GLYCEROL KINASE AND DIHYDROXYACETONE KINASE IN RAT BRAIN

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Abstract—The enzymatic phosphorylation of glycerol and dihydroxyacetone by ATP to *sn*-glycerol-3-phosphate and dihydroxyacetone phosphate respectively in various subcellular fractions of rat brain was studied. A sensitive radiochemical assay was used where the labelled phosphorylated products were separated from the radioactive substrates by high voltage paper electrophoresis and the radioactivity in these compounds determined. Using this assay the glycerol kinase (EC 2.7.1.30) activity was found to be associated with the mitochondrial fraction of the brain. Under optimum conditions 2·45 nmol of glycerol was phosphorylated/min per mg of protein. The K_m for glycerol was 70 μM at pH 7. This mitochondrial enzyme, like other glycerol kinases from different sources, also phosphorylated dihydroxyacetone. Under optimum conditions 1·7 nmol of dihydroxyacetone phosphate was formed/min per mg of mitochondrial protein. The K_m for dihydroxyacetone was 0·6 mM.

Glycerol kinase activity was also present in the cytoplasm of brain. However, the specific activity of this enzyme in cytosol is about 15% of the mitochondrial glycerol kinase. Compared to glycerol, dihydroxyacetone was phosphorylated by ATP in cytoplasm at a much higher rate. The pH optimum for this soluble dihydroxyacetone kinase was much lower (pH 6·5) than that of the soluble or mitochondrial glycerol kinase (pH 10·0). Using ammonium sulfate, brain cytoplasm was fractionated to yield a fraction in which the dihydroxyacetone kinase was enriched 2–3 fold with no glycerol kinase activity. Under optimum conditions 1·0 nmol of dihydroxyacetone was phosphorylated/min per mg protein. The K_m for dihydroxyacetone was 60 μM. This cytosol fraction was also found to phosphorylate D-glyceraldehyde and L-glyceraldehyde at a rate of 30–40% to that of the dihydroxyacetone phosphorylation. The properties and the possible metabolic role of these enzymes in brain are discussed.

Glycerol kinase (ATP: glycerol-3-phosphotransferase EC 2.7.1.30) catalyzes the phosphorylation of glycerol by ATP to *sn*-glycerol-3-phosphate (G-3-P). This enzyme was discovered by Kalckar (1939) in kidney. The properties of the enzyme were first described in detail by Bublitz & Kennedy (1954); Wieland & Suyter (1957). Using an enzymatic assay, Wieland & Suyter (1957) concluded that this kinase activity is present only in liver and kidney but not in other mammalian organs. However, later workers using more sensitive assays showed the presence of this enzyme in a wide variety of tissues (for a review see Thorner & Paulus, 1973). To date glycerol kinase has not been described in the brain, although there is indirect evidence to suggest its presence in that organ. A number of workers showed that when radioactive glycerol was injected intracranially into rats it was incorporated into the brain glycerolipids (Hokin & Hokin, 1958; Lapetina *et al.*, 1969; Benjamins & McKhann, 1973; O'Brien & Geison, 1974). This suggests that glycerol is phosphorylated in brain to G-3-P and subsequently acylated and converted to different lipids (Kennedy, 1962).

Glycerol kinase is also known to catalyze the phosphorylation of dihydroxyacetone and L-glyceraldehyde with ATP to form dihydroxyacetone phosphate (DHAP) and L-glyceraldehyde-3-phosphate, respectively (Bublitz & Kennedy, 1954; Wieland & Suyter, 1957). During the course of the investigations on the pathways for the metabolism of glycero-lipids in brain, we studied the glycerol kinase and dihydroxyacetone kinase activity in different subcellular fractions of brain by using a sensitive radiochemical assay. The results indicated that glycerol kinase activity is confined primarily to the mitochondrial fraction. Less activity was found in the 100,000 *g* supernatant fraction; however the conversion of dihydroxyacetone to DHAP was noted to be significantly higher in this fraction than could be accounted for by the glycerol kinase activity alone. Further studies showed the presence of another enzyme in the cytosol which phosphorylates dihydroxyacetone to DHAP by ATP. The properties of these enzymes are described in this paper.

MATERIALS AND METHODS

Materials

[1-^{14}C]glycerol and ^{32}P$_i$ were obtained from New England Nuclear (Cambridge, Mass.). [U-^{14}C]dihydroxyacetone was obtained from ICN (Irvine, Calif.). NAD$^+$,

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NADH, dihydroxyacetone, glycerol kinase, triose phosphate isomerase and G-3-P dehydrogenase were from Boehringer Mannheim Corp. (New York, N.Y.) and from Sigma (St. Louis, Mo). [γ-^{32}P]ATP was prepared from ^{32}P$_i$ and ATP by an enzymatic exchange reaction (GLYNN & CHAPPEL, 1964). 1-Chloro, 3-hydroxyacetone phosphate (chloroacetol phosphate) was a kind gift of Dr. F. Hartman (Oak Ridge, Tenn.). D-Glyceraldehyde and L-glyceraldehyde were obtained from Fluka, A. G. (Basel, Switzerland). Other materials were the same as previously described (LABELLE & HAJRA 1972a,b).

Purification of substrates

The [1-^{14}C]glycerol and [U-^{14}C]dihydroxyacetone were assayed for purity by ascending paper chromatography with a solvent system of ethyl acetate–pyridine–water (120:50:40). Dihydroxyacetone ($R_f = 0.74$), glycerol ($R_f = 0.57$) and DL-glyceraldehyde ($R_f = 0.34$) are separable from each other in this system. The [1-^{14}C]glycerol showed contamination with compounds which migrated with dihydroxyacetone (3%) and glyceraldehyde (1%). No impurities, especially in the glycerol and glyceraldehyde regions, were detected in the [U-^{14}C]dihydroxyacetone.

The impurities in glycerol could be removed by reduction with NaBH$_4$. The [1-^{14}C]glycerol (100 μCi/0.8 μmol/0.1 ml) in water was treated with 0.05 ml of an aqueous solution of NaBH$_4$ (0.1 M) for 30 min at room temperature. The excess borohydride was decomposed by 5 μl of 6 N-HCl and the pH was then adjusted to 7.0. When examined by paper chromatography, the NaBH$_4$-treated glycerol showed a single radioactive spot migrating with glycerol, and negligible radioactivity in the dihydroxyacetone or glyceraldehyde regions. This reduced [1-^{14}C]glycerol contained a small amount of borate, however this had no effect on the enzyme activity and the labelled glycerol was used directly for the assays.

Subcellular fractionation of the brain

Fifteen-day old male rats were sacrificed by CO$_2$ inhalation and the brains were quickly removed. Fractionation of the rat brains into crude mitochondria, microsomes and a 100,000 g supernatant was accomplished by differential centrifugation of the 10% homogenate in 0.25 M-sucrose containing EDTA (1 mM) (BRODY & BAIN, 1952). The crude mitochondrial fraction was further resolved into mitochondrial, synaptosomal, and myelin fractions by a discontinuous density gradient centrifugation (GRAY & WHITTAKER, 1962; SCHACHT & AGRANOFF, 1972). The mitochondrial, synaptosomal and microsomal fractions were washed twice with 0.25 M-sucrose and resuspended in 0.25 M-sucrose to give a concentration of 2 to 8 mg protein per ml. These fractions and the 100,000 g supernatant were kept frozen at −20°C in small batches and thawed immediately preceding their use.

The protein in the 100,000 g supernatant was first concentrated by precipitation with ammonium sulfate and the concentrated supernatant (10 mg protein/ml) was used for the ammonium sulphate fractionation. The cytosol was dialyzed overnight against 2 litres of 10 mM-citrate–phosphate buffer (pH 6.1) to remove the sucrose and then brought to 90% saturation with powdered ammonium sulphate. The precipitate was collected by centrifugation (13,000 g, 20 min) and then resuspended in a minimum vol of water and again dialyzed against 2 litres of 1 mM-citrate–phosphate buffer (pH 6.1). The concentrated supernatant was then centrifuged once again to remove insoluble protein and was then used for further fractionation with ammonium sulphate. An S$_1$ fraction was prepared containing protein which precipitated at a level of 50% saturation with ammonium sulphate. The dialyzed S$_1$ fraction (10 mg protein/ml) was treated with ammonium sulphate to 20% saturation and the precipitate was removed. The supernatant (S$_2$), which contained most of the dihydroxyacetone kinase, was dialyzed to remove the salt and kept frozen at −20°C.

Assay of the kinases

The enzymes were assayed radiometrically using labelled glycerol and dihydroxyacetone. The assay mixture contained ATP (5 mM), MgCl$_2$ (5 mM), 2-mercaptoethanol (5 mM), NaF (20 mM), buffer (various types and pH, 60 mM), labelled glycerol or dihydroxyacetone (0.2 mM), and enzyme from different subcellular fractions (0.01–0.3 mg protein) in a total vol of 0.1 ml. On various occasions, when the enzymes from the 100,000 g supernatant were used, 1-chloro, 3-hydroxyacetone phosphate (0.025 mM) was also included in the reaction mixture to inhibit the triose-P isomerase (HARTMAN, 1970). Because the brain cytosol contains triose-P isomerase and aldolase, a part of the product formed from dihydroxyacetone (i.e. DHAP) is converted to D-fructose 1.6-diphosphate. Addition of the chloroacetol phosphate prevented the conversion of DHAP to fructose diphosphate. The concentrations of the reagents varied slightly from one experiment to another and are included for the appropriate cases. The reaction mixture was incubated for 15–30 min at 37°C in a shaking water bath, and the reaction was stopped by placing the reaction tubes on ice and immediately adding 30 μl of 0.3 M-oxalic acid to each incubation mixture. Addition of oxalic acid decreased the pH to 1.5 thereby stopping the enzymatic reaction and also adjusting the pH of the reaction mixture to that used for electrophoresis. A 20 μl aliquot was removed from each reaction vessel and spotted as a 1–1.5 cm band on Whatman No. 1 paper for electrophoresis at pH 1.5 (0.08 M-oxalate buffer), at 4000 V for 30 min (HAJRA & AGRANOFF, 1968). In this electrophoretic system, DHAP, G-3-P and DL-glyceraldehyde-3-P migrate at different rates and are separable from each other. In a typical run (Fig. 1) DHAP migrated 21 cm from the origin compared to 18 cm for G-3-P and 17 cm for DL-glyceraldehyde-3-P. The finished electrophoretograms were radioautographed and the zones of interest cut out and counted directly in a liquid scintillation counter (Beckman LS-133) by immersing the paper in 10 ml of scintillation fluid in vials. The scintillation fluid contained toluene (950 ml), ethanol (50 ml), 2,5-diphenyl oxazole (PPO) (5 g) and 7,4-bis[2,4 methyl-5-phenyloxazolyl] benzene (dimethyl POPOP) (0.2 g). Counting efficiency of the vials containing the paper was 75% for ^{14}C and 90% for ^{32}P.

A different procedure was used when [γ-^{32}P]ATP was used as the labelled substrate. The incubation mixture was the same as described above except that [γ-^{32}P]ATP (0.2 mM) was incubated with non-radioactive glycerol or dihydroxyacetone or D- or L-glyceraldehyde (0.4 mM). Because ATP migrated very close to G-3-P ($R_{G-3-P} = 0.95$) and glyceraldehyde-3-phosphate in the electrophoretic system, it was necessary to hydrolyze radioactive ATP to ^{32}P$_i$ before electrophoresis. When DHAP or glyceraldehyde-3-P were the products, they were first reduced to the acid stable G-3-P before the hydrolysis of ATP as described below. The enzymatic reaction was stopped by adding 10 μl of 0.05 M-NaBH$_4$ to the incubation mixture and the solution

was kept at 0°C. After 10 min another 10 μl of $NaBH_4$ (0·05 M) solution was added and the reaction was allowed to continue for 10 min more prior to the addition of 1·0 ml of 1·0 N-HCl to each tube. The tubes were heated at 100°C for 15 min to hydrolyze the ATP and then evaporated to dryness. The residue was dissolved in a small volume of water and the solution was used for the electrophoretic separation of labelled G-3-P and $^{32}P_i$ as described before.

Protein was determined by the method of LOWRY et al. (1951). Other methods were the same as described previously (LABELLE & HAJRA, 1972a,b).

RESULTS

Enzymatic phosphorylation of glycerol and dihydroxyacetone in brain subcellular fractions

Both [1-^{14}C]glycerol and [U-^{14}C]dihydroxyacetone were shown to be phosphorylated in whole brain homogenate to form radioactive G-3-P and DHAP respectively. Under the assay conditions described in Table 1, about 3·1 μmol of glycerol and 1·7 μmol of dihydroxyacetone were phosphorylated/h per g of brain. Table 1 shows the distribution of enzyme activity for the phosphorylation of glycerol and dihydroxyacetone in various subcellular fractions of brain. Glycerol kinase activity was primarily found in the mitochondrial and the 100,000 g supernatant fractions. Very little enzymatic activity was found in other fractions. Dihydroxyacetone kinase activity was also found in the fractions showing activity for the phosphorylation of glycerol. However, whereas the rate of dihydroxyacetone phosphorylation is lower in mitochondria than the rate of glycerol phosphorylation, in the 100,000 g supernatant the rate is almost 4 times higher for dihydroxyacetone than for glycerol.

Table 2 shows the requirements for different cofactors of the mitochondrial glycerol kinase and the soluble dihydroxyacetone kinase. It was seen that ATP is absolutely required for both kinases. Magnesium was essential for dihydroxyacetone kinase activity,

but the mitochondrial glycerol kinase was fairly active in the absence of Mg^{2+}. This was probably due to the presence of endogenous Mg^{2+} because the addition of EDTA completely inhibited the glycerokinase activity (Table 2). Both kinases were found to be stimulated by mercaptoethanol (Table 2). NaF stimulated the mitochondrial glycerol kinase activity, probably by inhibiting the ATPase and phosphatase activity. It had no effect on the soluble dihydroxyacetone kinase activity.

Characterization of the products

The properties of the labelled products formed from [1-^{14}C]glycerol and [U-^{14}C]dihydroxyacetone in brain mitochondria and cytosol resemble those of G-3-P and DHAP respectively. For example, the product formed from labelled glycerol migrated with standard G-3-P on high voltage paper electrophoresis at pH 1·5 (Fig. 1), and also at pH 4·3 and pH 8. The labelled product was found to be stable to hydrolysis by acid (1 N-HCl, 100°C, 10 min) and alkali (1 N-KOH, 10 min, 40°C). When unlabelled glycerol, [γ-^{32}P]ATP and other cofactors were used, the ^{32}P-labelled product had the same migration rate on high voltage electrophoresis at pH 4·3 as the ^{14}C-labelled product described above.

The labelled product formed from ^{14}C-labelled glycerol and ATP in the brain mitochondrial fraction was found to be converted to labelled phosphatidic acid after incubation with acyl CoA and brain microsomes. The phosphatidic acid was characterized by solvent extraction followed by TLC (LABELLE & HAJRA 1972a).

Similarly, the product formed from labelled dihydroxyacetone was found to migrate with DHAP at different pH on high voltage paper electrophoresis (see Fig. 1). The same product was formed when unlabelled dihydroxyacetone and [γ-^{32}P]ATP were used. The product formed from dihydroxyacetone, like DHAP (MEYERHOF & LOHMANN, 1934; HAJRA &

TABLE 1. ENZYMATIC PHOSPHORYLATION OF DIHYDROXYACETONE AND GLYCEROL IN VARIOUS SUBCELLULAR FRACTIONS OF BRAIN

Subcellular fractions	Protein (μg)	[^{14}C]G-3-P from [1-^{14}C]glycerol (c.p.m.)	(nmol/mg protein)	[^{14}C]DHAP from [U-^{14}C]dihydroxyacetone (c.p.m.)	(nmol/mg protein)
Mitochondria	70	2770	11·0	1890	5·1
Mitochondria*	70	15	—	20	—
Synaptosomes	130	320	0·7	360	0·5
Microsomes	85	45	0·1	300	0·6
100,000 g supernatant	55	370	1·9	2190	7·6
100,000 g supernatant*	55	0	—	0	—

The incubation mixture contained citrate–phosphate buffer (60 mM, pH 7·1), ATP (5 mM), $MgCl_2$ (5 mM), 2-mercaptoethanol (5 mM), NaF (20 mM), either [1-^{14}C]glycerol (0·2 mM, 4·68 × 10^5 c.p.m.) or [U-^{14}C]dihydroxyacetone (0·2 mM, 6·9 × 10^5 c.p.m.) and different brain subcellular fractions as noted below in a total vol of 0·1 ml. The mixtures were incubated for 15 min at 37°C and the reaction was stopped by adding 30 μl of 0·3 M-oxalic acid to each tube. A 20 μl aliquot from each tube was used for separating the products from the substrate by high voltage paper electrophoresis as described in the text. The radioactivity reported here is the count recovered in the DHAP or G-3-P spots on the electrophoretogram which is approx 15% of the total product formed in the incubation mixture.

* These enzyme fractions were heated at 100°C for 10 min before use.

TABLE 2. REQUIREMENTS FOR THE ENZYMATIC PHOSPHORYLATION OF GLYCEROL AND DIHYD-
ROXYACETONE IN BRAIN

System	Expt. 1 [1-^{14}C]G-3-P formed nmol	Expt. 2 [U-^{14}C]DHAP formed nmol
Whole system	0·71	1·48
Whole −ATP	0·01	0·02
Whole −MgCl$_2$	0·46	0·06
Whole −MgCl$_2$ + EDTA (2·5 mM)	0·09	0·02
Whole −mercaptoethanol	0·65	1·01
Whole −NaF	0·55	1·49
Whole system (boiled)*	0·00	0·02

The whole incubation mixture is the same as described under Table 1. In Experiment 1 the phosphorylation of [1-^{14}C]glycerol (0·2 mM, 4·6 × 10^5 c.p.m.) by brain mitochondria (60 μg protein) is studied. In Experiment 2 the phosphorylation of [U-^{14}C]dihydroxyacetone (0·2 mM, 5·98 × 10^5 c.p.m.) with brain cytosol ammonium sulfate (20–50% saturation, see later in the text) fraction (110 μg protein) to labelled DHAP at pH 6·1 (Citrate–phosphate buffers) was studied. The incubation period was 30 min.
* These enzyme fractions were heated at 100°C for 10 min before use.

AGRANOFF, 1968), was found to be extremely labile to alkali. After incubation of [U-^{14}C]dihydroxyacetone with ATP and other cofactors in liver mitochondrial or supernatant fractions, aliquots of the mixture were treated with NaOH (pH 12) at 37°C for 10 min. The resulting mixture on high voltage electrophoresis showed no radioactive band corresponding to DHAP when compared to the sample not treated with alkali. Reduction of the reaction product (from [^{14}C]dihydroxyacetone) with NaBH$_4$ yielded a labelled product which migrated with G-3-P (Fig. 1). This reduced product was found to be stable to acid (1 N-HCl, 100°C, 10 min) and alkaline hydrolysis (0·05 N-NaOH, 15 min at 25°C). The product formed from labelled dihydroxyacetone in the 100,000 g supernatant fraction was also reduced enzymatically to G-3-P by adding NADH and glycerol-3-phosphate dehydrogenase to the reaction mixture. Finally, the ^{14}C-labelled product was shown to be converted to labelled acyl DHAP after enzymatic acylation in brain microsomes with acyl CoA as shown by TLC (LaBELLE & HAJRA, 1972a). These results confirmed the identity of the labelled product formed from labelled dihydroxyacetone and ATP as DHAP.

Properties of the mitochondrial enzyme

The mitochondrial fraction phosphorylates both dihydroxyacetone and glycerol to DHAP and G-3-P respectively. For both these substrates the enzymatic phosphorylation was found to have activity over a broad pH range between pH 7 and pH 11 (Fig. 2). The abnormal shape of these curves is not due to any non-enzymatic reaction because the controls containing heated enzyme or no enzyme did not show any glycerol phosphorylation. It is of interest to note that the general shape of these curves is in agreement with those of glycerol kinases from other tissue sources (BUBLITZ & KENNEDY, 1954; HAESSLER & ISSELBACHER, 1963; HAYASHI & LIN, 1963). The pH optimum for dihydroxyacetone kinase was 10·0 and for the glycerol kinase 10·5 (Fig. 2). There was a sharp drop of dihyd-

roxyacetone kinase activity beyond pH 10·0, probably due to the instability of DHAP at high pH. The enzyme activities with both substrates were found to be linear with time to an incubation period of 10 min (Fig. 3) and also linear with increasing protein concentration up to 0·2 mg protein (Fig. 3). The effect of increasing the substrate concentration on the enzyme activity is shown in Fig. 4A and 4B. The rate of reaction with increasing concentration of dihydroxyacetone appears to be only of first order kinetics (Fig. 4B) because the concentration of the substrate used was below the K_m. However, the enzymatic reaction followed the Michaelis–Menten Kinetics as shown by the double reciprocal plot (Fig. 4B). The K_m for dihydroxyacetone phosphorylation was 0·6 mM. The K_m for glycerol phosphorylation was 70 μM. The corresponding V_{max} values were 1·0 nmol/min per mg protein for the formation of DHAP and
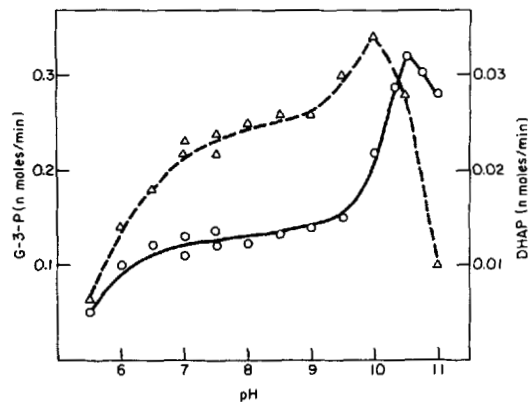

FIG. 2. Rates of glycerol phosphorylation and dihydroxyacetone phosphorylation in mitochondria as a function of pH. The incubation mixtures and assay conditions were the same as described in Table 1 except the pH and composition of the buffers varied. The buffers (60 mM) used in the incubation mixtures were phosphate (pH 5·5–7·5), triethanolamine–HCl (pH 7·0–8·5), glycine–NaOH (pH 8·5–11·0). Rates of G-3-P (O——O) and DHAP (△--- △) formation in mitochondria (40 μg protein) are shown.
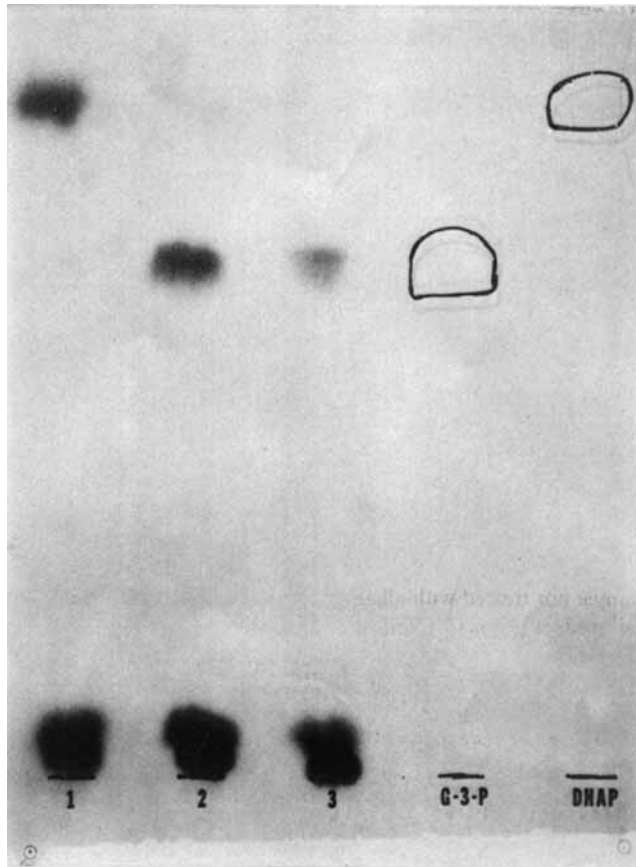
FIG. 1. Autoradiogram of the electrophoretogram showing labelled products formed enzymatically from radioactive glycerol or dihydroxyacetone. 1. The incubation mixture contained citrate–phosphate buffer (60 mM, pH 6·1), ATP (5 mM), MgCl$_2$ (5 mM), mercaptoethanol (5 mM), NaF (20 mM), 1-chloro, 3-hydroxyacetone phosphate (25 μM), and [U-^{14}C] dihydroxyacetone (0·1 mM, 3·5 × 105 c.p.m.) in a total vol of 0·1 ml. The mixture was incubated at 37°C for 15 min and then 30 μl of 0·3 M-oxalic acid was added to stop the reaction as described in the text. An aliquot (20 μl) of the resulting mixture was spotted on Whatman No. 1 paper which was subjected to high voltage paper electrophoresis (4000 V, 30 min at pH 1·5) to separate the phosphorylated product(s) from labelled substrate. The radioactive spots were located by autoradiography as shown above.
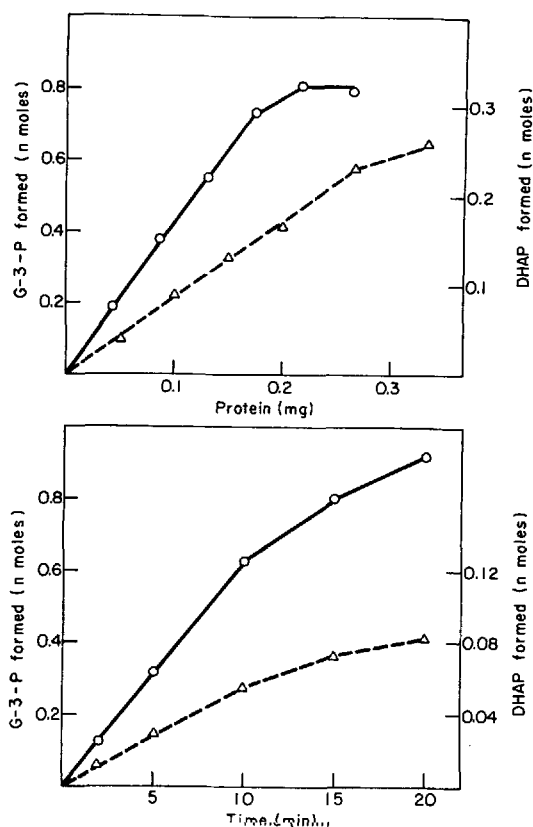
FIG. 3. Enzymatic phosphorylation of glycerol and dihydroxyacetone in mitochondria as a function of enzyme titer (upper) and time (lower). The conditions were the same as described in Table 1. A crude mitochondrial fraction containing synaptosome and myelin (0·17 mg protein for the lower figure) was used. A 10 min incubation time was used for the upper figure. The amount of G-3-P (O———O) and DHAP (△----△) formed are shown.
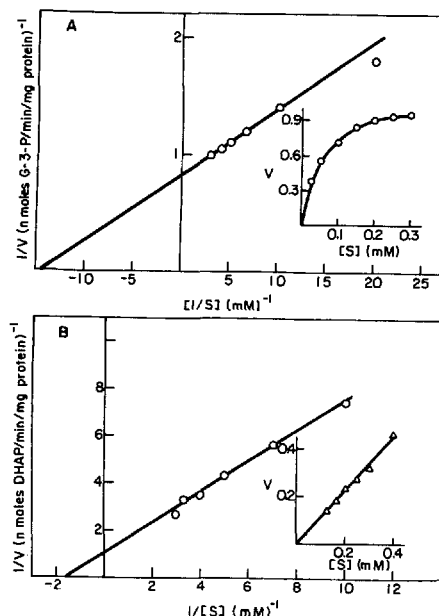

FIG. 4. Effect of increasing concentration of glycerol (A) and dihydroxyacetone (B) on the mitochondrial kinase activity. The reaction conditions were same as in Table 1 with the substrate concentration varying as indicated. The reaction time was 15 min and 0·17 mg mitochondrial protein was used. The double reciprocal plot ($1/s$ vs $1/v$) is shown in this figure with the effect of increasing substrate concentration (v vs s) in the insets.

0·8 nmol/min per mg protein for the phosphorylation of glycerol. An attempt was made to determine the K_m for ATP at fixed Mg^{2+} (0·01 M) and dihydroxyacetone (0·2 mM) or glycerol (0·2 mM) concentrations. The data are somewhat more variable, probably because of the presence of ATPase activity in mitochondria. An approximate K_m for ATP of 0·15 mM with glycerol and 3 mM with dihydroxyacetone was obtained.

The above results were obtained when the enzymatic reactions were carried out at physiological pH (pH 7). When similar experiments were done at the pH optimum of the enzyme (pH 10·0), the K_m for glycerol was found to be 20 μM and 2·45 nmol of glycerol was phosphorylated/min per mg of the mitochondrial protein. The corresponding values for dihydroxyacetone were 0·2 mM (K_m) and 1·5 nmol/min per mg of protein at pH 10.

Properties of the cytosol enzyme

The pH optimum of the enzyme from the 100,000 g supernatant was found to be quite different for dihydroxyacetone as compared to that of glycerol. Whereas the pH optimum for the formation of DHAP from

dihydroxyacetone was 6·0 (Fig. 5), the pH optimum for glycerol was 10·4 (Fig. 5), the same as the mitochondrial enzyme. The activity towards glycerol phosphorylation was much less in this fraction than the dihydroxyacetone kinase activity. These and other properties indicate that more than one enzyme is involved in the phosphorylation of glycerol and dihydroxyacetone in the cytosol fraction. This was verified by the separation of dihydroxyacetone kinase activity from the glycerol kinase activity by ammonium sulphate fractionation of the 100,000 g supernatant (Table 3). By this method the dihydroxyacetone kinase activity could be completely separated from the glycerol kinase activity. Because of the low activity, no further study was made of glycerol kinase in the cytosol. The dihydroxyacetone kinase activity in the ammonium sulphate purified fraction (S_2 fraction, Table 3) was studied in detail. The formation of DHAP from dihydroxyacetone and ATP was found to be linear with time and with the increasing amount of protein (Fig. 6). The effect of increasing the concentrations of dihydroxyacetone and ATP on the enzyme activity is shown in Fig. 7. The K_m for dihydroxyacetone was found to be 60 μM, and a V_{max} of 1·0 nmol of DHAP formed/min per mg of protein was obtained (Fig. 7A). At high magnesium concentration (10 mM) the K_m for ATP was 0·7 mM (Fig. 7B). The dihydroxyacetone phosphorylation, however, was inhibited by the high Mg^{2+} concentration.

TABLE 3. FRACTIONATION OF GLYCEROL KINASE AND DIHYDROXYACETONE KINASE ACTIVITY IN 100,000 g BRAIN SUPERNATANT BY AMMONIUM SULPHATE

Fraction	Glycerol kinase activity nmol G-3-P/min per mg protein	Dihydroxyacetone kinase activity nmol DHAP/min per mg protein
100,000 g supernatant	0·109	0·439
S_t	0·083	0·184
S_1	0·048	0·394
S_2	0·000	0·770

Dialyzed 100,000 g brain supernatant was fractionated with solid powdered ammonium sulfate as described in the text. The S_t fraction contained all proteins which were precipitated when cytosol was 90% saturated with ammonium sulfate. the S_t fraction was fractionated with ammonium sulphate to S_1 (0–50% saturated with ammonium sulphate) and S_2 (20–50% saturated with ammonium sulphate) as described in the text. The glycerol kinase and dihydroxyacetone kinase activities of various fractions are determined by the procedure as described in Tables 1 and 2.
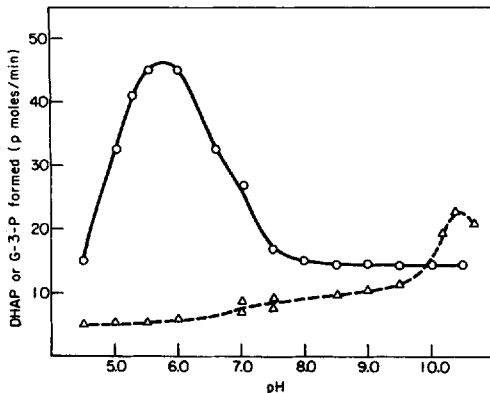

FIG. 5. The rate of phosphorylation of glycerol ($\triangle$ --- $\triangle$) and dihydroxyacetone (O—O) in the 100,000 g supernatant fraction as a function of pH. The incubation mixtures were identical to those used for Fig. 2 except that brain 100,000 g supernatant (0·14 mg protein) was used instead of the mitochondria protein. Citrate buffer was used for pH range 4·5–5·5.

FIG. 6. Effect of increasing time and enzyme concentration on the phosphorylation of dihydroxyacetone by the S_2 supernatant fraction. The reaction mixture contained citrate phosphate buffer (60 mM, pH 6·1), ATP (5 mM), Mg Cl$_2$ (5 mM), 2-mercaptoethanol (5 mM), NaF (20 mM), [U-^{14}C]dihydroxyacetone (0·2 mM, 60 μCi/μmol) and S_2 fraction in a total vol of 0·1 ml. The assay condition was same as described in Table 1. The reaction time was 10 min for the protein concentration study. The time course study was done with 0·12 mg of S_2 fraction protein.

Effect of other analogs on the phosphorylation of dihydroxyacetone and glycerol

Glycerol kinase is known to phosphorylate dihydroxyacetone and L-glyceraldehyde (BUBLITZ & KENNEDY, 1954; WIELAND & SUYTER, 1957). Also the dihydroxyacetone kinase activity in the supernatant may be similar to the triokinase activity described in liver (HERS & KUSAKA, 1953). To establish the identity of these kinases the effects of D- and L-glyceraldehyde, D-fructose, D-fructose-6-phosphate, dihydroxyacetone and glycerol on the phosphorylation of labelled dihydroxyacetone and glycerol were studied. The assumption was that if these compounds were common substrates for the enzyme, they would inhibit the phosphorylation of the labelled substrates by the same enzyme. The effects of different compounds at low concentration are shown in Table 4. At low concentration dihydroxyacetone did not inhibit the mitochondrial glycerol kinase activity. Slight inhibition of the mitochondrial glycerokinase activity was seen with D- and L-glyceraldehyde. A similar result was found with the soluble glycerokinase activity (Table 4). The mitochondrial dihydroxyacetone kinase
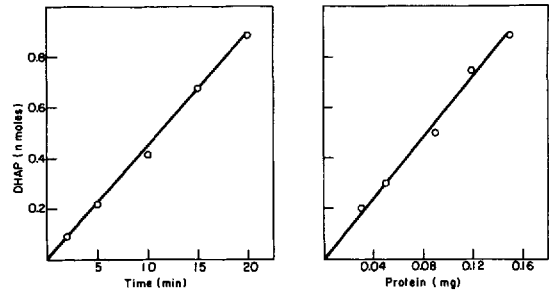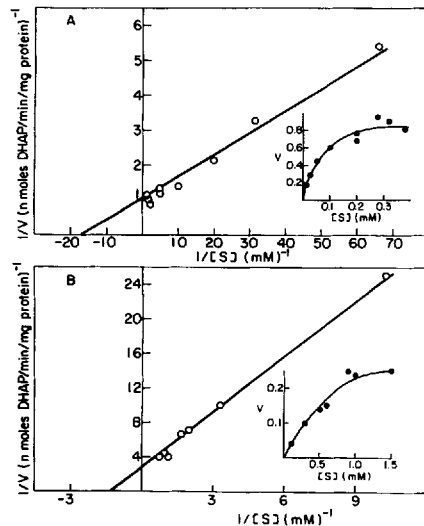

FIG. 7. Effect of changing the concentration of dihydroxyacetone (A) and ATP (B) on the phosphorylation of dihydroxyacetone by the supernatant S_2 fraction. The assay conditions were similar to those described under Fig. 6. The double reciprocal plot ($1/s$ vs $1/v$) is shown in the figure, with the effect of increasing substrate concentration indicated in the insets.

TABLE 4. EFFECT OF DIFFERENT COMPOUNDS ON THE ENZYMATIC PHOSPHORYLATION OF
GLYCEROL AND DIHYDROXYACETONE

| Additions | Relative enzyme activity and source | | | |
| | Glycerol kinase | | Dihydroxyacetone kinase | |
	mitochondria	cytosol	mitochondria	cytosol
None	100	100	100	100
Dihydroxyacetone (0·9 mM)	100	102	—	—
D-glyceraldehyde (0·9 mM)	80	70	11	55
L-glyceraldehyde (0·9 mM)	80	85	85	80
D-fructose (0·2 mM)	—	100	—	95
D-fructose-6-P (0·2 mM)	—	—	—	92
glycerol (0·2 mM)	—	—	96	120

The conversions of $[1-^{14}C]$glycerol and $[U-^{14}C]$dihydroxyacetone to radioactive
G-3-P and DHAP respectively were followed under conditions described in Tables
1 and 2. The glycerol kinase and dihydroxyacetone kinase activities in the absence
of any other substrate were arbitrarily set at 100 and the relative rates in the presence
of other non-radioactive substrates are given below. The concentrations of the non-
radioactive carbohydrates present in the incubation mixture are given above.

activity was strongly inhibited by glycerol and L-gly-
ceraldehyde. On the other hand, no inhibition by gly-
cerol of the soluble dihydroxyacetone kinase activity
was seen. This soluble dihydroxyacetone kinase ac-
tivity was inhibited by D-glyceraldehyde and to a
slight extent by L-glyceraldehyde. D-fructose and D-
fructose-6-phosphate did not inhibit the dihydroxy-
acetone kinase activity.

*Phosphorylation of different substrates by the superna-
tant enzyme with $[\gamma-^{32}P]ATP$*

The phosphorylation of glycerol, D- and L-glyceral-

TABLE 5. ENZYMATIC PHOSPHORYLATION OF DIFFERENT SUB-
STRATES BY $[\gamma-^{32}P]ATP$

	Substrate	^{32}P-labelled product formed pmol
1	none	45
2	dihydroxyacetone	240
3	$[U-^{14}C]$dihydroxyacetone	245 (210)*
4	D-glyceraldehyde	70
5	L-glyceraldehyde	100
6	glycerol	25
7	dihydroxyacetone†	0
8	dihydroxyacetone	255‡

The incubation mixture contained citrate–phosphate
buffer (60 mM, pH 6·1), 2-mercaptoethanol (5 mM), NaF
(20 mM), MgCl$_2$ (5 mM), chlorohydroxyacetone-P
(0·025 mM), $[\gamma-^{32}P]ATP$ (0·2 mM, 1·2 × 10^5 c.p.m./nmol),
different substrates (0·4 mM) as indicated above and brain
S$_2$ fraction (0·12 mg protein) in a total vol of 0·1 ml. The
mixtures were incubated at 37°C for 30 min. The products
formed were first reduced with NaBH$_4$ and the excess
$[^{32}P]ATP$ was hydrolyzed by 1 N-HCl at 100°C as de-
scribed in the text. The radioactive compounds were separ-
ated by high voltage paper electrophoresis and the radio-
activity present in the G-3-P spot was determined.
  * The figure in the parentheses shows the amount of
^{14}C-labelled product formed.
  † The enzyme was heated at 100°C for 10 min before
use.
  ‡ In this tube the $[^{32}P]DHAP$ formed was directly
measured without reduction and acid hydrolysis.

dehyde and dihydroxyacetone was studied by using
labelled ATP and the S$_2$ fraction of the cytosol (Table
5). As mentioned before, the acid labile products were
first reduced with NaBH$_4$ to acid-stable G-3-P, and
the excess $[\gamma-^{32}P]ATP$ was hydrolyzed to $^{32}P_i$ by
acid hydrolysis before separation by electrophoresis.
The resulting $^{32}P_i$ was well separated from G-3-P.
Using this assay system it was seen that dihydroxy-
acetone was the best substrate for the enzyme (Table
5). This was verified also by using ^{14}C-labelled dihyd-
roxyacetone and by measuring DHAP without reduc-
tion and acid hydrolysis (Table 5). The enzyme frac-
tion phosphorylated D-glyceraldehyde and L-glyceral-
dehyde. As shown before with labelled glycerol, gly-
cerol was not phosphorylated in this system.

*Change in brain glycerol kinase and dihydroxyacetone
kinase activity with age*

During myelination there is an accelerated rate of
lipid synthesis in the brain (WELLS & DITTMER, 1967).
To check whether there is any change in the activity
of these kinases during myelination, the specific ac-
tivities of glycerol kinase and dihydroxyacetone
kinase in the mitochondrial and the cytosol fractions
in rat brains before myelination (7-day old rats), dur-
ing active myelination (15-day old rats) and after mye-
lination (2 months and 1-year old rats) were
measured. In the 100,000 g supernatant fraction of
brain there appears to be a slight increase (10–15%)
in specific activity for both glycerol and dihydroxy-
acetone phosphorylation in the 15-day old rats, com-
pared to that of 7-day old rats. However, the activity
is not significantly different from that of adult rats.
Activity for glycerol phosphorylation in the mito-
chondrial fraction appears to be highest at 7 days
and decreases slightly thereafter.

DISCUSSION

A direct optical assay for the glycerol kinase and
dihydroxyacetone kinase activities based on the oxi-
dation or reduction of pyridine nucleotides was not

possible due to the low activity of these enzymes in brain. Consequently, a radioassay was employed which yielded the desired sensitivity. An alternative method for the separation of glycerol from G-3-P by adsorption to discs of DEAE-cellulose paper has been used for a sensitive radioassay (Newsholme et al., 1967). It was found that the glycerokinase activity in brain could be measured accurately by this method. However, the dihydroxyacetone kinase activity could not be measured reliably in this way because a large fraction of labelled dihydroxyacetone was also adsorbed on the DEAE-filter paper discs. The electrophoretic method used here was found to be accurate, reliable, and also useful in separating G-3-P from DHAP. Using this assay, brain glycerokinase activity was found primarily in the mitochondrial fraction. This enzyme escaped detection in brain (Wieland & Suyter, 1957; Vernon & Walker, 1970) in the past probably due to its low activity and its association with brain mitochondria. Though in most tissues this enzyme is present in the cytosol fraction, in insect flight muscle and bull spermatozoa glycerol kinase has been described to be primarily present in mitochondria (Newsholme & Taylor, 1969; Mohri & Masaki, 1967). A similar enzyme, hexokinase, which is present in the cytosol in most organs, has been shown to be associated primarily with brain mitochondria (Crane, 1962). The properties of the brain glycerokinase are similar to those of glycerol kinase found in other organs (Thorner & Paulus, 1973). The brain enzyme, like other glycerol kinases, has a high pH optimum and is stimulated by thiols. This enzyme also phosphorylates dihydroxyacetone and possibly L-glyceraldehyde. However, like the liver enzyme (Thorner & Paulus, 1973), the K_m for glycerol phosphorylation by the brain enzyme is much lower than the K_m for dihydroxyacetone phosphorylation (70 μM vs 0·6 mM). This difference in K_m probably explains the fact that at low concentration glycerol inhibits the dihydroxyacetone phosphorylation in mitochondria, while the converse is not true (Table 4). L- and D-glyceraldehyde partially inhibit the phosphorylation of glycerol (Table 4). L-glyceraldehyde inhibits glycerol phosphorylation by being a substrate for the enzyme. Hayashi & Lin (1967) showed that D-glyceraldehyde also acts as a substrate for glycerokinase. However, the product is an unstable hemiacetal phosphate which is hydrolyzed by water to P_i. Thus D-glyceraldehyde would inhibit the phosphorylation of glycerol by competing for the enzyme, and also by stimulating the hydrolysis of ATP in the reaction mixture.

As mentioned before, the K_m for ATP could not be accurately determined with the crude mitochondrial enzyme and the values obtained are probably higher than the real K_m. Actually, the substrate for the kinase is not free ATP but the $(Mg-ATP)^{2-}$ complex (Thorner & Paulus, 1973). However, because the Mg^{2+} concentration (10 mM) was much higher than the ATP^{4-} concentration (0·1–5 mM) and the

formation constant of the $(Mg-ATP)^{2-}$ complex is high (5·8 $\times$ 10⁴, see Burton, 1959), the ATP in the reaction mixture was mainly present as the $(Mg-ATP)^{2-}$ complex.

The properties of the dihydroxyacetone kinase activity in the cytoplasm are quite different than those of the glycerol kinase. The pH optima differ greatly (6·0 for dihydroxyacetone kinase vs 10·5 for glycerol kinase) and the K_m for dihydroxyacetone is much lower for this enzyme than it is with the mitochondrial enzyme. The partially purified enzyme did not have any glycerol kinase activity, and glycerol at high concentration did not compete for the phosphorylation of dihydroxyacetone with this enzyme. These results suggest that the soluble dihydroxyacetone kinase is an enzyme distinct from the glycerol kinase. This enzyme is probably similar to the triokinase of liver. Triokinase has been shown to phosphorylate dihydroxyacetone and D-glyceraldehyde, but not glycerol or L-glyceraldehyde (Hers, 1962). D-glyceraldehyde inhibits the phosphorylation of dihydroxyacetone by this enzyme and is also phosphorylated to D-glyceraldehyde-3-phosphate. However, D-glyceraldehyde kinase activity is much lower than the dihydroxyacetone kinase activity, and L-glyceraldehyde is also phosphorylated by this enzyme fraction. It is possible that some other enzyme is present in this fraction which phosphorylates L-glyceraldehyde. Because the enzyme is fairly crude and has a very low specific activity, nothing definite can be established about the substrate specificity until the enzyme is further purified. Recently, triokinase activity has been demonstrated in erythrocytes (Beutler & Guinto, 1973). The specific activity of the erythrocyte enzyme is somewhat lower than the brain enzyme; and the kinetic properties of the erythrocyte enzyme are different than the enzyme described here.

The metabolic role of these enzymes is not very clear. Glycerol kinase is believed to be important for the reutilization of free glycerol formed by lipolysis in tissues. The free glycerol concentration in brain is reported to be 0·4–0·7 mM (Wieland, 1957). Because the K_m of the brain mitochondrial glycerokinase is low (70 μM), the brain would be able to utilize the endogenous glycerol very effectively. Glycerol may also diffuse from blood (0·1–0·2 mM-glycerol) to brain and would be utilized in that tissue. However, Crone (1965) showed that glycerol is not easily transported across the blood–brain barrier. In such a case, glycerol kinase may be important only for the reutilization of free glycerol which is formed by the breakdown of glycerolipid in the brain. Similarly, dihydroxyacetone kinase may be important for the reutilization of dihydroxyacetone or D-glyceraldehyde in the brain. Dihydroxyacetone may be formed by the enzymatic oxidation of glycerol (glycerol dehydrogenase EC 1.1.1.6) or by the dephosphorylation of DHAP.

One important consequence of the presence of these enzymes in the brain is that in vivo conversion of labelled glycerol and dihydroxyacetone to different

glycerolipids can be easily studied. As reported by different workers (LAPETINA et al., 1969; BENJAMINS & MCKHANN, 1973; O'BRIEN & GEISON, 1974), we also found that [^{3}H]- or [^{14}C]glycerol, when injected intracranially is rapidly incorporated into brain lipid. We also found that [^{14}C]dihydroxyacetone is incorporated into brain glycerolipid when administered by the same route. A detailed study of the conversion of these precursors into brain lipid might be useful to determine the importance of different pathways (glycerol-3-phosphate and acyl dihydroxyacetone phosphate pathways) in the brain for the biosynthesis of glycerolipid.

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