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THE RELATIVE UTILIZATION OF THE ACYL DIHYDROXYACETONE PHOSPHATE AND GLYCEROL PHOSPHATE PATHWAYS FOR SYNTHESIS OF GLYCEROLIPIDS IN VARIOUS TUMORS AND NORMAL TISSUES

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Summary

Rates of phosphatidate synthesis from dihydroxyacetone phosphate via acyl dihydroxyacetone phosphate or glycerol phosphate are compared in homogenates of 13 tissues, most of which are deficient in glycerol phosphate dehydrogenase (EC 1.1.1.8).

In all tissues examined, dihydroxyacetone phosphate entered phosphatidate more rapidly via acyl dihydroxyacetone phosphate than via glycerol phosphate. Tissues with a relatively low rate of phosphatidate synthesis via glycerol phosphate, showed no compensating increase in the rate of synthesis via acyl dihydroxyacetone phosphate. The rates at which tissue homogenates synthesize phosphatidate from dihydroxyacetone phosphate via glycerol phosphate increase as glycerol phosphate dehydrogenase increases.

Both glycerol phosphate dehydrogenase and glycerol phosphate: acyl CoA acyltransferase (EC 2.3.1.15) are more active than dihydroxyacetone phosphate : acyl CoA acyltransferase (EC 2.3.1.42). Thus, all the tissue homogenates possessed an apparently greater capability to synthesize phosphatidate via glycerol phosphate than via acyl dihydroxyacetone phosphate, but did not express this potential. This result is discussed in relation to in vivo substrate limitations.

Introduction

The studies reported here concern the relative importance of the acyl dihydroxyacetone phosphate and *sn*-glycerol 3-phosphate pathways in the de novo synthesis of glycerolipids. These pathways are illustrated in Fig. 1.

The following is known about the acyl dihydroxyacetone phosphate pathway: (1) several tissues can acylate dihydroxyacetone phosphate with long-

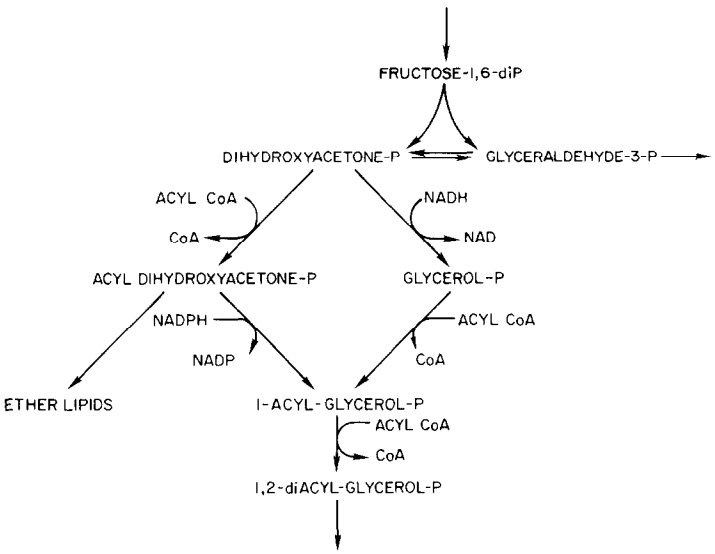

Fig. 1. The glycerol phosphate and acyl dihydroxyacetone phosphate pathways for the de novo synthesis of glycerolipids.

chain acyl CoAs [1]; (2) acyl dihydroxyacetone phosphate is a direct precursor of ether glycerolipids [2]; (3) subcellular fractions of several tissues can reduce acyl dihydroxyacetone phosphate with NADPH to form 1-acyl-*sn*-glycerol 3-phosphate [3]; (4) acyl glycerol phosphate is a direct precursor of phosphatidic acid and thus of glycerolipids in general [4]; (5) since tissues are potentially able to acylate dihydroxyacetone phosphate, then reduce acyl dihydroxyacetone phosphate, an alternate pathway to acyl glycerol phosphate and thus to glycerolipids is possible [5].

The glycerol phosphate pathway for phosphatidic acid synthesis consists of the NADH-dependent reduction of dihydroxyacetone phosphate by glycerol phosphate dehydrogenase (EC 1.1.1.8), then acylation by glycerol phosphate : acyl CoA acyltransferase (EC 2.3.1.15). The activity of glycerol phosphate dehydrogenase, the first step in the glycerol phosphate pathway, differs greatly among tissues, and it is especially low in tumors [6]. This diversity suggests that the relative importance of the two pathways in phosphatidic acid synthesis may also differ, depending on the activity of glycerol phosphate dehydrogenase. In Morris hepatomas, the concentration of ether glycerolipids increases with decreasing activity of glycerol phosphate dehydrogenase [7]. Since ether glycerolipids are produced from acyl dihydroxyacetone phosphate, this finding suggests the relative carbon flow through the acyl dihydroxyacetone phosphate pathway might also increase as the glycerol phosphate dehydrogenase activity decreases. We have previously provided evidence that one tumor, Ehrlich ascites, uses the acyl dihydroxyacetone phosphate pathway relatively more than a normal tissue, liver, which has a higher activity of glycerol phosphate dehydrogenase [2,8].

The present study examines the relative utilization of the glycerol phosphate and acyl dihydroxyacetone phosphate pathways in de novo synthesis of

glycerolipids. We chose tissues in which glycerol phosphate dehydrogenase activity is expected to be low, such as tumors, so that a greater dependence on the acyl dihydroxyacetone phosphate pathway might be anticipated.

A preliminary report of this work has been published [9].

Materials and Methods

Tissues

Transformed and normal astroglia were kindly provided by H.M. Shein. Nitrosourea-transformed rat astroglia, lines C-6 and C-2_1, were prepared by multiple intravenous injections of *N*-nitrosomethylurea [10]. Line C-6 is differentiated (Grades I and II), while line C-2_1 is pleomorphic (Grade III) [10]. Normal astroglia were prepared from newborn Syrian hamsters by culturing dispersed brain fragments for 14 days [11]. Neurons and oligodendroglia do not survive this culture period [11]. Astroglia transformed by SV-40 were derived from astroglial cultures of either fetal (2/3 gestational) or newborn hamsters, lines THA 15 and NT 1, respectively [12]. The transformed astroglia from fetal hamsters are poorly differentiated (Grade IV), and those from newborn hamsters are a mixture of well- and poorly differentiated cells [12]. These various tissues were stored at −70° C for two years before being used.

Neuroblastoma C1300 was donated by R.W. Ruddon. This tumor was grown subcutaneously in A/J mice.

The line I_b leukemia cells were contributed by W.H. Murphy. They were prepared from the spleens of leukemic C58/wm mice [13].

Leukocytes were isolated from crushed spleens of normal C58 mice. After the reticular material settled, the supernatant extract was centrifuged twice for 2 min at 50 × *g* to collect the leukocytes. Blood lymphocytes were isolated from fresh human peripheral blood [14]. We prepared thymocytes from thymus glands by grinding minced glands from male Spraque-Dawley rats in Krebs-Ringers phosphate buffer [15] with a Duall tissue grinder, then sedimenting reticular material by a 3 min centrifugation at 100 × *g*. The supernatant was then used without further purification. The lymphocytes and thymocytes were identified after treatment with Wright's strain [16].

Mouse brain and liver were taken from male Swiss mice. The fetal brain sample (12 brains) was prepared from a pregnant Spraque-Dawley rat. The average fetus body and brain weighed 4.8 and 0.19 g, respectively.

Isotopes

D-[1-^{3}H] Glucose and carrier-free $^{32}P_i$ were purchased from New England Nuclear.

[γ-^{32}P] ATP was synthesized by a P_i : ATP exchange reaction [17]. To produce dihydroxyacetone [^{32}P] phosphate, we then added dihydroxyacetone and glycerokinase (EC 2.7.1.30), without purifying the [γ-^{32}P] ATP [1]. Glycerol [^{32}P] phosphate was prepared similarly by substituting glycerol for dihydroxyacetone. Dihydroxyacetone [^{32}P] phosphate and glycerol [^{32}P] phosphate concentrations were measured with glycerol phosphate dehydrogenase [18,19]. Purity was determined by autoradiography after high voltage electrophoresis [20], both before and after reaction with glycerol phosphate dehydrogenase.

B-[4-^{3}H]NADPH was formed from D-[1-^{3}H]glucose with the hexokinase (EC 2.7.1.1) plus glucose-6-phosphate dehydrogenase (EC 1.1.1.49) reactions [8]. It was then purified by DEAE-cellulose chromatography [21]. The purity was measured after high voltage electrophoresis, both before and after transfer of the ^{3}H to glutamate with glutamate dehydrogenase (EC 1.4.1.3) [3].

Enzyme assays

The enzymes of the glycerol phosphate and the proposed acyl dihydroxyacetone phosphate pathways for phosphatidic acid synthesis were assayed in cell-free homogenates prepared by centrifuging for 10 min at 800 × *g* a 10% tissue homogenate in 0.25 M sucrose. The basis for this assay is the reported difference between the two pathways in specificity for pyridine nucleotide : glycerol phosphate dehydrogenase, in the glycerol phosphate pathway, is NADH-specific [22] but acyl dihydroxyacetone phosphate reductase (EC 1.1.1.101) is NADPH-specific [3]. [^{32}P]Phosphatidic acid synthesis from dihydroxyacetone [^{32}P]phosphate and NADH measures the rate of the glycerol phosphate pathway whereas synthesis from dihydroxyacetone [^{32}P]phosphate and NADPH measures the rate of the acyl dihydroxyacetone phosphate pathway. The rate of the acyl dihydroxyacetone phosphate pathway was also determined from the amount of [^{3}H]phosphatidic acid produced from dihydroxyacetone phosphate and B-[4-^{3}H]NADPH.

The following components were present in every incubation: 40 mM potassium phosphate (pH 7.5), 8 mM reduced glutathione, 8 mM NaF, 8 mM ATP, 8 mM $MgCl_2$, 1 mM dihydroxyacetone [^{32}P]phosphate (spec. act. 3—6 Ci per mol), 0.4 mM potassium palmitate, 0.04 mM CoA and homogenate, in a final volume of 1.2 ml. Unless otherwise stated, 1.0 mg of homogenate protein [23] was used. To correct for the possible presence of labeled impurities, we included a blank containing heat-denatured (10 min, 100°C) homogenate. The incubations also contained 0.1 mM NADH or 0.1 mM B-[4-^{3}H]NADPH (spec. act. about 20 Ci per mol) for the glycerol phosphate and acyl dihydroxyacetone phosphate pathways respectively. The incubation mixture was prewarmed 4 min at 37°C then the reaction started by the addition of homogenate. Unless otherwise stated, we terminated the incubations after an additional 4 min at 37°C by adding 4.5 ml of chloroform/methanol (1 : 2). The lipids were then extracted under acidic conditions [3] in order to completely extract acyl glycerol phosphate and acyl dihydroxyacetone phosphate [24]. We isolated the phosphatidic acid by thin-layer chromatography with the solvent chloroform/methanol/acetic acid/5% freshly-made aqueous busulfite (100 : 40 : 12 : 4, by vol.) [3,5]. The lipids were located by autoradiography and scraped from the plate. Before being counted, the scrapings were dispersed by sonication in a mixture of 0.6 ml of water and 10 ml of toluene-based scintillant containing 26% (by vol.) Triton X-100.

To measure dihydroxyacetone phosphate : acyl CoA acyltransferase (EC 2.3.1.42) we omitted the reduced pyridine nucleotides from the above described incubation. Essentially no phosphatidic acid is synthesized in this case (about 13 pmol). To measure glycerol phosphate : acyl CoA acyltransferase, we omitted the reduced pyridine nucleotides and replaced dihydroxyacetone [^{32}P]phosphate by glycerol [^{32}P]phosphate. The values given for dihydroxy-

acetone phosphate : acyl-CoA and glycerol phosphate : acyl CoA acyltransferase represent total ^{32}P incorporated into lipid. To measure the activity of glycerol phosphate dehydrogenase, we used the dihydroxyacetone phosphate-dependent oxidation of NADH [25]. The rate of NADH-oxidation that existed in the absence of dihydroxyacetone phosphate is subtracted.

Results

Assay conditions

In order to find suitable conditions for assaying the pathways for the tissue survey, we examined the effect of several assay variables. These include the dependence of phosphatidic acid synthesis upon the concentration of homogenate protein, the incubation period, the substrate concentration, the method for providing acyl CoA and the storage history of the tissue.

The formation of phosphatidic acid from either dihydroxyacetone phosphate or glycerol phosphate is roughly proportional to the amount of homogenate protein present (Fig. 2), which demonstrates that the assay system adequately measures enzyme concentration. There may be slightly less than the expected rate of phosphatidic acid synthesis from dihydroxyacetone phosphate via the glycerol phosphate pathway when small amounts of homogenate pro-

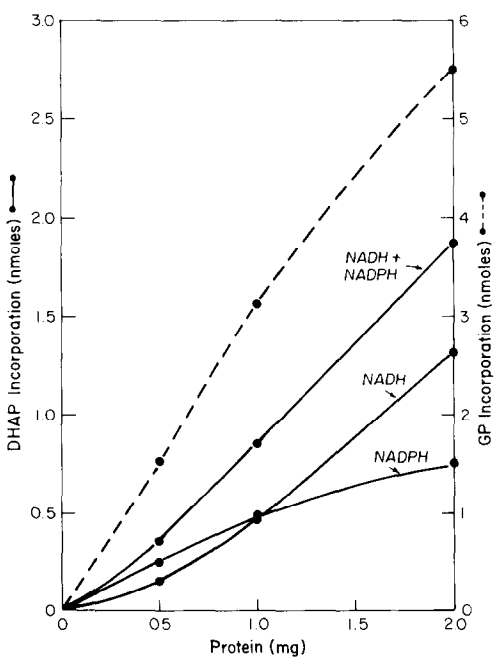

Fig. 2. Dependence of phosphatidic acid synthesis on the amount of homogenate protein present in the incubation. The indicated amounts of mouse brain homogenate protein were incubated 4 min at 37°C with 40 mM potassium phosphate (pH 7.5), 8 mM ATP, 8 mM $MgCl_2$, 8 mM reduced glutathione, 8 mM NaF, 0.4 mM potassium palmitate, 0.04 mM CoA, 1 mM dihydroxyacetone [^{32}P]phosphate and 0.1 mM NADH and 0.1 mM NADPH, as indicated, in a final volume of 1.2 ml. The dashed line represents [^{32}P]phosphatidic acid synthesized when 1 mM glycerol [^{32}P]phosphate replaced dihydroxyacetone [^{32}P]phosphate and reduced pyridine nucleotides were absent. Lipid was extracted and then analyzed by thin layer chromatography. Abbreviations: DHAP, dihydroxyacetone phosphate; GP, glycerol phosphate.

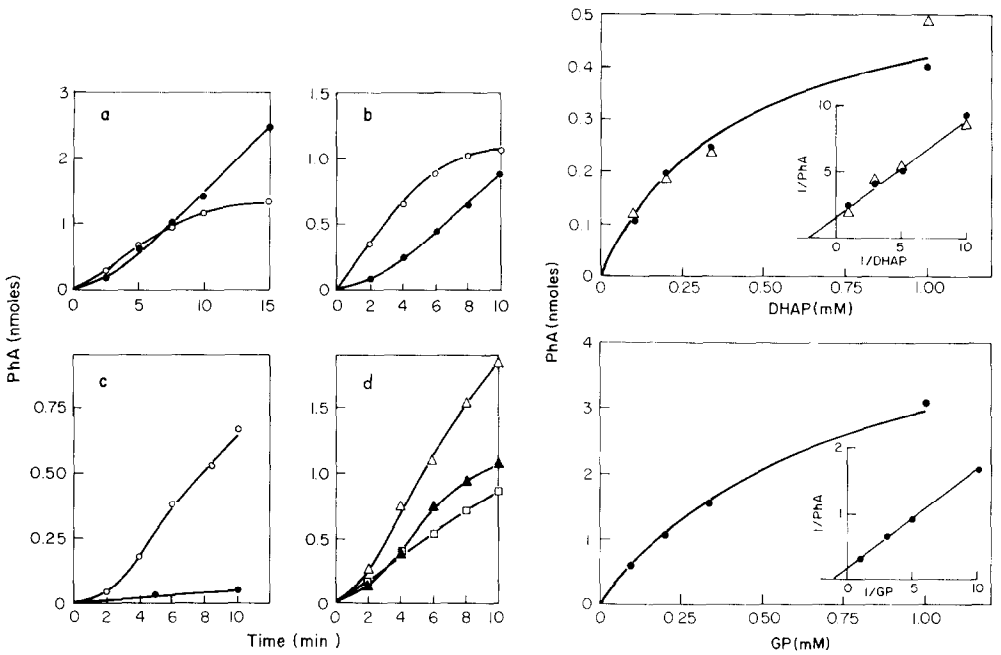

Fig. 3. Dependence of phosphatidic acid synthesis on the incubation period. The legend to Fig. 2 describes the incubation conditions. We used 1.0 mg of homogenate protein from the following sources: (a) and (d) mouse brain, (b) neuroblastoma C1300 and (c) rat thymocytes. The symbols are as follows: (●) [^{32}P]phosphatidic acid (PhA) synthesized from dihydroxyacetone [^{32}P]phosphate plus NADH; (○) [^{32}P]phosphatidic acid synthesized from dihydroxyacetone [^{32}P]phosphate plus NADPH; (△) [^{32}P]-phosphatidic acid synthesized from dihydroxyacetone [^{32}P]phosphate when both NADH and NADPH are present; (▲) [^{3}H]phosphatidic acid synthesized from dihydroxyacetone phosphate plus B-[4-^{3}H]-NADPH and (□) [^{3}H] phosphatidic acid synthesized from dihydroxyacetone phosphate when both B-[4-^{3}H] NADPH and NADPH are present.

Fig. 4. Dependence of phosphatidic acid synthesis on the concentration of the glyceride-glycerol precursor. 1.0 mg homogenate protein from mouse brain was incubated with the indicated concentrations of dihydroxyacetone [^{32}P]phosphate or glycerol [^{32}P]phosphate. Conditions are in other respects identical to those described for Fig. 2 except that 0.1 mM NADH was included in the incubation containing glycerol [^{32}P]phosphate. (●) represents use of 0.1 mM NADH whereas (△) represents use of 0.1 mM NADPH. Abbreviations: DHAP, dihydroxyacetone phosphate; GP, glycerol phosphate; PhA, phosphatidic acid.

tein are used. We chose to use 1.0 mg of homogenate protein in subsequent assays.

The time-dependent incorporation of dihydroxyacetone [^{32}P]phosphate into phosphatidic acid by the two pathways is shown for three tissues that appear to differ greatly in their relative activities of the two pathways (Fig. 3). There is a lag in the rate of appearance of phosphatidic acid, presumably reflecting the sequential accumulation and conversion of intermediate products. In thymocytes, the rate of phosphatidic acid formation via glycerol phosphate remains very low during the 10 min incubation, and it does not increase if the incubation time is extended to 30 min. This suggests that the low rate of phosphatidic acid synthesis via the glycerol phosphate pathway does not result from a prolonged lag, as might be due, for example, to glycerol phosphate accumulation. The time-dependence of ^{3}H incorporation into phos-

phatidic acid from B-[4-^{3}H]NADPH in the presence and absence of NADH is shown in Fig. 3d. An incubation period of 4 min was used for further studies.

The effect of dihydroxyacetone phosphate concentration on the rates of incorporation into phosphatidic acid via glycerol phosphate and acyl dihydroxyacetone phosphate is shown in Fig. 4. The apparent K_m for dihydroxyacetone phosphate is the same for the two pathways in brain homogenate. This suggests that the relative rates of dihydroxyacetone phosphate incorporation into phosphatidic acid via the two pathways should not depend on the dihydroxyacetone phosphate concentration. A dihydroxyacetone phosphate concentration of 1 mM was chosen for subsequent assays. Also shown in Fig. 4 is the dependence of phosphatidic acid synthesis on glycerol phosphate concentration. The K_m values for glycerol precursors are relatively large, about 0.5 mM and 0.8 mM for dihydroxyacetone phosphate and glycerol phosphate, respectively. This indicates that, for the glycerol phosphate pathway, the rate of the glycerol phosphate : acyl CoA acyltransferase step might increase with time as glycerol phosphate, formed from dihydroxyacetone phosphate, accumulates. The data of Fig. 3 suggests this is not very significant. A steady state for the glycerol phosphate pathway is apparently attained within 2—4 min.

The linear dependence of phosphatidic acid synthesis upon both protein concentration (Fig. 2) and incubation period (Fig. 3) implies that none of the substrates are depleted. To confirm this, we determined the substrate concentrations at the end of the brain and liver assays (Table I). Except for NADH in the liver assay, the substrates were not depleted. In both the brain and liver

TABLE I

METABOLITE CONCENTRATIONS AFTER INCUBATION WITH BRAIN AND LIVER HOMOGENATES

Brain and liver homogenates containing 1.0 mg protein were incubated 4 min at 37°C in the following medium: 40 mM potassium phosphate (pH 7.5), 8 mM ATP, 8 mM $MgCl_2$, 8 mM reduced glutathione, 8 mM NaF, 0.4 mM potassium palmitate and 0.04 mM CoA in a final volume of 1.2 ml. Where indicated, the following substrates were also present: 1 mM dihydroxyacetone phosphate (0.68 mM for liver), 1 mM glycerol phosphate (0.86 mM for brain), 0.09 mM NADH and 0.09 mM NADPH. For measurement of dihydroxyacetone phosphate and glycerol phosphate, the assays were terminated with perchloric acid [19,26], whereas, for measurement of NADH and NADPH, they were terminated with ethanolic KOH [27]. The metabolite concentrations were determined by published procedures [19,26,27].

Assay conditions	Metabolite concentration (μM)*					
	DHAP	Fru-1,6-P_2	Gald-P	NADH	NADPH	GP
Brain						
DHAP	790	94	33	—		0
DHAP + NADH	630	70	24	30	0	120
DHAP + NADPH	810	82	33	2	55	3
GP	24	4	0	—	—	850
Liver						
DHAP	410	20	17	—	—	0
DHAP + NADH	150	6	4	2	0	270
DHAP + NADPH	440	22	18	0	12	0
GP	8	0	0	—	—	1030

* Abbreviations: Fru-1,6-P_2, fructose 1,6-diphosphate; Gald-P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; GP, glycerol phosphate.

assays, glycerol phosphate is practically inert with little being converted to dihydroxyacetone phosphate. Dihydroxyacetone phosphate, glycerol phosphate, fructose 1,6-diphosphate and glyceraldehyde 3-phosphate account for 85—95% and 60—70% of the dihydroxyacetone phosphate added to the brain and liver assays, respectively.

In both the brain and liver assays, apparently more glycerol phosphate is formed from dihydroxyacetone phosphate plus NADH than permitted by the limiting precursor, NADH. This suggests that NAD can be reduced to NADH, perhaps with dihydroxyacetone phosphate through the reaction sequence catalyzed by triose phosphate isomerase (EC 5.3.1.1), glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) and 3-phosphoglycerate kinase (EC 2.7.2.3). Supporting this explanation are the observations that (1) with either dialyzed or undialyzed rat brain homogenate, NAD is as effective as NADH for phosphatidic acid synthesis from dihydroxyacetone phosphate and (2) the triose phosphate isomerase inhibitor, 1-hydroxy-3-chloro-2-propanone phosphate [28], and the glyceraldehyde phosphate dehydrogenase inhibitor, tetrose phosphate [29], prevent this non-stoichiometric formation of glycerol phosphate in rat brain homogenate. Glycerol phosphate dehydrogenase is probably fully active under the conditions used for assaying lipid synthesis, since the amount of glycerol phosphate formed from dihydroxyacetone phosphate plus NADH is about that expected from the measured glycerol phosphate dehydrogenase activity of brain and liver (See Table V). As expected, no glycerol phosphate was formed when NADH was replaced by NADPH.

The procedure chosen for assaying the various lipid-synthesizing activities uses endogenous fatty acid thiokinase (EC 6.2.1.3) to generate acyl CoA. To check whether the relative rates of the two pathways reflect the method for providing acyl CoA, the rates of dihydroxyacetone phosphate : acyl CoA and

TABLE II

COMPARISON OF THE RATES OF GLYCEROL PHOSPHATE:ACYL CoA ACYLTRANSFERASE AND DIHYDROXYACETONE PHOSPHATE:ACYL CoA ACYLTRANSFERASE UNDER DIFFERENT CONDITIONS

The incubation medium for the acyl CoA-generating system is that described for Fig. 2 but reduced pyridine nucleotides were omitted. 1.0 mg protein in brain homogenates from either adult or fetal rats was used. Where acyl CoA was added the incubation medium included 100 mM imidazole formate (pH 7.4), 40 mM NaF, 5 mM $MgCl_2$ 2 mM dithiothreitol, 0.5% (w/v) fatty acid-poor bovine serum albumin, 5 mM dihydroxyacetone [^{32}P]phosphate or 5 mM glycerol [^{32}P]phosphate and 0.1 mM palmitoyl CoA, in a final volume of 1.0 ml. Also present were 1.8 mg or 1.3 mg protein in brain homogenates from adult or fetal rats, respectively. Each incubation lasted 4 min at 37°C.

Conditions	Acyltransferase*	Rate (pmol · min^{-1} · mg^{-1})	
		Adult	Fetus
Acyl CoA-generating system	GP	470	240
	DHAP	79	86
	Ratio:	5.9	2.8
Acyl CoA added	GP	960	1300
	DHAP	260	440
	Ratio:	3.7	3.0

* Abbreviations: GP, glycerol phosphate; DHAP, dihydroxyacetone phosphate.

TABLE III

THE EFFECT OF STORAGE ON ENZYME ACTIVITIES

Neuroblastoma C1300 was stored in 500 mg pieces at −70°C for the indicated times. Activities were measured as described for Tables IV and V. Rates are given in pmol · min^{-1} · mg^{-1} except for glycerol phosphate dehydrogenase which is given in nmol · min^{-1} · mg^{-1}.

Storage	[^{32}P]DHAP → [^{32}P]PhA		Acyl transferase		Glycerol phosphate dehydrogenase
	via Acyl-DHAP	via GP	to DHAP	to GP	
Fresh	160	60	190	1060	14
One month	180	60	190	970	16
Ten months	190	50	150	1050	14

Abbreviations: DHAP, dihydroxyacetone phosphate; GP, glycerol phosphate; Acyl-DHAP, acyl dihydroxyacetone phosphate; PhA, phosphatidic acid.

glycerol phosphate : acyl CoA acyltransferase with the acyl CoA generating system were compared to those with added palmitoyl CoA (Table II). Although greater rate of phosphatidic acid synthesis from glycerol [^{32}P] phosphate (the acyltransferase activities is not dependent on the source of acyl CoA. The much greater rate of phosphatidic acid synthesis from glycerol[^{32}P] phosphate (the glycerol phosphate : acyl CoA acyltransferase assay) than from dihydroxyacetone [^{32}P] phosphate plus NADH (the glycerol phosphate pathway), seen in Tables IV and V, confirms that the fatty acid thiokinase step is not seriously rate-limiting for the glycerol phosphate pathway under our assay conditions.

The brain tumors and cultured glial cells were stored about two years at −70°C before use. To test the possibility that some of the enzymes had denatured during storage, we assayed fresh neuroblastoma and reassayed additional samples after storage at −70°C (Table III). The stability of this tissue to storage suggests that the other stored tissue samples were not significantly denatured. We also measured the lactate dehydrogenase (EC 1.1.1.27) activities of these stored tissues to determine whether their low glycerol phosphate dehydrogenase activities reflect extensive denaturation of soluble enzymes, in general. Although the glycerol phosphate dehydrogenase activities of the stored tissues were usually lower (See Table V), their lactate dehydrogenase activities were higher than that of fresh mouse brain.

Tissue survey

A summary of the glycerol phosphate and acyl dihydroxyacetone phosphate pathway activities found with a variety of tissue homogenates is shown in Table IV. The relative ability of these tissues to form phosphatidic acid from dihydroxyacetone phosphate via acyl dihydroxyacetone phosphate is greater than via glycerol phosphate. Although the rates of both pathways tend to decrease together, the differences between pathway ratios are mainly due to changes in the rate of the glycerol phosphate pathway. Thus, the tissue homogenates that showed a high pathway ratio had a low rate of phosphatidate synthesis via glycerol phosphate rather than a high rate of synthesis via acyl dihydroxyacetone phosphate.

The two methods for measuring the acyl dihydroxyacetone phosphate

TABLE IV

THE RELATIVE RATES OF THE TWO PATHWAYS IN TISSUE HOMOGENATES

Tissue homogenates containing 1.0 mg protein were incubated 4 min at 37°C with 1 mM dihydroxyacetone [^{32}P]phosphate. Either 0.1 mM NADH or 0.1 mM B-[4-^{3}H]NADPH was added for measuring the glycerol phosphate and acyl dihydroxyacetone phosphate pathways, respectively. In the absence of added reduced pyridine nucleotide, very little phosphatidic acid was formed (about 13 pmol). Other incubation conditions are as described for Fig. 2. Rates are expressed as pmol · min^{-1} · mg^{-1} and are given ± S.E. Unless otherwise indicated, the number of times each tissue was assayed is given in parentheses before the name of the tissue.

(*n*) Source	Pathway ratio[d]	[^{32}P]DHAP → [^{32}P]PhA		DHAP $\xrightarrow{[^{3}H]NADPH}$ [^{3}H]PhA	
		via Acyl-DHAP	via GP	Acyl-DHAP path only	Both paths operating[e]
(3) Nitrosourea-transformed astroglia (50 C-2$_1$)	1.1 ± 0.2	110 ± 31	100 ± 16	140 ± 41	130 ± 48
(18) Brain	1.3 ± 0.1	81 ± 3	64 ± 5	79 ± 4	70 ± 4
(2) Liver	1.8 ± 0.2	310 ± 70	170 ± 20	215[a]	213[a]
(2) Neuroblastoma (C1300)	2.9 ± 0.1	170 ± 10	59 ± 1	146 ± 5	146 ± 6
(3) SV-40 Transformed fetal astroglia (32 THA-15)	6 ± 2	60 ± 17	13 ± 4.4	60 ± 17	60 ± 13
(1) Fetal brain	6	76	12	77	83
(4) Nitrosourea-transformed astroglia (44 C-6)	7 ± 3	80 ± 26	30 ± 23	135 ± 9.1	130[a]
(2) Leukemia cells (I_b)	7.4 ± 0.7	140 ± 12	18 ± 1	112 ± 2	114 ± 4
(3) Splenic leukocytes	10 ± 1.3	90 ± 32	8 ± 2.5	80 ± 28	61 ± 3[b]
(6) SV-40 Transformed newborn astroglia (61 NT-1)	10 ± 1	58 ± 5	6 ± 1.7	68 ± 3.7	66 ± 5[c]
(1) Lymphocytes	12	20	1.7	19	19
(5) Cultured astroglia	14 ± 3	40 ± 7	3 ± 0.7	70 ± 15	70 ± 15
(2) Thymocytes	20 ± 9	56 ± 11	3 ± 1.2	61 ± 4	64[a]

[a] n = 1, [b] n = 2, [c] n = 5. [d] Pathway ratio = rate of Acyl-DHAP pathway : rate of GP pathway.

[e] The rate at which B-[4-^{3}H]NADPH labels PhA when NADH is also present.

Abbreviations: DHAP, dihydroxyacetone phosphate; GP, glycerol phosphate; Acyl-DHAP, acyl dihydroxyacetone phosphate; PhA, phosphatidic acid.

TABLE V

THE DISCREPANCY BETWEEN THE PATHWAY RATIO AND THE ACTIVITY OF THE INDIVIDUAL PATHWAY ENZYMES

Glycerol phosphate dehydrogenase activity of the tissue homogenate was assayed using dihydroxyacetone phosphate-dependent oxidation of NADH. Glycerol phosphate and dihydroxyacetone phosphate acyltransferases were assayed by measuring the incorporation into total lipids of glycerol [^{32}P]phosphate and dihydroxyacetone [^{32}P]phosphate, respectively. 1 mM glycerol [^{32}P]phosphate or dihydroxyacetone [^{32}P]phosphate was incubated 4 min at 37°C with 1 mg homogenate protein in the medium described in Fig. 2. Reduced pyridine nucleotides were not added. Analysis of the labeled lipids by thin-layer chromatography indicated that 94% of the glycerol [^{32}P]phosphate was incorporated into phosphatidic acid and acylglycerol phosphate. 75% of the dihydroxyacetone [^{32}P]phosphate was incorporated into acyldihydroxyacetone phosphate. Rates are given ± S.E. where the value of n is the same as for Table IV except as indicated.

Source	Pathway ratio	Glycerol phosphate dehydrogenase (nmol · min^{-1} · mg^{-1})	Acyl transferase	
			to GP (pmol · min^{-1} · mg^{-1})	to DHAP
Nitrosourea-transformed astroglia (50 C-2$_1$)	1.1	71 ± 10	400 ± 130	120 ± 50**
Brain	1.3	44 ± 2	590 ± 29	96 ± 5
Liver	1.8	260 ± 20	1200 ± 400	210 ± 40
Neuroblastoma (C1300)	2.9	14 ± 1	1010 ± 43	188 ± 2
SV-40 Transformed fetal astroglia (32 THA-15)	6	7 ± 4	390 ± 49	80 ± 18
Fetal brain	6	3	240	86
Nitrosourea-transformed astroglia (44 C-6)	7	30 ± 20***	200 ± 170**	160*
Leukemia cells (I$_b$)	7.4	3 ± 0.6	600 ± 140	130 ± 12
Splenic leukocytes	10	8 ± 4	170 ± 60	80 ± 25**
SV-40 transformed newborn astroglia (61 NT-1)	10	5.6 ± 0.8	250 ± 29	72 ± 4
Lymphocytes	12	0.4	74	47
Cultured astroglia	14	1.8 ± 0.6	106 ± 8	50 ± 12
Thymocytes	20	4.5 ± 0.4	300 ± 250	61 ± 5

*n = 1, **n = 2, ***n = 3.

Abbreviations: DHAP, dihydroxyacetone phosphate; GP, glycerol phosphate.

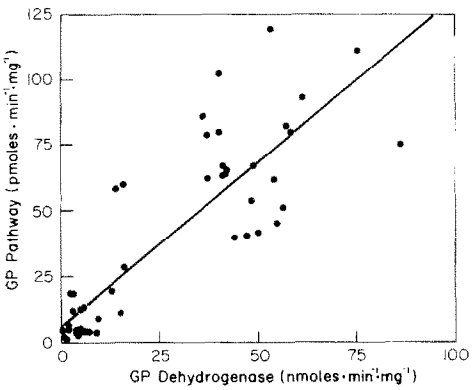

Fig. 5. Dependence of the rate through the glycerol phosphate (GP) pathway on glycerol phosphate dehydrogenase activity. The points are the individual values whose averages are given in Tables IV and V. The correlation coefficient is significant ($P < 0.001$).

pathway gave similar values*. ^{3}H entered phosphatidic acid from B-[4-^{3}H]-NADPH as rapidly as ^{32}P enters from dihydroxyacetone [^{32}P]phosphate. Furthermore, when both pathways are operating, ^{3}H enters phosphatidic acid at 91% the rate observed when only the acyl dihydroxyacetone phosphate pathway is active. Thus, the two pathways seem to operate independently. This independence shows that NADPH is not supporting phosphatidic acid synthesis by first forming NADH via a transhydrogenase.

The rates of the separate enzyme steps in the two pathways are summarized in Table V. As indicated by previous work [3], the rate-limiting step in the acyl dihydroxyacetone phosphate pathway is dihydroxyacetone phosphate : acyl CoA acyltransferase. As either glycerol phosphate : acyl CoA acyltransferase or glycerol phosphate dehydrogenase increases, the rate of the glycerol phosphate pathway also increases (Fig. 5). Thus, these two steps act rate-limiting to the glycerol phosphate pathway. Since their individual rates are each much greater than the measured rate of the glycerol phosphate pathway, this is unexpected. Table V also shows that the enzymes of the glycerol phosphate pathway are more active than those of the acyl dihydroxyacetone phosphate pathway. Thus the data in Table V imply a much more active glycerol phosphate than acyl dihydroxyacetone phosphate pathway, whereas the data in Table IV support the opposite conclusion.

Discussion

The purpose of this study is to help answer the question: do tissues differ in their dependence on acyl dihydroxyacetone phosphate for synthesizing glycerolipids? The data in Table IV show that homogenates of several tissues, including experimental tumors, have a strong capacity for glycerolipid synthesis

* B-[4-^{3}H]NADH was not used to assay the glycerol phosphate pathway [8] since it was found that in rat brain the $^{3}H/^{32}P$ ratio in phosphatidic acid is less than one. The deficiency in ^{3}H incorporation from B-[4-^{3}H]NADH may be due to NADH recycling.

via acyl dihydroxyacetone phosphate. There did not seem to be an unusual reliance by tumors upon the acyl dihydroxyacetone phosphate pathway, since the homogenates showing the highest pathway ratios were those of normal tissues. The effect of transformation upon the pathway ratio was not rigorously examined, however.

Tissue homogenates differ in their dependence on the acyl dihydroxyacetone phosphate pathway, but these differences are primarily due to changes in the rate of the glycerol phosphate pathway. The measured activities of the acyl dihydroxyacetone phosphate pathway do not increase as those of the glycerol phosphate pathway decrease. Thus, those tissues which show little glycerol phosphate pathway activity should have a decreased ability to synthesize glycerolipids. As suggested by previous work [7,8], the apparent utilization by a tissue of the acyl dihydroxyacetone phosphate pathway is greater for those tissues with little glycerol phosphate dehydrogenase.

The relative rates of dihydroxyacetone phosphate incorporation via the two pathways (Table IV) are difficult to reconcile with the observed activities of the separate steps (Table V). The measured enzyme activities of limiting steps in the glycerol phosphate pathway are several fold higher than the limiting step in the acyl dihydroxyacetone phosphate pathway.

The way in which the pathways are compared (Table IV) offers one possible explanation for this discrepancy. Our choice of dihydroxyacetone phosphate as the precursor and use of a short incubation period may bias the estimate of the pathways in favor of the acyl dihydroxyacetone phosphate pathway. We have previously reported that the use of dihydroxyacetone phosphate as the precursor gives a higher acyl dihydroxyacetone phosphate/glycerol phosphate pathway ratio than does the use of fructose 1,6-diphosphate or glucose [8]. With dihydroxyacetone phosphate as the precursor, dihydroxyacetone phosphate : acyl CoA acyltransferase is exposed to a high dihydroxyacetone phosphate concentration from the start of the incubation but glycerol phosphate : acyl CoA acyltransferase is exposed to no glycerol phosphate. As the incubation proceeds, the glycerol phosphate concentration increases and the rate of the glycerol phosphate pathway should increase. For example, the expected rate of the glycerol phosphate pathway in thymocyte homogenate can be calculated from the measured rates of glycerol phosphate dehydrogenase and glycerol phosphate : acyl CoA acyltransferase in the homogenate and the K_m for glycerol phosphate of glycerol phosphate : acyl CoA acyltransferase seen in brain homogenate (Fig. 4). However, the observed rate of the glycerol phosphate pathway (Fig. 3c) fails to increase. The observed rate is ten-fold less than the calculated rate after ten min of incubation.

A second possible explanation for the discrepancy between the measured pathway ratio (Table IV) and the activity of the pathway enzymes (Table V) is that we are misinterpreting the measured glycerol phosphate dehydrogenase activity. Perhaps only a fraction of the total enzyme is active in glycerolipid synthesis. It has been reported [30] that a significant percentage (20—60%) of the total glycerol phosphate dehydrogenase in liver and kidney may be peroxisomal and therefore, presumably, not used for glycerolipid synthesis. Also, there is evidence that much of the liver glycerol phosphate dehydrogenase may be used in catabolism rather than in glycerolipid synthesis [31]. Finally, a

membrane-bound glycerol phosphate dehydrogenase has been observed but its function is unknown [3,30].

It is reported that the physiological glycerol phosphate concentration is much greater than the dihydroxyacetone phosphate concentration. In liver and brain, the glycerol phosphate/dihydroxyacetone phosphate ratio is about ten [32,33]. Even in cells with very little glycerol phosphate dehydrogenase activity, such as Ehrlich ascites, the glycerol phosphate/dihydroxyacetone phosphate ratio is also reported to be about ten [34]. In addition, the glycerol phosphate concentration in a mammary gland tumor is as great as that in normal gland, yet the former has much less glycerol phosphate dehydrogenase [35]. Given the greater activity of glycerol phosphate acyltransferase than dihydroxyacetone phosphate acyltransferase in the tissues used (Table V) and a glycerol phosphate concentration greater than dihydroxyacetone phosphate, it remains probable that in vivo the glycerol phosphate pathway to glycerolipids is more active than the acyl dihydroxyacetone phosphate pathway.

On the other hand, two observations are consistent with a significant contribution by the acyl dihydroxyacetone phosphate pathway to glycerolipid synthesis. First, most reductive, synthetic pathways use NADPH rather than NADH [36]. The 10^5-fold higher ratio of NADPH/NADP than NADH/NAD facilitates synthesis using NADPH [37]. The acyl dihydroxyacetone phosphate pathway fits this generalization, but the glycerol phosphate pathway does not. Second, an assay of the glycerol phosphate pathway in liver slices implied that about one-half of the glycerolipid is synthesized from acyl dihydroxyacetone phosphate [38]. This assay uses glycerol and, thus, it does not include the normal branch point of the metabolic sequence (Fig. 1). Glucose is the usual glyceride glycerol precursor [39], and its conversion to glycerol phosphate is subject to possible regulation by the intracellular NADH/NAD ratio. Thus, the acyl dihydroxyacetone phosphate pathway in the liver in vivo could be even more active than that reported.

We are currently examining this assay of liver slices, since it is not yet established how much the acyl dihydroxyacetone phosphate pathway contributes to lipid synthesis in liver. It has recently been reported [40] that isolated liver cells show less dependence on the acyl dihydroxyacetone phosphate pathway than that claimed for liver slices [38]. The two reports are not readily comparable because of the different incubation conditions used, but, even with the liver cells [40], there is evidence for some lipid synthesis via the acyl dihydroxyacetone phosphate pathway (Experiment 2, ref. 40).

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