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Determination of Alpha-glycerophosphate Oxidation with Tetrazolium.* (26675)

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A variety of technics are presently employed for assay of alpha-glycerophosphate dehydrogenases, but none are entirely satisfactory in terms of precision or reproducibility. Growing emphasis on the significance of glycerophosphate oxidation in muscle metabolism(1), endocrine dysfunction(2), and the biochemical characteristics of tumors(3) indicates the need for a simple and reliable technic for assaying the enzyme systems involved in oxidation of glycerophosphate. INT (2-p-iodo-3-p-nitro-5-phenyl tetrazolium chloride), a monotetrazolium salt, has been used successfully for measuring a number of enzyme systems(4,5). The present communication describes and evaluates a simplified technic utilizing INT for determination of the activities of the soluble (DPN-dependent) and insoluble (cytochrome-dependent) alpha-glycerophosphate oxidase complex (AGPO) and the corresponding specific alpha-glycerophosphate dehydrogenases (AGPD) in homogenates of animal tissues.

Method. Excised tissues of V stock house mice (Bar Harbor) were placed in iced Ringer solution, and homogenates of appropriate strengths made up with deionized distilled water. Homogenization was carried out with motor-driven teflon pestles in glass tubes. Total AGPO was assayed in 10 ml test tubes containing 0.2 ml of 0.5M AGP (alpha-glycerophosphate, Sigma), 0.3 ml of 0.4% INT (Dajac), 0.2 ml of 1% DPN (Sigma), and 0.2 ml of 0.5% or 1.0% homogenate. M/15 phosphate buffer (pH 8.0) was added to bring the total volume to 2.0 ml. In assays for insoluble AGPO, DPN was omitted. Corresponding assays for total and insoluble AGPD were carried out by adding 0.2 ml of 10^{-4}M phenazine methosulfate (Sigma) and 0.2 ml of 10^{-3}M NaCN to reaction mixtures before bringing to a final volume of 2.0 ml. This step establishes direct electron transfer between the dehydrogenase and INT. Appropriate blanks containing no substrate were run in all experiments. After uniform agitation (by holding against a rotating cuboidal rubber stopper for 10 seconds) the tubes were placed in a water bath at 37°C for 15 minutes. The reaction was stopped with 0.2 ml of 30% TCA. Prolonged contact with the acid should be avoided, as a 5% loss of formazan was found after 24 hours exposure to the TCA. The iodoformazan (IF) produced during incubation was extracted with 5 ml ethyl acetate, shaken vigorously 50 times, centrifuged, and the decanted ethyl acetate extract read spectrophotometrically (Coleman Jr.) at 490 mμ against a calibration curve of chemically reduced (hydrosulfite) IF in ethyl acetate. Total nitrogen values in equivalent aliquots of homogenate were determined by the micro-Kjeldahl method and enzyme activities expressed as μg IF per μg tissue N. Values for DPN-dependent enzyme activity were obtained by subtracting the values for the in-

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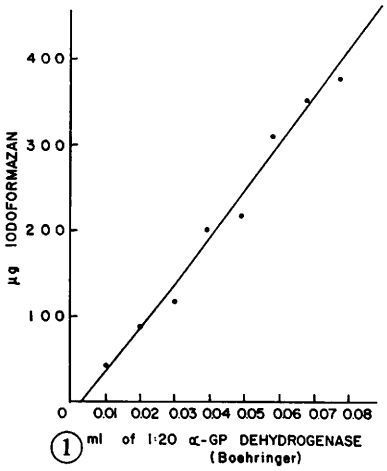

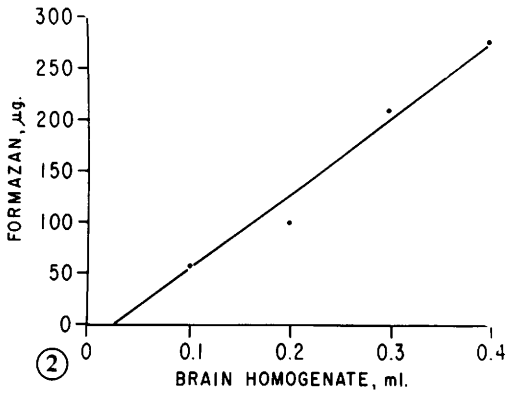

FIG. 1. Production of iodoformazan by varying amounts of crystalline alpha-glycerophosphate dehydrogenase.

FIG. 2. Increase in formazan production with increasing volumes of 5% rat brain homogenate.

soluble component from total activity levels.

Varying amounts of a commercial (Boehringer) crystalline preparation of AGPD (soluble) were assayed and linearity established between IF produced and amount of enzyme used (Fig. 1). Linearity of a similar order was also obtained with a series of varying levels of tissue homogenates (Fig. 2). The course of the reaction was linear up to 30 minutes, except at very low levels of enzyme activity. In several runs iodoacetate was added to a final concentration of 0.001M in order to block any phosphoglyceraldehyde dehydrogenase activity which might conceivably be contributing to the enzyme activities measured. Since no appreciable effect was observed with this inhibitor it can be assumed that the assay is specific for the enzymes involved in glycerophosphate oxidation only.

Results. In experiments with homogenates from male V stock house mice the order of decreasing activity of total (soluble and insoluble) AGPO was liver>kidney>brain>muscle (Table I). For total AGPD the order of activity was kidney>liver>muscle>brain. The insoluble AGPO and AGPD levels were negligible or low in all tissues except brain. In tissues other than brain the total AGPO and AGPD activities reflect the soluble enzyme components almost completely. In brain the insoluble enzyme constituted about 70% of total AGPO and AGPD activity. In selected instances determinations of tissue activity were performed at least in triplicate, to test the reproducibility of the technic. Values within any one group agreed with each other in orders ranging from 2% to 12%. In tissues with very low levels of activity the percentage variations were greatest.

Discussion. Glycerophosphate oxidation is appreciable in all of the tissues studied although variations are encountered in terms of specific enzymes involved. The high levels of insoluble AGPO and AGPD in brain may be related to its high content of DPNase (6) since the insoluble enzymes are not DPN-dependent. Although only moderate increase in activity was elicited by addition of phenazine methosulfate and cyanide in kidney, liver, and brain, a striking increase (8-fold) was observed for the total enzyme

TABLE I. AGPO and AGPD Levels in Tissues of Mice.

Organ	AGPO total	AGPO insoluble	AGPD total	AGPD insoluble
Kidney (11)	6.3 ± .32*	1.0 ± .13	8.9 ± .46	1.1 ± .19
Liver (11)	7.5 ± .49	negligible	8.1 ± .55	negligible
Brain (11)	5.0 ± .43	3.4 ± .30	5.5 ± .39	4.2 ± .32
Muscle (11)	.8 ± .25	negligible	6.9 ± .86	.4 ± .19

* Stand. error of mean.

of muscle. This may prehaps be explained by low levels of some flavoprotein moiety in muscle which is shunted in the assay for AGPD.

It has been reported(7) that female rat livers contain 4 times as much soluble AGPD as male rat livers, with both groups demonstrating extremely low activity. However, in a preliminary study in this laboratory with 3 female and 5 male rats (Sprague-Dawley), essentially similar mean values for soluble AGPD were found. These data [5.5 (5.2-5.9) in female and 5.4 (3.9-7.5) in male animals] are more consistent with usual findings of similarity of enzyme levels in the two sexes of the same species. The routine use of high levels of iodoacetate in the spectrophotometric method may account for the low levels of measurable AGPD activity described earlier.

Summary. AGP oxidation has been determined and characterized in kidney, liver, brain and muscle of the mouse. The assay technic utilized is simple, results are reproducible and only readily available laboratory equipment is required. In addition, enzyme inhibitors which might interfere with the accuracy of the determination are not used. It is felt that the present method may serve to broaden the limited lines of clinical investigation dealing with the significance of AGPD in regulation of metabolic processes.

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Lipid Formation by Fibroblasts Cultured on Serum from Stressed Animals.* (26676)

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Tissue cells cultivated *in vitro* are particularly sensitive to altered environment(1). An example is the tendency toward fat accumulation characteristic of most tissue culture cells, particularly those grown in presence of certain hormones(2). Preliminary investigations into possible physiological and morphological changes in tissue culture cells cultured on media containing serum from stressed chickens revealed an apparent alteration in cell physiology. Mouse fibroblasts were seen to accumulate lipid as intracytoplasmic droplets when cultured in the presence of serum from stressed chickens. This was not true in the presence of serum from non-stressed chickens or horses. The present paper constitutes a report on several experiments undertaken to establish and elaborate on the earlier preliminary observation.

Materials and methods. Young Leghorn cockerels were chosen as the test animals because of their availability, their notoriously labile emotional nature, and because of the ease with which their blood could be drawn. Early experiments revealed that hens could not be employed, evidently because of the toxicity of high serum calcium levels. Control animals were maintained in an isolated, enclosed, temperature-controlled ($24 \pm 2°C$)

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