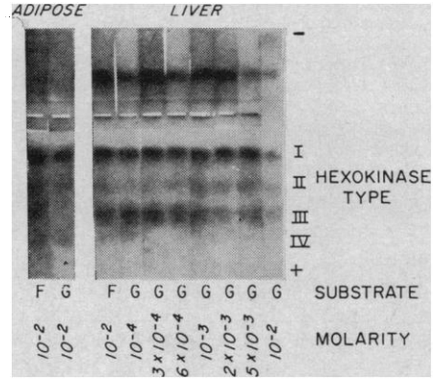

Fig. 2. Starch-gel electrophoresis of isoenzymes of hexokinase in extracts of liver and adipose tissue from a fed dog. The substrate included in the staining solution was glucose (*G*) or fructose (*F*). Conditions as in Fig. 1. Glucokinase (type IV) was not seen in the extract from this dog.

Confirmation of the presence of glucokinase in human liver is provided by determination of total hexokinase and glucokinase activities by the method of Sharma, Manjeshwar, and Weinhouse (*3*), with minor modifications. Extracts of liver obtained from three well-nourished patients showed the fourth anodal band on starch-gel electrophoresis, and contained twice as much glucokinase as hexokinase activity (Table 1). In contrast, extracts from livers of four poorly nourished patients showed no fourth band and contained little or no glucokinase. Hexokinase activity was not significantly different in the extracts of liver from the two groups of patients. Similar observations made in extracts of liver from fed and fasted dogs confirmed the presence of glucokinase in the dog as well as in man (*11*).

Of the hexokinases with low K_m that are present in extracts of human liver, the band migrating toward the cathode had the greatest affinity for glucose and stained maximally at $10^{-6}M$. The first anodal band was next in order in decreasing affinity for glucose and was followed by anodal III. Then came a sharp change at glucokinase, which did not stain until glucose was present at 0.01 to 0.1*M* concentrations. Estimation of the K_m of the second anodal band in human liver is prevented by faintness, but in adipose tissue this isoenzyme stains well when glucose is present at $5 \times 10^{-4}M$ (Fig. 1). The pattern of staining of the hexokinase bands in dog liver generally resembles that in man except for anodal III, which in glucose affinity appears to be more like that in the rat (K_m, 5 to 7 $\times$ 10^{-6}) than in man (*5*, *6*, *11*). Thus these isoenzymes, which migrate at the same rate on starch-gel electrophoresis, have widely different affinities for glucose. On the other hand, type III hexokinase of human and dog are both similar to that of the rat in being inhibited by high concentrations of glucose but not by fructose. The affinity for fructose in three of the isoenzymes of human hexokinase is indicated by

maximal staining at $5 \times 10^{-3}M$ (Fig. 1). This finding is in good agreement with the K_m for fructose of rat hexokinases which are all similar in the range 3.1 to $4.0 \times 10^{-3}M$ (*12*).

The presence of glucokinase in human and dog liver and its responsiveness to the nutritional state emphasize the importance of this enzyme in the regulation of glucose utilization by the liver. The greater ease of demonstration of glucokinase in rat liver than in human liver may partly result from the eating habits of rats, which consume most of their food in the course of several hours during the night and thus present large amounts of substrate to the liver at one time. Moreover, the ordinary diet of rats contains only a small amount of fat and is chiefly carbohydrate, whereas the usual human diet contains considerable fat and less carbohydrate. Since glucokinase makes up most of the phosphorylating capacity of the liver in well-fed humans and dogs, as well as in rats, the level of this enzyme determines the capacity of the liver to dispose of glucose; thus the level determines the character of the glucose-tolerance curve. It is known that glucose tolerance declines with fasting, advancing age, and the presence of malignant disease. Our study has demonstrated that glucokinase is low in or absent from poorly nourished humans and dogs, and we anticipate that similar correlation may be possible with advancing age or malignancy in man.

JOSIAH BROWN, DONALD M. MILLER
MARY T. HOLLOWAY, GERALD D. LEVE
Departments of Medicine and Zoology, University of California, Los Angeles

References and Notes

1. D. L. Di Pietro and S. Weinhouse, *J. Biol. Chem.* **235**, 2542 (1960); E. Vinuela, M. Salas, A. Sols, *ibid.* **238**, 1175 (1963); D. G. Walker, *Biochim. Biophys. Acta* **77**, 209 (1963).
2. M. Salas, E. Vinuela, A. Sols, *J. Biol. Chem.* **238**, 3535 (1963).
3. C. Sharma, R. Manjeshwar, S. Weinhouse, *ibid.*, p. 3840.
4. C. Gonzáles, T. Ureta, R. Sanchez, H. Niemeyer, *Biochem. Biophys. Res. Commun.* **16**, 347 (1964).
5. H. M. Katzen and R. T. Schimke, *Proc. Nat. Acad. Sci. U.S.* **54**, 1218 (1965).
6. L. Grossbard, M. Weksler, R. T. Schimke, *Biochem. Biophys. Res. Commun.* **24**, 32 (1966).
7. P. McLean, J. Brown, K. Greenslade, K. Brew, *ibid.* **23**, 117 (1966).
8. G. E. Boxer, in G. Weber, *Science* **151**, 479 (1966).
9. V. Lauris and G. F. Cahill, *Diabetes* **15**, 475 (1966).
10. An alternative medium, giving the same results, contained: Hepes buffer, 0.02*M*, *p*H 7.4; 1-cysteine, 30 m*M*; $MgSO_4$, 5 m*M*; K_2SO_4, 15 m*M*. In addition 0.5m*M* glucose was sometimes added to the homogenizing and dialyzing media and to the starch.
11. F. J. Ballard, *Comp. Biochem. Physiol.* **14**, 437 (1965).
12. L. Grossbard and R. T. Schimke, *J. Biol. Chem.* **241**, 3546 (1966).
13. O. H. Lowry, H. J. Rosebrough, A. L. Farr, R. J. Randall, *ibid.* **193**, 264 (1951).

3 October 1966

Psilocybin: Reaction with a Fraction of Rat Brain

Abstract. *Psilocybin, a hallucinogen, formed a blue color with a subfraction of rat-brain mitochondria believed to contain nerve-ending particles. Color formation increased with pH, did not require oxygen, and involved a component that could not be solubilized. The effect was not shown by chemically related neuroactive compounds, such as bufotenine and serotonin, and was antagonized by only tyramine or ethylenediaminetetraacetic acid.*

Psilocybin is a hallucinogen whose mode of action is unknown and whose potency is 130 times less than that of LSD-25 (*1*). While investigating the binding of norepinephrine to the crude mitochondrial fraction of rat brain, Herblin (*2*) noted that a blue color developed when psilocybin was present along with the norepinephrine. We now report investigation of the nature of this phenomenon. By use of a method similar to that of De Robertis (*3*), a crude mitochondrial fraction was prepared from rat brain and suspended in 0.32*M* sucrose at (fresh weight) 0.5 g/ml. Samples (1-ml) were each mixed with 1 ml of tris buffer, *p*H 7.4 and 0.15*M*; 0.5 ml of psilocybin, $1.5 \times 10^{-3}M$; and 0.5 ml of water or another reagent where indicated.

Subcellular fractions of the crude mitochondria were prepared by use of sucrose-density gradient of 0.8 ml of 0.7*M*, 0.8 ml of 1.0*M*, 0.8 ml of 1.2*M*, and 1.5 ml of 1.3*M* sucrose. One milliliter of homogenate in 0.32*M* sucrose was layered on top and centrifuged for 45 minutes at 48,000 rev/min in a Beckman L-2 ultracentrifuge with an SW50 head. Five fractions were visible; they were separated with a tube cutter. Microsomal, nuclear, and soluble fractions also were prepared (*3*).

The color, an intense Wedgewood blue, formed only after standing for 16 to 20 hours at 5°C (*p*H 7.4) and was restricted to the precipitate produced by centrifuging for 15 minutes at 3400*g*. Because the blue was in a solid phase, only qualitative statements can be made about its intensity; intensity depended on concentration of psilocybin, being weak at $10^{-4}M$ final concentration and intense at $5 \times 10^{-4}M$. The *p*H was important, for no color was produced at *p*H 6.0; at *p*H 7.4, color was first visible after 8 hours and required about 18 hours for complete development; at *p*H *9* the color was first visible after 1 hour and complete after about 9 hours. Oxygen was apparently not required, for blue formation was not affected by prior passage of N_2 through the crude mitochondria and subsequent incubation with psilocybin under N_2.

Work with brain mitochondria, subfractionated in such a sucrose-density gradient, showed that virtually the only active fraction was that found in 1.2*M* sucrose. This was equally true whether one added the psilocybin to the subfractions or to the crude mitochondria before subfractionation. The 1.2*M* fraction should correspond to De Robertis's fraction C, said to be cholinergic nerve-ending particles; but De Robertis found fraction C to be the richest fraction in serotonin (*4*), while we found most serotonin in the 1.0*M* fraction—0.9 μg per gram of original brain, by the method of Uchida and O'Brien (*5*). Color development by the 1.2*M* subfraction was slower than by crude mitochondria; color was nil at 18 hours and fully developed at 36 hours. No color was developed by the microsomal or soluble fractions. A faint blue color developed by the nuclear fraction may have resulted from incomplete separation. When the crude mitochondria were shaken with water and centrifuged, the precipitate, which should have been free of synaptic vesicles (*6*), showed no loss in ability to develop a blue color with psilocybin. Examination of rat-liver mitochondria, prepared by the same procedure as crude brain mitochondria, showed only a thin light blue on the top of the precipitate after incubation with psilocybin.

We tried to solubilize the color in order to measure its intensity, but the color remained in the precipitate when any of the following reagents was used: butanol, chloroform-methanol (2:1), ether, petroleum ether, hexane, benzene, toluene, Triton X-100 (1 percent in water), acetone, trichloroacetic acid, sodium taurocholate, 0.05*N*