

UNUSUALLY HIGH MITOCHONDRIAL ALPHA GLYCEROPHOSPHATE DEHYDROGENASE ACTIVITY IN RAT BROWN ADIPOSE TISSUE

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ABSTRACT

Brown adipose tissue of the rat has been found to have an unusually high activity of mitochondrial α -glycerophosphate dehydrogenase (α -GPD) when assayed both by a histochemical staining procedure and by a quantitative biochemical method with isolated mitochondria. In contrast to succinic, glutamic, and β -hydroxybutyrate dehydrogenases, all mitochondrial enzymes, the activity of α -GPD in brown fat was 10 times that in liver, more than 20 times that in white adipose tissue, and 9 times that in kidney. The soluble NAD-linked α -GPD was also higher in brown adipose tissue than in white adipose tissue, liver, or kidney, but the differences were much less marked. The possible importance of the high activity of mitochondrial α -GPD in the regulation of synthesis of esterified lipid and in thermogenesis in brown fat is discussed.

INTRODUCTION

Histochemical observations of the distribution of mitochondrial non-NAD-linked α -glycerophosphate dehydrogenase activity in various rat organs disclosed an unusually intense staining of adipose tissue at the hilus of the kidney (1). Analysis of this observation revealed that the heavy staining was localized in brown adipose tissue near the kidney and in various sites elsewhere in the body, and that white adipose tissue showed little or no positive staining for this oxidative enzyme. Since α -glycerophosphate is known to be a precursor of glycerophosphatides and triglycerides (2-9), variations in the oxidative metabolism of this substrate by mitochondrial α -glycerophosphate dehydrogenase (GPD) could play an important regulatory role in lipid metabolism in the cells of adipose tissue.

The present study is concerned with the GPD

activity in a variety of tissues as observed both by staining and by quantitative assay. For comparison, the activities of soluble NAD-linked GPD and of several mitochondrial oxidative enzymes have been studied in several tissues. The unusual metabolic pattern of brown adipose tissue as compared to white adipose tissue, liver, and kidney is the subject of this paper.

MATERIALS AND METHODS

Male Wistar albino rats (Carworth Farms, New City, N.Y.), weighing between 300 and 380 g, were used for both histochemical and biochemical investigations. Sodium succinate, disodium DL- α -glycerophosphate hexahydrate, and sodium adenosine-5'-diphosphate (from equine muscle) (ADP) were purchased from Sigma Chemical Co., St. Louis, Mo., sodium glutamate and sodium β -hydroxybutyrate, from Nutritional Biochemicals Corporation, Cleveland, Ohio;

and nitro blue tetrazolium, from Calbiochem, Los Angeles, Calif.

Staining Procedures

Paraffin sections for histochemical observations were prepared by a previously described technique (10). The incubation media for non-NAD-linked GPD, succinic dehydrogenase (SD), β -hydroxybutyric dehydrogenase (HBD), and glutamic dehydrogenase (GD) were standard (11).

Biochemical Procedures

PREPARATION OF MITOCHONDRIA: All operations were performed and all solutions were used at 4°C. 1 g of liver or kidney was homogenized in 10 ml of 0.3 M sucrose in a glass-Teflon homogenizer. Nuclei, whole cells, and cell debris were removed by centrifugation at 2600 *g* for 10 min. Mitochondria were obtained from the supernatant by centrifugation at 7500 *g* for 15 min. The resulting pellet was washed once with 0.3 M sucrose. The first postmitochondrial supernatant was used for assay of soluble NAD-linked GPD.

In the case of the adipose tissues, 2 g of brown fat or 15 g of white fat were homogenized in 0.3 M sucrose solution. 10 ml of sucrose were used in the case of brown adipose tissue. In the case of white adipose tissue the 15 g aliquot was divided into three equal portions, and each portion was homogenized in 30 ml of 0.3 M sucrose solution. After an initial centrifugation at 2600 *g* for 10 min, the bulk of the fat was found floating on the top, while the nuclei, whole cells, and debris were sedimented. The slightly turbid middle layer, aspirated by means of a needle, was centrifuged at 7500 *g* for 15 min to remove mitochondria which were prepared as described above for liver and kidney.

For enzyme assays, mitochondrial pellets from each of the tissues were suspended in 2 ml of 0.3 M sucrose containing 0.2 ml of bovine serum albumin (10 mg/ml). All the serum albumin used in this study was extracted with cold methanol and purified to remove fatty acids (12). The final fatty acid concentration was less than 5 μ moles/g albumin.

The postmitochondrial supernatants were centrifuged at 105,000 *g* for 60 min, and the resulting supernatant was used for assay of soluble NAD-linked GPD.

In some experiments, mitochondria of brown adipose tissue were prepared as follows: 2 g of brown adipose tissue were homogenized in 10 ml of cold 0.3 M sucrose containing bovine serum albumin (10 mg/ml). This 0.3 M sucrose containing albumin was used through the whole process, and the resulting mitochondrial pellet was suspended in 2 ml of the same solution for enzyme assays. The mitochondrial preparations were frequently examined for gross con-

tamination by phase-contrast microscopy and were periodically checked by electron microscopy. The preparations were consistently free of nuclear and whole cell contamination and were only minimally contaminated with rough endoplasmic reticulum.

ENZYME ASSAYS: Mitochondrial enzymes (non-NAD-linked GPD, succinic dehydrogenase, glutamic dehydrogenase, and β -hydroxybutyric dehydrogenase) were assayed by measuring oxygen consumption polarographically with a Gilson Medical Electronics (Middleton, Wisc.) oxygraph. The rates of mitochondrial respiration in the presence of substrates and adenosine diphosphate (ADP), i.e. state 3 respiration rate, were used as a measure of the dehydrogenase activities. State 3 respiration was initiated by addition of 2 μ l of ADP containing 250–300 μ moles. The total volume was 1.8 ml, and the temperature was 25°C.

For GPD, the incubation medium contained 25 mM K_2PO_4 (pH 7.6), 5 mM $MgCl_2$, 0.2 M Na-EDTA (ethylenediaminetetraacetate), 62.5 mM sucrose, 183.5 mM mannitol, and 50 mM Na α -glycerophosphate. The final medium contained 0.1–0.4 ml of mitochondrial suspension. For SD, GD, and HBD, the media were prepared as described by Hagihara (13) and contained 62.5 mM sucrose, 183.5 mM mannitol, 10 mM KCl, 10 mM Tris-HCl (pH 7.2), 5 mM K_2PO_4 buffer (pH 7.2), 5 mM $MgCl_2$, and 0.2 mM Na-EDTA. The substrates were as follows: 10 mM Na-succinate, pH 7.2; 10 mM Na-glutamate, pH 7.2; or 10 mM Na- β -hydroxybutyrate, pH 7.2.

Soluble α -glycerophosphate dehydrogenase was assayed as described by Beisenherz et al. (14) with a Carey 15 recording spectrophotometer. The assay mixture contained 50 mM Tris-HCl (pH 7.5), 0.4 mM dihydroxyacetone phosphate, 0.3 mg β -NADH₂, and varying volumes of the microsomal supernatant in a total volume of 3.0 ml. One unit of enzyme activity was defined as 100 divided by the number of seconds required for an optical density change of 0.10 at 340 μ . Protein determinations were made on the mitochondrial suspensions or on the soluble fraction by the use of a modified biuret method (15).

RESULTS

Histochemical Observations

On staining for non-NAD-linked GPD, the brown adipose tissue shows rapid, intense staining in contrast to white adipose tissue. For example, obvious staining is regularly observed in brown adipose tissue after 5–10 min of incubation (Figs. 1 and 2) while essentially no staining is obtained in white adipose tissue even after 80–120 min of incubation. The kidney shows moderate staining of portions of the proximal and distal

convoluted tubule and of segments of the collecting ducts after incubation periods of 40–50 min (Fig. 3). The liver showed only minimal staining. The staining in all positive cells was cytoplasmic. In brown adipose tissue, cells with multilocular lipid inclusions showed more intensive staining than did those cells with unilocular inclusions in the same sections. However, the latter cells showed much more rapid and intensive staining than did the unilocular cells in white adipose tissue. This would suggest that the presence or absence of staining in adipose tissue is not merely a result of whether the lipid in the cell exists as a single or as a multiple inclusion.

The intensive staining which brown adipose tissue shows for mitochondrial GPD is not seen for some other mitochondrial oxidative enzymes. Although brown adipose tissue shows more rapid staining for SD, GD, and HBD than does white adipose tissue, the staining activities are far less

than for GPD (Figs. 4 and 5). Thus, it would appear that the intense reaction for GPD is not a general phenomenon of the oxidative enzymes of mitochondria of the cells of brown adipose tissue but is rather specific for this enzyme. Unpublished experiments with NADH₂-tetrazolium reductase indicate that this enzyme is, likewise, not especially active in brown adipose tissue.

Biochemical Studies

The histochemical observations on the activity of GPD in brown adipose tissue are confirmed by the quantitative biochemical data recorded in Table I. The mitochondria from brown adipose tissue have more than 10 times the non-NAD-linked α -glycerophosphate dehydrogenase activity observed in liver, more than 20 times that of white adipose tissue, and about 9 times that of kidney mitochondria. Although the GPD activity

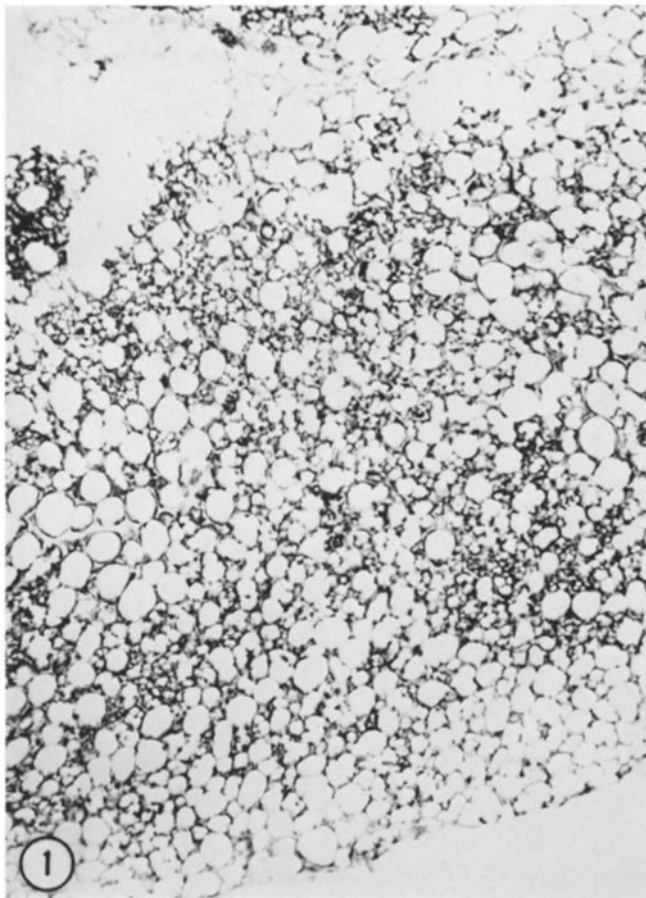


FIGURE 1 Brown adipose tissue from the interscapular region. Non-NAD-linked GPD activity. Incubation for 15 min. The multilocular cells show a stronger staining than the unilocular cells. The staining is cytoplasmic. $\times 120$.

of the kidney mitochondria is the mean activity of highly active and poorly active mitochondrial populations from different segments of the nephron, it is probable that few if any portions of the nephron would show a GPD activity that approaches that seen in brown adipose tissue.

The soluble NAD-linked α -glycerophosphate dehydrogenase activity is also higher in brown adipose tissue than in liver, kidney, or white adipose tissue (Table II). However, the difference between brown adipose tissue and the other tissues is considerably less than in the case of mitochondrial GPD. The soluble NAD-linked α -GPD activity in both liver and white adipose tissue is about 50% of that in brown adipose tissue; activity of this enzyme in whole kidney is approximately 10% of the level found in brown adipose tissue.

The activities of three mitochondrial enzymes other than GPD in brown and white adipose tissues and liver and kidney are also recorded in Table I.

The SD activity is considerably higher in kidney than in liver and brown and white adipose tissues. The SD activities in brown and white adipose tissues are roughly the same. In the case of GD and HBD, the liver and kidney have similar levels of enzyme activity, whereas both brown and white adipose tissues have low levels of enzyme activity.

In Table I are also recorded the effects of ADP addition upon the oxygen consumption of mitochondria in the presence of the four substrates. The mitochondria from both liver and kidney showed the expected increase in respiratory rate upon addition of ADP (state 3). In contrast, mitochondria from each of the types of adipose tissue showed no significant increase in respiratory rate in the presence of ADP. However, oxidative phosphorylation as measured directly with hexokinase and glucose was observed with all four substrates, in agreement with several recent reports on brown fat (16-20), although it was not observed in previous studies (21-23).

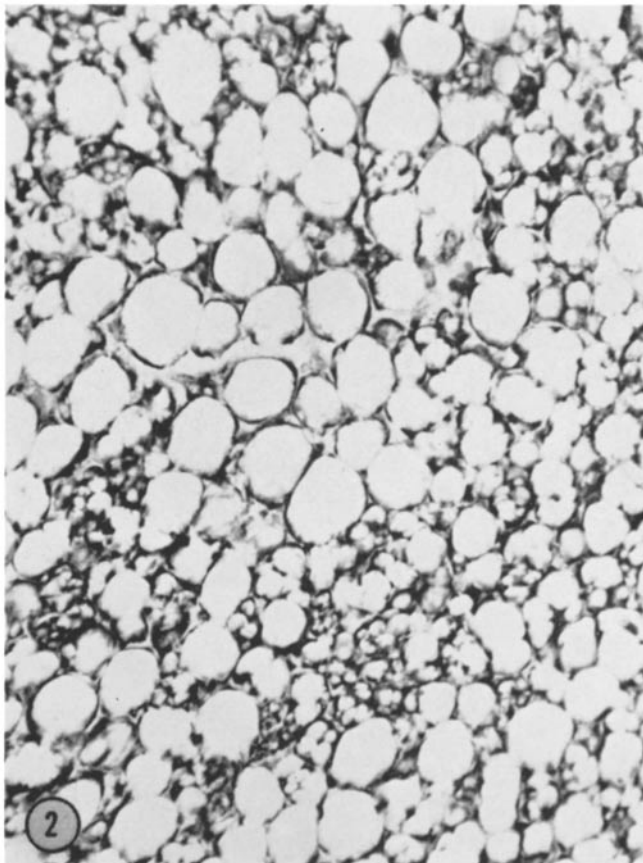


FIGURE 2 Brown adipose tissue from the interscapular region. Non-NAD-linked GPD activity. Incubation for 15 min. The staining is stronger in the multilocular cells than in the unilocular cells. $\times 240$.

DISCUSSION

It is evident from the results of the present study that the cells of brown adipose tissue, in contrast to those of white adipose tissue, liver, or kidney, have an unusually high activity of mitochondrial non-NAD-linked α -GPD. Chaffee et al. (24) and Kornacker and Ball (25) also reported a relatively high rate of α -glycerophosphate oxidation by mitochondria or homogenates of brown adipose tissue but did not include a comparison between the activities of mitochondrial α -GPD of different tissues.

The possible role of mitochondrial α -GPD as an important regulatory factor in the control of lipid synthesis and metabolism is worthy of consideration. Since α -glycerophosphate is an important substrate for the synthesis of both glycerophosphatides and triglycerides (2-9), and since its

availability may be a regulatory component in the synthesis of triglycerides (26), an enzyme that can oxidatively change glycerophosphate could be in a key position to have a major influence upon lipid metabolism in the multilocular cells of brown adipose tissue. In mitochondria, α -glycerophosphate is oxidized to dihydroxyacetone phosphate by non-NAD-linked α -GPD as follows: Glycerol-1-phosphate + $\frac{1}{2}$ O₂ \rightarrow dihydroxyacetone phosphate + H₂O. Therefore, it is reasonable to think that the higher the non-NAD-linked α -GPD activity, the more α -glycerophosphate will be converted to dihydroxyacetone phosphate; the availability of α -glycerophosphate for the esterification of fatty acids will decrease, resulting in a decrease of triglyceride synthesis in brown adipose tissue (27). Thus, non-NAD-linked α -GPD of mitochondria could play a role in controlling the triglyceride synthesis by regulating the availability

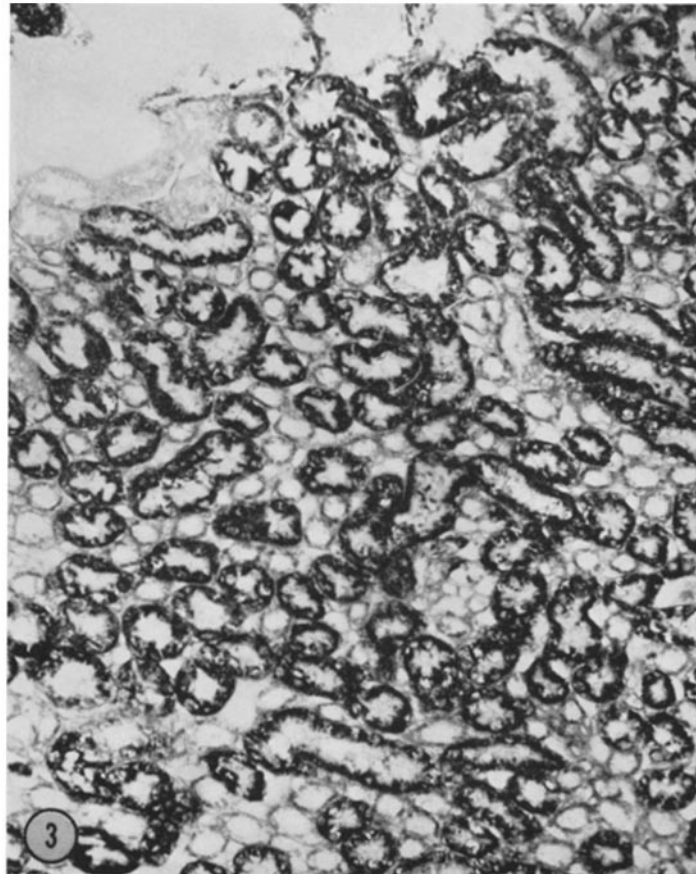


FIGURE 3 Kidney. Non-NAD-linked GDP activity. A strong staining is seen in the epithelial cells lining the proximal convoluted tubules. Nuclei do not show any staining. Incubation for 40 min. $\times 120$.

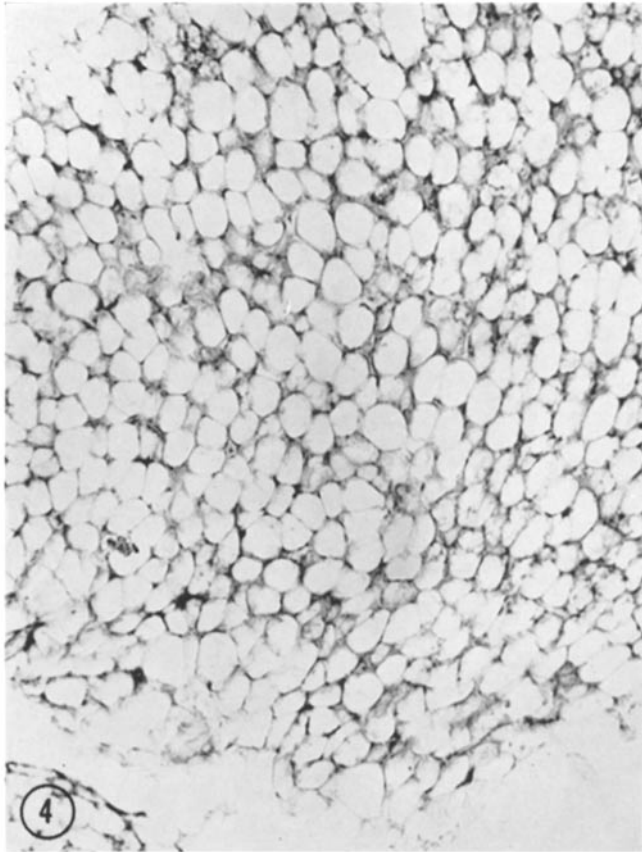


FIGURE 4 Brown adipose tissue from the interscapular region. Succinic dehydrogenase activity. Incubation for 80 min. A moderate staining is seen. $\times 120$.

of α -glycerophosphate for fatty acid esterification in adipose tissues. The close apposition of lipid droplets to mitochondria, observed in brown fat cells by Napolitano and Fawcett (28), could conceivably be a morphologic expression of a close link between mitochondria and triglyceride metabolism.

The brown adipose tissue cells have also a higher activity of soluble α -GPD than the white adipose tissue cells. This finding suggests that the formation of α -glycerophosphate may be greater in brown adipose tissue cells than in white adipose tissue cells. In brown adipose tissue cells, however, with high activity of non-NAD-linked GPD, more of the α -glycerophosphate might be metabolized inside the mitochondria, resulting in limited biosynthesis of triglycerides. In white adipose tissue cells the reverse would be expected. The total amount of α -glycerophosphate formed extramitochondrially would be considerably lower in white adipose tissues, but almost none of this will be taken up into the mitochondria. Therefore,

white adipose tissue may well have relatively larger amounts of α -glycerophosphate available for extramitochondria triglyceride synthesis. The difference between the two types of adipose tissue is much greater with respect to the activity of non-NAD-linked α -GPD than with respect to the activity of soluble α -GPD. Therefore, the major role of regulation of triglyceride synthesis in adipose tissues might reside in the mitochondrial non-NAD-linked α -GPD activity. Parenthetically, it should be mentioned that mitochondrial α -GPD from brown fat, in contrast to that from liver (29), failed to show any response to induced hypo- or hyperthyroid states in repeated experiments.

The storage of lipid substances has long been regarded as the most important physiological role of the adipose tissues. For the brown adipose tissue, however, there are many disputes about its physiological role. The heat production prior to the arousal from hibernation has been suggested as an important physiological role of brown adipose tissue in hibernators (30). The physiological roles

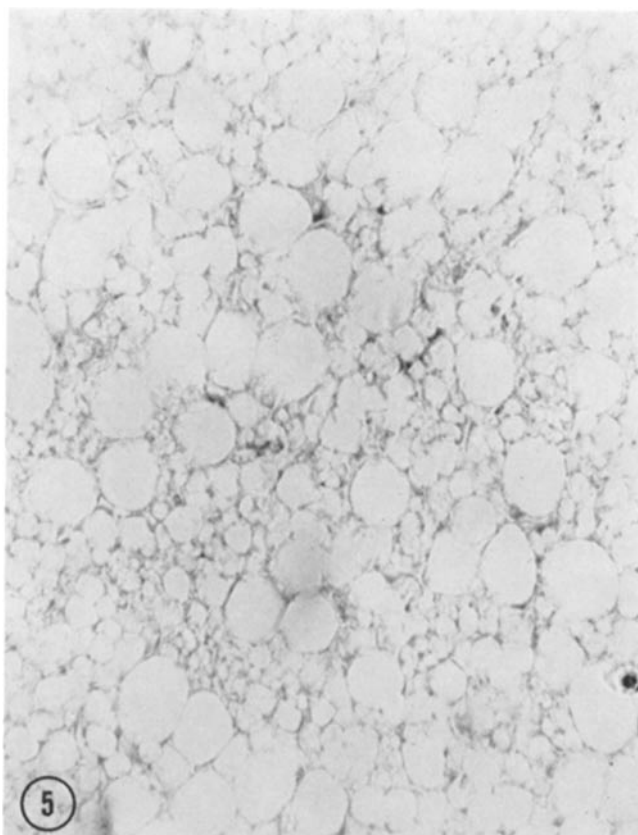


FIGURE 5 Brown adipose tissue from the interscapular region. Glutamic dehydrogenase activity. Incubation for 80 min. A slight staining is seen. $\times 240$.

TABLE I
Activities of Mitochondrial α -Glycerophosphate, Succinic, Glutamic, and β -Hydroxybutyric Dehydrogenases in Various Tissues

Tissue	Dehydrogenase							
	α -Glycerophosphate		Succinic		Glutamic		β -Hydroxybutyric	
	State 4*	State 3*	State 4	State 3	State 4	State 3	State 4	State 3
Brown adipose tissue (4)†	188 \pm 3§	176 \pm 2	56 \pm 10	62 \pm 13	8 \pm 1	8 \pm 1	7 \pm 2	7 \pm 2
White adipose tissue (4)	8 \pm 1	9 \pm 0.2	48 \pm 7	53 \pm 7	5 \pm 1	5 \pm 2	7 \pm 2	7 \pm 2
Liver (4)	7 \pm 0.2	16 \pm 0.2	39 \pm 3	153 \pm 15	16 \pm 2	68 \pm 5	14 \pm 0.2	67 \pm 4
Kidney (4)	20 \pm 3	33 \pm 0.4	89 \pm 8	288 \pm 15	31 \pm 0.5	57 \pm 4	26 \pm 6	50 \pm 7

* Respiration is expressed as μ atoms oxygen uptake/min/mg protein in the absence (state 4) and in the presence (state 3) of ADP.

† Number of experiments.

§ Mean \pm standard error of the mean.

TABLE II
The Activity of Soluble NAD-linked α -Glycerophosphate Dehydrogenase in Various Tissues

Source of enzyme	Units/mg protein*
Brown adipose tissue (6)†	93.5 \pm 3.0§
White adipose tissue (7)	45.5 \pm 6.7
Liver (6)	40.7 \pm 4.0
Kidney (6)	11.2 \pm 0.6

* Definition of unit: the time for Δ optical density (OD) of 0.1. Reaction is allowed to proceed for 30 sec before timing, e.g. Unit = 100/(Seconds needed for Δ OD of 0.1).

† Number of experiments.

§ Mean \pm standard error of the mean.

of brown adipose tissue in nonhibernating animals are not yet well understood. Recently, Smith and coworkers (21) have reported the nonphosphorylating oxidation in the mitochondria of brown adipose tissue of cold-acclimated rats and suggested the physiological significance of the nonphosphorylating oxidation in thermogenesis of brown adipose tissue. However, several authors have reported recently that brown fat mitochondria may be normally coupled and perform oxidative phosphorylation (16-20).

In a critical analysis of thermogenesis in brown fat, Prusiner et al. (31) suggested that major control of heat production in brown fat might be greatly dependent upon variation in rates of oxidation of fatty acids rather than on a stimulated ATPase activity. It is conceivable that one factor in the regulation of the supply of fatty acids for thermogenesis may reside in the availability of α -glycerophosphate, either from glycolysis or as a result of the relatively high activity of glycerokinase (32). Thus, the concentration of glycerophosphate generated either from glycolytic activity or from glycerol released during lipolysis may be subject to control by the high mitochondrial α -GPD. Even though the mitochondrial

oxidation of glycerophosphate might generate some heat through hydrolysis of the ATP so formed, the amount of heat would be far less than that available through the oxidation of the fatty acids remaining unesterified (31). It is perhaps noteworthy that high mitochondrial α -GPD activities appear to be associated with at least two physiological conditions, thermogenesis in brown fat and hyperthyroidism (20), both of which are associated with a high rate of heat production.

A third possible role of α -GPD in the regulation of cell metabolism is concerned with the electron shuttle suggested by Estabrook and Sacktor (33) to permit the rapid mitochondrial oxidation of NADH generated in the cytoplasm. This phenomenon has been discussed in detail by Chaffee et al. (24) and by Kornacker and Ball (25), as well as by Dryer and Paulsrud (34). Hopefully, further studies on brown fat may clarify it.

Thus, on theoretical grounds, the unusually high mitochondrial α -GPD could play three roles in the over-all metabolic control of brown adipose tissue, depending upon the physiological conditions at the time: (a) regulation of the synthesis of esterified lipid, (b) participation in thermogenesis of brown fat during either arousal from hibernation or cold adaptation, and (c) controlling the level of glycolysis through participation in an electron shuttle for NADH.

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