A Microassay for Mitochondrial α-Glycerophosphate Dehydrogenase[1] (36785)

R. S. GARDNER[2] AND D. M. KIPNIS

Metabolism Division, Washington University, School of Medicine, St. Louis, Missouri 63110

A number of semimicroassays for mitochondrial α-glycerophosphate dehydrogenase (α-GPD) have previously been described (1–3). In attempting to adapt one of these procedures for use with micro-quantities of tissue, it was found that none was entirely satisfactory. The colorimetric assays (1, 2) make use of the dye, phenazine methosulfate (PMS), as an intermediate electron acceptor between the dehydrogenase and the tetrazolium dye, 2-*p*-iodo-3-*p*-nitro-5-phenyl tetrazolium chloride (INT). However, PMS has the property of being reduced on exposure to light and temperatures above 4° (4), and the reduced PMS will then transfer electrons to INT forming iodoformazan, the same colored product measured in the dehydrogenase reaction (4). Since this nonenzymatic reaction results in unacceptably high blank values, the present study was undertaken to study the possible use of INT as a direct electron acceptor for mitochondrial α-GPD. In this regard, INT has been shown to accept electrons directly from succinic dehydrogenase (4), and both this enzyme and GPD appear to be similarly linked to the mitochondrial electron transport system (3).

Methods. Buffalo male rats (180–200 g) obtained from the Department of Biochemistry, Howard University School of Medicine, Washington, DC, were made hyperthyroid to increase hepatic mitochondrial α-GPD activity (1) by feeding the animals 2% dessicated thyroid extract mixed with Purina lab chow. After 2 wk on this diet, the animals were sacrificed by decapitation. Rat liver mitochondrial acetone powder was prepared and stored as the dried powder at −20° (5) without significant loss of activity over 1 yr. For each experiment, a 4% (w/v) homogenate was prepared in 0.03 *M* potassium phosphate buffer (pH 8.0) (5), and sonicated for 20–30 sec at 4° in a Bronwill Biosonik II sonicator in order to establish a more uniform particulate suspension. Sonication did not alter enzyme activity. Particulate enzyme was solubilized by treatment with the phospholipase A present in cobra venom. After ultracentrifuging the clarified suspension for 1 hr at 100,000*g*, the supernatant was used as the source of solubilized mitochondrial enzyme and corresponded to the Step 3 preparation described by Ringler (5).

The assay for α-GPD is based on the ability of INT to accept directly electrons from the dehydrogenase with reduction of the INT to iodoformazan. After extraction into ethyl acetate, the color developed is read at 500 mμ using a molar extinction coefficient of $1.85 \times 10^4\ M^{-1}\ cm^{-1}$ (1). The enzyme was assayed in tubes containing 0.01 ml of 0.5 *M* α-glycerophosphate (Sigma), 0.05 ml of 0.4% INT (Sigma), and 0.01 ml of 0.4 *M* potassium phosphate buffer, (pH 8.0) containing 0.01 *M* KCN, and mitochondria and/or 0.03 *M* phosphate buffer to bring the reaction volume to 0.1 ml. When PMS was used, 0.01 ml of 1% PMS was added last to the reaction mixture. After incubating at 37° for 30 min, the reaction was stopped by the addition of 0.4 ml of 1 *M* acetic acid. The iodoformazan was extracted into 1 ml of ethyl acetate by agitating with a Vortex Genie for 30 to 45 sec and then centrifuging at low speed for 2 min to separate the organic and aqueous phases. The organic phase was aspirated and its color was read at 500 mμ in a Hitachi Perkin-Elmer spectrophotometer

[1] Supported in part by U.S. Public Health Service Grant AM 1921 and RR-36.

[2] Trainee in Metabolism-U.S. Public Health Service AMO-5027.

TABLE I. Assay of α-Glycerophosphate Dehydrogenase Using PMS and INT as Direct Electron Acceptors.

Mitochondrial preparation	(Iodoformazan μg/30 min)					
	Complete System	—α-GP	Net α-GPD	—PMS	α-GP —PMS	Net α-GPD
Particulate enzyme (50 μg protein)	30.6	17.0	13.6	7.4	0.9	6.5
Soluble enzyme (100 μg protein)	33.7	19.2	14.5	8.3	1.3	7.0
None	17.0	17.0	—	0.5	0.5	—

using a 1 ml semimicrocell. The extraction procedure significantly reduced the blank reading. A control containing no substrate was run at each mitochondrial concentration used. Protein concentration was measured by the method of Lowry *et al.* (6).

Results. There is significant nonenzymatic formation of iodoformazan in the presence of PMS sufficient to constitute a blank of 50–60% in the assay of particulate α-GPD activity (Table I). Using INT as a direct electron acceptor, nonenzymatic reduction of INT is markedly reduced and represents a blank of $< 10\%$. Using this colorimetric assay however, PMS is a more efficient electron acceptor from a α-GPD than is INT. In a typical experiment using 50 μg of mitochondrial protein, the PMS coupled dehydrogenase activity was 13.6 μg iodoformazan generated in 30 min compared to an INT coupled activity of 6.5 μg indoformazan or a PMS/INT activity ratio of 2.1:1 (Table I). Thus, in the absence of PMS there is less dehydrogenase activity as measured in this assay, but the nonenzymatic conversion of INT to iodoformazan is markedly reduced and consequently the assay is more precise as well as more reproducible.

Mitochondrial α-GPD, solubilized as described in Methods, behaves like the particulate enzyme in respect to PMS and INT activity. The PMS/INT activity ratio on solubilization was 2.0, virtually unchanged from the particulate enzyme ratio. This suggests that both INT and PMS accept electrons directly from α-GPD rather than from another site in the electron transport chain. As shown in Fig. 1, the reaction is proportional to enzyme concentration over at least a

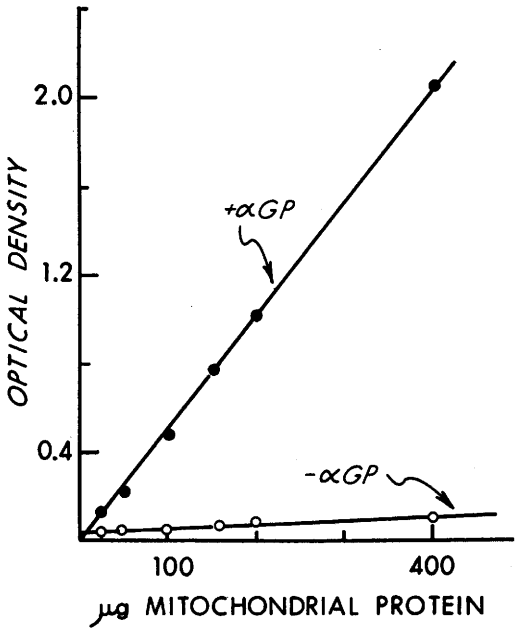

FIG. 1. Variation in α-glycerophosphate dehydrogenase activity with protein concentration.

20-fold concentration range. The kinetics of the dehydrogenase reaction are illustrated in Fig. 2. The reaction proceeds linearly for at least 30 min.

A Lineweaver-Burk plot (Fig. 3) was made to determine the K_m for INT. The value determined by this method was 1.9×10^{-3} M. In the enzyme assay, INT is used at twice this concentration with the limiting factor being the relative insolubility of INT in aqueous solutions. The K_m for α-GP in this assay was 1.1×10^{-2} M, a value in close agreement with that determined for the pig brain mitochondrial enzyme (5).

This assay should not be used with crude

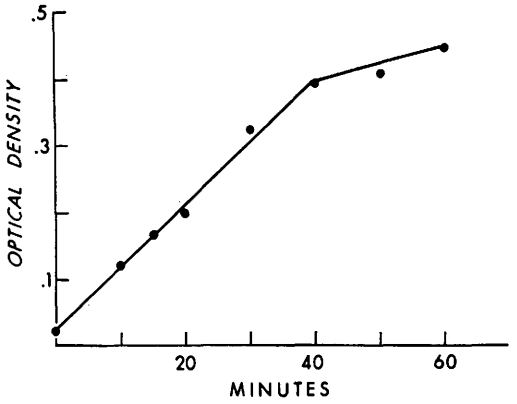

FIG. 2. Time course of the α-glycerophosphate dehydrogenase reaction.

cell extracts containing mitochondria because soluble α-GPD, which is NAD-dependent, would also be measured using the INT assay in the presence of even small amounts of NAD. Under these conditions, the NADH generated by the soluble α-GPD enzyme is reoxidized to NAD as evidenced by INT conversion to iodoformazan (Table II). This reduction in all probability is due to NADH

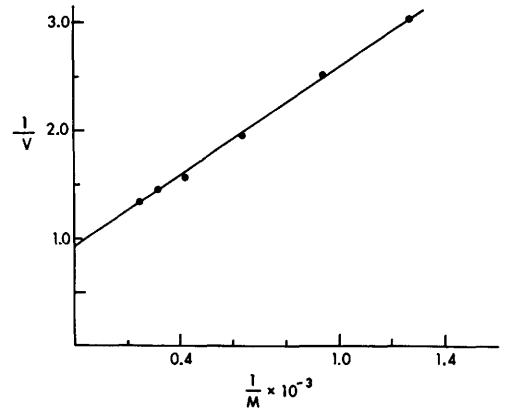

FIG. 3. Variation in α-glycerophosphate dehydrogenase activity with INT.

cytochrome *c* reductase, a mitochondrial enzyme which readily transfers electrons to tetrazolium compounds (7).

TABLE II. Oxidation of NADH by Mitochondria as Measured by Iodoformazan Formation.

Mitochondrial preparation (mg)	NADH (μmoles)	PMS (μg)	Iodoformazan formed (μmoles)
0.75	0.26	—	0.26
0.38	0.26	—	0.24
—	0.26	10	0.25

[a] Assay conditions as described in methods section but volume of assay increased to 0.3 ml and incubated for 45 min at 37°.

Summary. A simple reproducible assay for mitochondrial α-GPD has been described. It takes advantage of the ability of INT to accept directly electrons from the dehydrogenase. The assay as described measures accurately enzyme activity using 20 μg of mitochondrial protein, but it could be scaled down for smaller quantities of protein by using smaller reaction volumes and microcuvettes.

1. Lee, Y. P., and Lardy, H. A., J. Biol. Chem. **240**, 1427 (1965).
2. Fried, G. H., Greenberg, N., and Antopol, W., Proc. Soc. Exp. Biol. Med. **107**, 523 (1961).
3. Ringler, R. L., and Singer, T. P., J. Biol. Chem. **234**, 2211 (1959).
4. Nachlas, M. M., Margulies, S. I., and Seligman, A. M., J. Biol. Chem. **235**, 499 (1960).
5. Ringler, R. L., J. Biol. Chem. **236**, 1192 (1961).
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1961).
7. Brodie, A. F., and Gots, J. S., Science **116**, 588 (1962).

Received June 2, 1972. P.S.E.B.M., 1972, Vol. 141.