PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 158, 255–259 (1978)

The Effects of Fasting, Diabetes, and Hypophysectomy on Rat Adipose Pyruvate Kinase (40183)

E. P. HERN[1] AND G. S. SERIF[2]

Department of Biochemistry, The Ohio State University, Columbus, Ohio 43210

Pyruvate kinase is often found as a mixture of isozymes in mammalian tissue (1). Adipose tissue pyruvate kinase, however, has been shown to be a single isozyme (2–5). This isozyme may be found in one of two interchangeable forms depending upon the effector substances present or the extraction medium used to obtain it (3–5). Although pyruvate kinase levels have been shown to be responsive to conditions of fasting and feeding in tissues such as liver and kidney (7), there is considerable confusion about the ability of adipose pyruvate kinase to respond to such physiological conditions. Both Pogson and Denton (8) and Orevi *et al.* (9) have reported that fasting and the induction of diabetes have no appreciable effect on adipose tissue pyruvate kinase. However, while Pogson and Denton (8) were able to demonstrate an elevation of the adipose tissue enzyme when rats were fed on a high carbohydrate diet, Orevi *et al.* (9) were unable to see this effect with normal rats on a high carbohydrate diet or with fasted rats refed a high carbohydrate diet.

Both groups used cell protein as a reference point for enzyme levels. Since adipose tissue protein levels have now been shown to vary appreciably under conditions where that tissue is undergoing metabolic shifts (10, 11), it is clear protein content is an unacceptable baseline against which to measure adipose tissue enzyme levels. We have, as a consequence, undertaken to reexamine whether adipose pyruvate kinase is responsive to hormonal and nutritional controls using cell DNA content as a more stable parameter against which to measure enzyme levels. We have also extended this study to examine some effects of hypophysectomy and of phosphate ion in this system.

[1] Current address: Fels Research Institute, Yellow Springs, OH.

[2] Person to whom reprint requests should be addressed.

Materials and methods. Albino male rats of the Sprague–Dawley strain were used in the fasting, refeeding and diabetic experiments and their weights were in the range of 160 ± 30 g. Hypophysectomized male rats of the same strain and age were obtained from Hormone Assay Laboratories, Chicago, IL. Hypophysectomized rats were stabilized for a minimum of 10 days prior to their use in experiments and were not used beyond four weeks following surgery. All animals were maintained *ad lib.* on Purina Rat Chow.

Rats were made diabetic by intravenous administration of streptozotocin (7 mg per 100 g body wt dissolved in 0.05 *M* citrate buffer at a pH of 4.5). Blood glucose level greater than 350 mg% at the time of sacrifice (4 days after streptozotocin injection) was used as the criterion of diabetes. Control rats in the diabetic experiments received equivalent volumes of citrate buffer by intravenous injection.

At the end of the experimental period, all rats were weighed and sacrificed by cervical dislocation. The epididymal fat pads were freed of major blood vessels and placed on ice. The pads were weighed and then homogenized in a cooled Potter-Elvehjem homogenizer in 1–3 vol of homogenizing fluid (145 m*M* KCl:5 m*M* $MgSO_4$:1 m*M* EDTA, pH 7.4). The homogenate was centrifuged at 30,000*g* for 30 min at 4° and, after the solidified lipid was removed, an aliquot of the clear supernatant solution was assayed for pyruvate kinase content.

Pyruvate kinase was measured by coupling the enzyme to lactic dehydrogenase and measuring the disappearance of NADH at 340 nm (5). The reaction mixture was composed of 2 m*M* PEP, 150 μM NADH and 0.25 units of LDH/ml in assay buffer (50 m*M* Tris:75 m*M* KCl:8 m*M* $MgSO_4$:1.3 m*M* ADP at pH 7.5). The reaction was initiated by the addition of 10 μl of supernatant enzyme to 1.99 ml of this reaction mixture for

0037-9727/78/1582-0255$01.00/0
Copyright © 1978 by the Society for Experimental Biology and Medicine
All rights reserved.

a total volume of 2.0 ml. The concentration of enzyme was such that the reaction was linear over the 90 sec assay period. A unit of pyruvate kinase activity is defined as an initial oxidation rate of one μmole of NADH per minute at 25°.

DNA measurements were performed on the 30,000*g* precipitated pellet by a modification of the method of Schneider (12) in which the tissue pellet was extracted three times with 5 vol of water saturated ether prior to TCA extraction. Ninety-two % of total DNA was recovered in this pellet as measured through recovery of standard samples.

Rats treated with cycloheximide received 25–40 μg of the antibiotic per 100 g body wt 2 times daily as indicated for the specific experiment. A 100-fold stock solution was made in 95% ethanol and kept refrigerated for no longer than 1 week. Injections were prepared by diluting the appropriate volume of stock solution to 1.0 ml with 0.9% NaCl. Control animals received an equivalent volume of 95% ethanol diluted with 0.9% NaCl. All injections were administered by the intraperitoneal route. The injections were started at the initiation of either fasting or diabetes and continued until the rats were sacrificed.

Animals receiving inorganic phosphate received 1.0 ml of 154 m*M* NaH_2PO_4 (or $KH_2PO_4 \cdot NaOH$, pH 7.5) two times daily by intraperitoneal injection. Control animals received an equivalent volume of 154 m*M* NaCl at the same time and by the same route. The injections were started at either the induction of fasting or diabetes or at the end of the stabilization period for the hypophysectomized rat and continued until the rats were sacrificed.

All injections were given at 9:00 AM and 4:00 PM during each day of treatment.

Oral glucose (500 mg/100 g body wt) was administered 5 times daily by stomach tube. The glucose was administered as a 50% solution (w/v) of D-glucose which was made fresh daily. These animals were maintained on 2.5% (w/v) glucose water overnight. Control animals received an equivalent volume of water orally.

Whole blood glucose was measured by the "Glucostat" method (7) from heparinized blood removed from either the tail vein or the heart.

Supernatant protein was measured by the method of Warburg and Christian (18).

Probability values were derived from Students' "t" test and the expression:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{\frac{S_1^1}{N_1} \frac{S_2^2}{N_2}}}$$

Results. Table I contains a summary of the effects of fasting, hypophysectomy, and streptozotocin-induced diabetes on adipose tissue pyruvate kinase levels expressed as units of enzyme per unit of cellular DNA. Clearly all three conditions cause a significant decrease in pyruvate kinase levels. Fasting for 72 and 96 hr results in a 55 and 60% loss, respectively, in total pyruvate kinase activity relative to their control animals. Oral glucose alone is seen to reverse the effects of fasting in that normal levels of pyruvate kinase are found in fasted animals refed with glucose for 48 hr. The loss of pyruvate kinase activity resulting from both hypophysectomy and diabetes is 42% and 61%, respectively, for each group of animals relative to their respective controls.

Previous studies in this laboratory with adipose lactic dehydrogenase (LDH) isozymes have demonstrated that an apparent loss in LDH enzyme levels due to fasting and diabetes could occur only if protein synthesis was permitted (11). In order to examine whether this condition is more general than with LDH isozymes alone we studied the effect of the protein synthesis inhibitor, cycloheximide, on adipose pyruvate kinase. The results of this study are presented in Table II. Cycloheximide alone is seen to have no effect on the normal fed animal. However, in fasting and diabetes the expected loss of pyruvate kinase is effectively prevented by this protein inhibitor.

Since we had noted in our previous study with adipose LDH isozymes (11) that the effects of fasting and hypophysectomy were modified when phosphate ion is injected into the rat, we examined this phosphate effect with fasted, diabetic, and hypophysectomized rats with respect to adipose pyruvate kinase. The diabetic animals were susceptible to phosphate injection and died or appeared moribund before the study concluded. Consequently only the data for fasted and hy-

TABLE I. THE EFFECTS OF FASTING, DIABETES, AND HYPOPHYSECTOMY ON ADIPOSE PYRUVATE KINASE.

Exp. No.	Treatment	No. animals	Total pyruvate kinase[a]	P Value
1	(i) Normal fed control	6	4.9 ± 0.4[b]	
	(ii) Fasted—72 hr	10	2.2 ± 0.3	<0.02 (ii vs i)
	(iii) Hypophysectomized	4	2.8 ± 0.2	<0.02 (iii vs i)
2	(i) Normal fed control	4	6.3 ± 0.7	
	(ii) Fasted—96 hr	4	2.5 ± 0.2	<0.01 (ii vs i)
	(iii) Fasted—glucose-refed[c]	6	4.3 ± 0.5	<0.05 (iii vs ii)
3	(i) Streptozotocin control	7	10.2 ± 0.7	
	(ii) Streptozotocin diabetic	10	4.0 ± 0.5	<0.05 (ii vs i)

[a] μMoles NADH/min/mg DNA.
[b] Average ± SE.
[c] Fasted 60 hr, refed glucose 48 hr.

TABLE II. THE EFFECTS OF CYCLOHEXIMIDE ON ADIPOSE PYRUVATE KINASE LEVELS.

Exp. No.	Treatment	No. animals	Total pyruvate kinase[a]	P Value
1	Normal fed + ethanol—48 hr[b]	4	6.3 ± 0.7[c]	
	Normal fed + cycloheximide[d]—48 hr	4	5.8 ± 0.7	N.S.
2	Fasted + ethanol—72 hr[b]	7	3.4 ± 0.6	
	Fasted + cycloheximide—72 hr[d]	7	8.1 ± 0.5	<0.01
3	Streptozotocin diabetic + ethanol—96 hr[b]	5	5.9 ± 0.3	
	Streptozotocin diabetic + cycloheximide—96 hr[d]	5	9.3 ± 0.4	<0.01

[a] μmole NADH/min/mg DNA.
[b] Controls injected with ethanol, the carrier for cycloheximide.
[c] Average ± SE.
[d] Normal-fed and fasted rats received 40 μg of cycloheximide per injection and streptozotocin diabetic rats received 25 μg (19).

pophysectomized animals are presented in Table III. Surprisingly phosphate ion alone elevates the total adipose pyruvate kinase levels 76% above fed control rats. Additionally, phosphate-treated fasted and hypophysectomized rats maintained normal kinase levels despite the fact that their uninjected controls showed markedly lower adipose pyruvate kinase levels.

As a comparative measure, some of the animals from the experiments of Table III were assayed for pyruvate kinase activities as a function of protein content in the supernatant extracts. Table IV contains the data from that study. Although significant differences are observed for phosphate effects, it is seen that the differences between the experimental pairs is markedly reduced. A direct comparison between the animals of experiments 1 and 2 of Table IV, i.e., normal-fed vs fasted is also of interest. It may be seen that using cell protein as a base to express enzyme units the differences between the two groups is reduced to 27%. A statistical evaluation of this difference indicates that it is not significant ($P > 0.05$).

Discussion. The results of studies conducted by Orevi *et al.* (9) and Pogson and Denton (8) would suggest that diabetes and fasting, conditions which produce pronounced alterations in liver pyruvate kinase levels, are without effect on adipose pyruvate kinase. The Orevi group proposed, as a consequence of their study, that regulatory adjustments in adipose tissue glycolytic pathways are effected through mechanisms other than those observed in liver tissue.

Our observations are not consistent with an unresponsive adipose pyruvate kinase. Rat adipose pyruvate kinase levels, in our hands, appear responsive to fasting, diabetes, and hypophysectomy in that under these metabolic stresses enzyme levels are reduced to approximately 50% of those of control animals. We believe that a partial reason for the discrepancy between our data and that of earlier workers may be due to the fact these workers expressed adipose enzymes levels as

TABLE III. THE EFFECTS OF PHOSPHATE INJECTION ON ADIPOSE PYRUVATE KINASE LEVELS.

Exp. no.	Treatment	No. animals	Total pyruvate kinase[a]	P Value
1	Normal fed + saline—72 hr[b]	6	4.9 ± 0.4[c]	
	Normal fed + phosphate—72 hr	6	8.6 ± 0.7	<0.02
2	Fasted + saline—72 hr	10	2.2 ± 0.3	
	Fasted + phosphate—72 hr	10	5.1 ± 0.5	<0.02
3	Hypophysectomized + saline—48 hr[b]	4	2.8 ± 0.2	
	Hypophysectomized + phosphate—48 hr	7	7.5 ± 0.7	<0.02

[a] μmoles NADH/min/mg DNA.
[b] Controls injected with 0.154 *M* NaCl.
[c] Average ± SE.

TABLE IV. THE EFFECTS OF PHOSPHATE INJECTION ON ADIPOSE PYRUVATE KINASE LEVELS BASED ON CELL PROTEIN.

Exp. no.	Treatment	No. animals	Total pyruvate kinase[a]	
1	Normal fed + saline—72 hr[b]	5	0.30 ± 0.05	$P < 0.03$
	Normal fed + phosphate—72 hr	6	0.44 ± 0.03	
2	Fasted + saline—72 hr[b]	4	0.22 ± 0.02	$P < 0.03$
	Fasted + phosphate—72 hr	4	0.32 ± 0.03	
3	Hypophysectomized + saline—48 hr[b]	4	0.33 ± 0.04	$P < 0.01$
	Hypophysectomized + phosphate—48 hr	4	0.55 ± 0.01	

[a] μmoles NADH/min/mg supernatant protein.
[b] Controls injected with 0.154 *M* NaCl.
[c] Average ± SE.

units of enzyme/unit of protein in the adipose tissue extract. Mersman and Brown (10) have shown recently that adipose tissue protein is an unreliable measure of cell number for this tissue since this parameter varies considerably with age and physiological state. These authors feel that actual cell counts or DNA content are the most appropriate measures of cell number against which to measure adipose cell enzyme content. Our studies suggest that differences observed with DNA as the cell reference are markedly reduced when protein is substituted as the standard cell reference.

The decrease in pyruvate kinase as a response to fasting and the diabetic state may be due to two mechanisms. It is possible that the rate of synthesis of pyruvate kinase is decreased, or alternatively that its rate of degradation is enhanced. Which of these mechanisms is actually involved is not available from the current data. The cycloheximide study, however, clearly implicates the synthesis of protein in the disappearance of pyruvate kinase under these conditions. That protein might be a repressor molecule which inhibits protein synthesis or a degradative factor such as a protease. Similar data has been observed with tyrosine transaminase in rat liver (13). With the latter system, the more recent evidence (13, 15, 16) discounts the repressor theory and suggests that enhanced proteolytic activity is responsible. The determination of whether enhanced proteolytic degradation of pyruvate kinase accounts for decreased levels of the adipose enzyme in fasting and diabetes awaits further study.

The interesting effects of phosphate ion in stimulating pyruvate kinase levels is similar to that observed with lactic dehydrogenase in previous studies (11) with the exception that phosphate ion elevates pyruvate kinase levels even in the normal fed animals. Berglund *et al.* (17) have observed that pyruvate kinase may be inactivated by protein kinases and reactivated by phosphatases in *in vitro* systems. These workers suggest that this action may reflect a physiological control of the enzyme by cAMP. Possibly such an activation-inactivation mechanism is operative in these studies with phosphate. Specifically how phosphate ion would induce these effects is not presently apparent. The phosphate ef-

fects, however, add additional support to the thesis that adipose pyruvate kinase is quite responsive to nutritional and hormonal environment.

Summary. Pyruvate kinase in rat adipose tissue, unlike liver pyruvate kinase, has been reported to be unresponsive to fasting and the diabetic state. Levels of this enzyme in liver tissue are diminished as a response to these conditions. We have reexamined the responsiveness of adipose pyruvate kinase using DNA as a measure of cell number rather than protein content as previously used. Under these conditions we observed a significant reduction in adipose cell pyruvate kinase levels in fasting, diabetes, and hypophysectomy. The protein synthesis inhibitor, cycloheximide, given concurrently with the fasting regimen or the induction of diabetes prevents the loss of pyruvate kinase which otherwise would be observed. Presumably protein, possibly a protease, must be synthesized to permit a decrease in pyruvate kinase under fasting and diabetic conditions. Phosphate ion is demonstrated to inhibit the reduction in pyruvate kinase levels observed under some of these metabolic conditions.

1. Strandholm, J. J., Dyson, R. D., and Cardenas, J. M., Arch. Biochem. Biophys. **173,** 125 (1976).
2. Tanaka, T., Harano, Y., Sue, F., and Morimua, H., J. Biochem. (Tokyo) **62,** 71 (1967).
3. Pogson, C. I., Biochem. Biophys. Res. Commun. **30,** 297 (1968).
4. Pogson, C. I., Biochem. J. **110,** 67 (1968).
5. Imemura, K. and Tanaka, T., J. Biochem. **71,** 1043 (1971).
6. Marco, R., Carbonell, J., and Llorente, P., Biochem. Biophys. Res. Comm. **43,** 126 (1971).
7. Washko, M. E., and Rice, E. W., Clin. Chem. **7,** 542 (1961).
8. Pogson, C. I., and Denton, R. M., Nature (London) **216,** 156 (1967).
9. Orevi, M., Gorin, E., and Shafrir, E., Eur. J. Biochem. **30,** 418 (1972).
10. Mersman, H. J., and Brown, L. J., Int. J. Biochem. **4,** 503 (1973).
11. Hern, E. P., Schultz, J., O'Dorisio, M. S., Moore, R. O., and Serif, G. S., Arch. Biochem. Biophys. **169,** 331 (1975).
12. Schneider, W. C., *in* Methods in Enzymology (S. Colowick, and N. Kaplan, eds.), Vol. 3, pp 680–684, Academic Press, New York (1957).
13. Garren, L. D., Howell, R. R., Tompkins, G. M., and Croceo, R. M., Proc. Nat. Acad. Sci. U.S.A. **52,** 1121 (1964).
14. Reel, J. R., and Kenney, F. T., Proc. Nat. Acad. Sci. U.S.A. **61,** 200 (1968).
15. Grossman, A., and Charalampos, M., J. Biol. Chem. **242,** 1398 (1967).
16. Levitan, I. B., and Webb, T. E., J. Mol. Biol. **48,** 339 (1970).
17. Berglund, L., Ljungstrom, O., and Engstrom, L., J. Biol. Chem. **252,** 613 (1977).
18. Layne, E., *in* Methods in Enzymology (S. P. Colowick and N. O. Kaplan, eds.) Vol. 3, pp 447–454, Academic Press, New York (1957).
19. Singhal, R. L., and Lafreniere, R., Endocrinology **87,** 1099 (1970).

Received November 2, 1977. P.S.E.B.M. 1978, Vol. 158.