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Proportional Activities of Glycerol Kinase and Glycerol 3-Phosphate Dehydrogenase in Rat Hepatomas

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1. The activities of glycerol 3-phosphate dehydrogenase (EC 1.1.1.8), glycerol kinase (EC 2.7.1.30), lactate dehydrogenase (EC 1.1.1.27), 'malic' enzyme (L-malate–$NADP^+$ oxidoreductase; EC 1.1.1.40) and the β-oxoacyl-(acyl-carrier protein) reductase component of the fatty acid synthetase complex were measured in nine hepatoma lines (8 in rats, 1 in mouse) and in the livers of host animals. 2. With the single exception of Morris hepatoma 16, which had unusually high glycerol 3-phosphate dehydrogenase activity, the activities of glycerol 3-phosphate dehydrogenase and glycerol kinase were highly correlated in normal livers and hepatomas ($r = 0.97$; $P<0.01$). The activities of these two enzymes were not strongly correlated with the activities of any of the other three enzymes. 3. The primary function of hepatic glycerol 3-phosphate dehydrogenase appears to be in gluconeogenesis from glycerol.

Glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) can serve three distinct metabolic functions. First, it can help maintain the cytoplasmic NAD^+ redox state during anaerobic glycolysis (Klingenberg & Bucher, 1960; Krebs & Woodford, 1965; White & Kaplan, 1972). Secondly, it can furnish the glycerol backbone for glyceride biosynthesis (Weiss & Kennedy, 1956; Lamb & Fallon, 1974; Rognstad *et al.*, 1974). Thirdly, glycerol 3-phosphate dehydrogenase can participate in gluconeogenesis from glycerol (Exton *et al.*, 1970). Although all these metabolic functions are documented, their relative importance for a particular enzyme or isoenzyme *in vivo* is not defined.

There are several reasons to question a major role for liver glycerol 3-phosphate dehydrogenase in glyceride biosynthesis. Glycerol 3-phosphate dehydrogenase represents one of the few exceptions to the generalization that NADPH rather than NADH is the cofactor used by enzymes involved in reductive biosynthesis (Lowenstein, 1961). Next, the acyl dihydroxyacetone phosphate pathway, which by-passes glycerol 3-phosphate dehydrogenase, provides an alternative for the synthesis of glycerides (Rao *et al.*, 1968; Hajra, 1968; Hajra & Agranoff, 1968). Finally, the change in activity of hepatic glycerol 3-phosphate dehydrogenase in chicken in response to dietary stress (Harding *et al.*, 1975) is opposite to that of enzymes involved in lipid biosynthesis (Goodridge, 1968; Pearce, 1968; Goodridge, 1973) and similar to that of enzymes with a gluconeogenic function (Ashmore & Weber, 1968).

Glycerol 3-phosphate dehydrogenase is usually absent or present in low amounts in most tumours (Boxer & Shonk, 1960). Rat hepatomas show a spectrum of glycerol 3-phosphate dehydrogenase activities ranging from very low activities in rapidly growing lines to near normal activities in the slowest growing lines (Shonk *et al.*, 1965; Weber & Lea, 1966). This pattern is similar to that observed for a variety of gluconeogenic enzymes (Weber, 1963; Weber *et al.*, 1961, 1965). In order for glycerol 3-phosphate dehydrogenase to serve as a gluconeogenic enzyme, glycerol kinase (EC 2.7.1.30) must be present to convert glycerol into glycerol 3-phosphate. The activity of glycerol kinase in a variety of hepatomas is reported here for the first time. In addition, we demonstrate that there is a very high positive correlation between glycerol kinase and glycerol 3-phosphate dehydrogenase activities in these rat hepatomas and also in the livers of host rats. There is very little correlation between the activities of either enzyme and 'malic' enzyme (L-malate–NAD^+ oxidoreductase, EC 1.1.1.40) the β-oxoacyl-(acyl-carrier protein) reductase component of the fatty acid synthetase complex, or lactate dehydrogenase (EC 1.1.1.27).

Materials and Methods

Chemicals

NADH, NADPH, $NADP^+$, ATP, creatine phosphate, creatine kinase (EC 2.7.3.2), sodium pyruvate and the dimethylketal bismonocyclohexylammonium salt of dihydroxyacetone phosphate were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The ketal of dihydroxyacetone phosphate was

hydrolysed and the salt converted into the free acid with Dowex-50 by using the procedure suggested by the manufacturer. 2-Mercaptoethanol and the disodium salt of EDTA were supplied by Eastman Organic Chemicals (Rochester, N.Y., U.S.A.). $MgSO_4$, NaF, $MnCl_2$ and DL-malic acid were from Fisher Chemical Co. (King of Prussia, Pa., U.S.A.).

The DE-81 ion-exchange filter discs used in the glycerol kinase assay were purchased from Reeve Angel (Clifton, N.J., U.S.A.). [1-^{14}C]Glycerol used in the same assay was from International Chemical and Nuclear Corp. (Waltham, Mass., U.S.A.). Aquasol supplied by New England Nuclear Corp. (Boston, Mass., U.S.A.) was the scintillation solution used.

(±)-*trans*-1,2,3,4,4a,5,8,8a,-Octahydronaphthalene-1,4-dione (m.p. 93°C) which was used as the substrate in the assay of the β-oxoacyl-(acyl-carrier protein)-reductase component of the fatty acid synthetase complex (Dutler *et al.*, 1971; Harding *et al.*, 1975) was synthesized from benzoquinone (Fisher Chemical Co.) and butadiene (Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.) by the method of Robins & Walker (1958).

Animals

The tumour-bearing rats used in this laboratory were provided by the laboratory of Dr. H. P. Morris at Howard University. The rats were either Buffalo or ACI/c strain. The hepatoma lines studied included 3924A, 5123D, 7793, 7787, 16, 21, 44 and 9618B. Transplantation was carried out at Howard University by the method of Morris & Wagner (1968). The rats were maintained on Purina rat chow at the University of Delaware until the tumours were large enough to be excised cleanly from surrounding thigh tissue.

The single mouse hepatoma studied, BW7756, was selected because it was reported to have near-normal activities of glycerol 3-phosphate dehydrogenase (Kopelovich & Sabine, 1970). Tumour-bearing mice and normal C57L/J mice, which were to be used as hosts, were supplied by Jackson Laboratory in Bar Harbor, Maine, U.S.A. Transplantation was carried out at the University of Delaware as mentioned above. The mice were also maintained on a standard commercial diet until the tumours were large enough to excise.

Methods

Preparation of homogenates. Animals were killed by asphyxiation with chloroform. Immediately after death, the tumours and the liver of each animal were excised and contaminating or necrotic tissue was removed. Solid well-defined tumour tissue was used for assays. The tissues were frozen and stored at −20°C. All tissues were assayed within 2 weeks, most on the same day as they were prepared.

Portions (0.7–1.2g) of each liver and tumour were homogenized with a motor-driven Teflon pestle in 4vol. (4.0ml/g wet wt. of tissue) of 5mM-Tris–HCl buffer, pH7.6, containing 1mM-EDTA and 10mM-2-mercaptoethanol. Crude homogenates were centrifuged at 4°C for 30min at 39000*g* in a Sorvall RC-2B centrifuge. The clear portion of the supernatant was used for the enzyme and protein assays.

Assay for NAD$^+$-linked glycerol 3-phosphate dehydrogenase. Glycerol 3-phosphate dehydrogenase was determined spectrophotometrically by the method of White & Kaplan (1969).

Assay for glycerol kinase. Glycerol kinase activity was measured by the radioactive method described by Newsholme *et al.* (1967) except that a creatine kinase system for regenerating ATP was used (Robinson & Newsholme, 1969). The DEAE filter-paper discs were placed into vials containing 10ml of scintillation fluid and counted for radioactivity in a Beckman LS-100 liquid-scintillation counter.

Assay for L-lactate dehydrogenase. Lactate dehydrogenase activity was measured by the method of Pesce *et al.* (1964).

Assay for 'malic' enzyme. 'Malic' enzyme activity was measured by the method of Hsu & Lardy (1969).

Assay for β-oxoacyl-(acyl-carrier protein) reductase activity of the fatty acid synthetase complex. The β-oxoacyl-(acyl-carrier protein) reductase activity of fatty acid synthetase was determined by a method similar to that of Dutler *et al.* (1971), except that (±)-*trans*-1,2,3,4,4a,5,8,8a-octahydronaphthalene-1,4-dione was used as a substrate (Harding *et al.*, 1975) rather than (±)-*trans*-decahydronaphthalene-1,4-dione. About 70% of the dione reductase activity is attributable to β-oxoacyl-(acyl-carrier protein) reductase (J. Harding, A. Ullman & H. White, unpublished work). The activities reported are not corrected for non-specific dione reductase activity.

Protein assay. Protein concentrations were determined by the methods of Warburg & Christian (1942) and Lowry *et al.* (1951) with bovine serum albumin as standard.

Electrophoresis. Polyacrylamide-gel electrophoresis was carried out on extracts of all the hepatoma lines studies and host livers. A Hoefer SE 600 vertical slab electrophoresis unit was used. The electrolyte buffer for all runs was Tris–glycine, pH8.3. A 10% separating gel, pH8.9, was used. Samples were applied in 20% (w/v) sucrose (Davis, 1964; Ornstein, 1964).

Enzyme-specific stains were used to detect lactate dehydrogenase and glycerol 3-phosphate dehydrogenase isoenzymes. Staining solutions were prepared as described by Fine & Costello (1963) except that a transhydrogenase is not required for the glycerol 3-phosphate dehydrogenase stain.

Table 1. *Activities of the β-oxoacyl-(acyl-carrier protein) reductase component of the fatty acid synthetase complex, 'malic' enzyme, lactate dehydrogenase, glycerol kinase and glycerol 3-phosphate dehydrogenase in rat and mouse hepatomas and their corresponding host livers*

The hepatomas are arranged in order of increasing time between transplants. The average time in months between transfers for the last ten transfers is indicated in parentheses next to each tumour line. The values for hepatomas 16 and 21 are based on the last eight and nine transfers respectively. Line BW7756 is a mouse hepatoma in strain C57L/J. The remaining hepatomas are maintained in Buffalo strain rats except for line 3924A which is in ACI/c strain rats. Host animals were female for hepatoma lines BW7756, 3924A, 7793 and 5123D and male for the remaining lines. The enzyme activity values are means±S.E.M. for the number of different samples in parentheses. The average values for livers from host rats include Buffalo strain livers only (3924A host livers are excluded). The activities reported for β-oxoacyl-(acyl-carrier protein) reductase component of fatty acid synthetase have not been corrected for the non-specific reduction of the dione used as an artificial substrate.

	Enzyme activity (μmol/min per mg of protein)								Average values from rat host livers
Tumour line ...	BW7756 (0.5)	3924A (0.9)	7793 (1.3)	5123D (1.6)	44 (4.9)	16 (5.6)	7787 (6.3)	21 (7.3)	9618B (8.4)	
10^2×β-Oxoacyl-(acyl-carrier protein) reductase activity										
Tumour	5.0±0.7 (6)	4.7±0.2 (4)	2.7±0.2 (4)	3.8±0.6 (4)	3.9±0.4 (4)	4.3±0.2 (4)	5.2±0.6 (4)	2.7±0.2 (4)	3.9±0.1 (4)	
Host	3.0±0.2 (6)	2.7±0.2 (4)	4.1±0.3 (4)	4.3±0.5 (4)	3.8±0.1 (2)	5.8±0.5 (2)	4.6±0.8 (4)	5.1±0.05 (2)	2.0±0.2 (2)	3.92
10^2×'Malic' enzyme activity										
Tumour	2.4±0.3 (6)	1.8±0.1 (4)	1.1±0.2 (4)	1.2±0.1 (4)	1.7±0.1 (4)	2.0±0.1 (4)	2.1±0.1 (4)	1.0±0.1 (4)	1.7±0.1 (4)	
Host	1.7±0.1 (6)	0.9±0.1 (4)	1.4±0.1 (4)	1.3±0.1 (4)	1.7±0.3 (2)	2.7±0.2 (2)	1.3±0.2 (2)	2.2±0.1 (2)	0.8±0.1 (2)	1.41
Lactate dehydrogenase										
Tumour	3.1±0.3 (6)	7.4±0.1 (4)	1.5±0.3 (4)	3.0±0.3 (4)	0.7±0.1 (4)	2.5±0.2 (4)	1.6±0.1 (4)	1.3±0.1 (4)	2.5±0.2 (4)	
Host	2.2±0.2 (6)	1.8±0.2 (4)	2.7±0.2 (4)	2.2±0.3 (4)	3.8±0.3 (2)	5.0±0.5 (2)	1.9±0.3 (4)	3.9±0.3 (2)	1.9±0.2 (2)	2.31
10^3×Glycerol kinase activity										
Tumour	1.4±0.1 (6)	0.2±0.01 (4)	1.6±0.3 (4)	2.2±0.2 (4)	1.8±0.1 (4)	3.6±0.5 (4)	4.2±0.5 (4)	1.6±0.1 (4)	4.9±0.5 (4)	
Host	6.0±0.4 (6)	10.2±0.9 (4)	12.4±0.6 (4)	13.7±0.6 (4)	22.1±0.2 (2)	25.9±0.3 (2)	11.6±0.6 (4)	28.3±0.3 (2)	8.1±0.1 (2)	16.8
10×Glycerol 3-phosphate dehydrogenase activity										
Tumour	0.71±0.07 (6)	0.08±0.01 (4)	0.52±0.07 (4)	0.51±0.04 (4)	0.66±0.08 (4)	14.5±0.2 (4)	0.91±0.08 (4)	0.97±0.06 (4)	1.7±0.4 (4)	
Host	1.5±0.1 (6)	2.0±0.3 (4)	3.7±0.2 (4)	3.9±0.4 (4)	5.8±0.1 (2)	5.4±0.4 (2)	2.0±0.3 (4)	5.8±0.2 (2)	3.6±0.3 (2)	4.04

Table 2. *Correlation coefficients for the activities of β-oxoacyl-(acyl-carrier protein) reductase, 'malic' enzyme, lactate dehydrogenase, glycerol kinase and glycerol 3-phosphate dehydrogenase versus each other in rat and mouse hepatomas and host livers*

All values were included except those for tumour 16 (see the Discussion section). $*P<0.1$; $**P<0.01$.

	β-Oxoacyl-(acyl-carrier protein) reductase	'Malic' enzyme	Lactate dehydrogenase	Glycerol kinase
Glycerol 3-phosphate dehydrogenase	0.14	0.05	0.19	0.97**
Glycerol kinase	0.11	0.08	0.18	—
Lactate dehydrogenase	0.43	0.37	—	—
'Malic' enzyme	0.79*	—	—	—

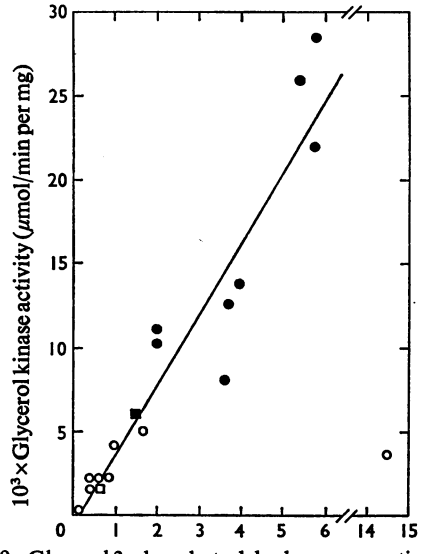

Fig. 1. *Activity of glycerol 3-phosphate dehydrogenase versus the activity of glycerol kinase in rat hepatomas* (○) *livers* (■)

Excluding Morris hepatoma 16, the activities of these enzymes are highly correlated ($r=0.97$, $P<0.01$). If hepatoma 16 is included, the correlation is still significant ($r=0.71$, $P<0.01$). The line represents a least-squares fit with tumour 16 excluded ($m=4.33$, $b=-0.87$).

Results

For the hepatomas studied, the activities of the β-oxoacyl-(acyl-carrier protein) reductase component of fatty acid synthetase and 'malic' enzyme varied from 50 to 200% of the activities in corresponding host livers. The highest specific activities of these two enzymes involved in lipid synthesis were in Morris hepatomas 3924A, 16 and 7787 and in mouse hepatoma BW7756 (Table 1). Their activities in the hepatomas and in host livers showed a positive correlation coefficient of 0.79 ($P<0.1$) (Table 2). There does not appear to be any clear relationship between the activity of either enzyme and tumour growth rate. The lack of correlation of 'malic' enzyme activity and growth rate has been noted by Weber (1966).

With one exception, glycerol 3-phosphate dehydrogenase and glycerol kinase activities in the rat and mouse hepatomas varied between 2 and 60% of the corresponding host livers (Table 1). The one exception is Morris hepatoma 16 where the activity of glycerol 3-phosphate dehydrogenase is more than 250% of that of the host liver. Exclusive of tumour 16, glycerol kinase and glycerol 3-phosphate dehydrogenase activities show a very strong positive correlation coefficient of 0.97 ($P<0.01$) (Table 2). This is clearly illustrated in Fig. 1 in which the activities of the two enzymes are plotted against each other. The lowest activities for both enzymes were found in the fast-growing Morris hepatoma 3924A and the highest activities were found in the slower growing rat hepatomas 7787, 9618B and the mouse tumour BW7756. In general the activities of both enzymes increase with decreasing growth rate (Table 1). This pattern of activity as a function of growth rate is typical of gluconeogenic enzymes (Weber, 1966) and had been reported for glycerol 3-phosphate dehydrogenase (Shonk *et al.*, 1965).

It is well established that many tumours have increased glycolysis (Elwood *et al.*, 1963). Among hepatomas increased glycolysis is associated with rapidly growing tumours (Elwood *et al.*, 1963; Lin *et al.*, 1962). In agreement with these observations, the specific activity of lactate dehydrogenase was near-normal for the slower growing hepatomas and only the rapidly growing lines showed greatly increased activity.

Electrophoretic patterns for glycerol 3-phosphate dehydrogenase in hepatoma extracts showed only one band of activity with the same mobility as that in host liver extracts. Lactate dehydrogenase electrophoretograms showed a fairly consistent pattern of isoenzymes from host liver and from most of the hepatomas studied. Only in the slowly growing

Morris hepatoma 21 was the 'heart-type' (H_4) lactate dehydrogenase strongly evident.

Discussion

In a variety of cancer tissues glycerol 3-phosphate dehydrogenase activity is quite low when compared with normal tissue (Boxer & Shonk, 1960; Sacktor & Dick, 1960). The fact that slower hepatoma growth rates are correlated with increased lipid oxidation (Bloch-Frankenthal *et al.*, 1965) and higher glycerol 3-phosphate dehydrogenase activity (Shonk *et al.*, 1965) might suggest a role for the liver enzyme in gluconeogenesis from lipolytically derived glycerol (Warkentin & Fondy, 1973). Its function in gluconeogenesis would be to oxidize glycerol 3-phosphate, formed from glycerol by glycerol kinase, to dihydroxyacetone phosphate which would then be converted into glucose. It is assumed that glyceride oxidation and glucose synthesis are complementary processes in hepatomas as they are in liver (Cahill, 1970) and therefore glycerol would be converted into glucose and not oxidized.

Consistent with a gluconeogenic function are the similar changes in hepatic glycerol 3-phosphate dehydrogenase and glycerol kinase activities in response to dietary stresses in chicks (Harding *et al.*, 1975) and the parallel increase in these two enzyme activities in rat liver during embryonic development (Burch *et al.*, 1974). In addition the activity of glycerol 3-phosphate dehydrogenase in relation to hepatoma growth rate is similar to that of gluconeogenic enzymes (Weber, 1963; Weber *et al.*, 1961, 1964, 1965; Weber & Morris, 1963; Weber & Cantero, 1957; Weber & Lea, 1966).

In an attempt to resolve whether hepatic glycerol 3-phosphate dehydrogenase has a lipogenic or gluconeogenic function, we have assayed glycerol 3-phosphate dehydrogenase in a variety of rat hepatomas. We have compared its activity with that of glycerol kinase, an enzyme which is necessary for glycerol 3-phosphate dehydrogenase to function in gluconeogenesis, and with the β-oxoacyl-(acyl-carrier protein) reductase component of the fatty acid synthetase, a lipogenic enzyme. In addition, the activities of 'malic' enzyme, presumably a lipogenic enzyme, and lactate dehydrogenase were measured and compared with glycerol 3-phosphate dehydrogenase. Our data indicate an inverse relationship between glycerol 3-phosphate dehydrogenase activity and tumour-growth rates in agreement with Shonk *et al.* (1965). Glycerol kinase is the rate-limiting step in glycerol metabolism by rat liver (Ackermann *et al.*, 1974). The activity of glycerol kinase, which was measured for the first time in a series of rat hepatomas, was shown to be directly proportional to the activity of glycerol 3-phosphate dehydrogenase not only among tumour lines but also among host livers (Fig. 1). The highly significant positive correlation between these enzymes (Table 2) and the similarity of their pattern of activity and that of gluconeogenic enzymes versus tumour-growth rate leads us to propose a coupled function for glycerol 3-phosphate dehydrogenase and glycerol kinase in hepatic gluconeogenesis. There is no strong correlation between the activities of either of these two enzymes and that of lactate dehydrogenase, 'malic' enzyme, or the β-oxoacyl-(acyl-carrier protein) reductase component of the fatty acid synthetase complex.

The one exception to the strong positive correlation between glycerol 3-phosphate dehydrogenase and glycerol kinase activities was observed in Morris hepatoma 16 which had glycerol 3-phosphate dehydrogenase activities over 250% of that in host liver. This tumour was not included in the correlation calculations as it seemed to be a distinct phenomenon from that being measured in the other hepatomas and host livers. We have no satisfactory explanation for the unusually high activity in this tumour. Surrounding tissue contamination can be discounted since the glycerol 3-phosphate dehydrogenase activity in surrounding thigh muscle tissue was only 60–70% of that in normal liver. There does not seem to be any obvious connection between the unusually high amounts of glycerol 3-phosphate dehydrogenase reported here and the relatively small mitochondria found in Morris hepatoma 16 (Cornbleet *et. al.*, 1974).

Glycerol 3-phosphate dehydrogenase has been purified and characterized from normal rat liver (Fondy *et al.*, 1971) and multiple molecular forms have been observed (Ross *et al.*, 1971). Ono (1966) observed qualitative changes in the isoenzyme patterns of lactate dehydrogenase, glucose 6-phosphate dehydrogenase (EC 1.1.1.49), and aspartate aminotransferase (EC 2.6.1.1) in different tumours which were correlated with growth rate and disturbances in major pathways. Our examination of phosphate dehydrogenase revealed that the electrophoretic mobility of the enzyme in all the hepatomas studied here is identical with that in normal rat liver. Therefore the unusual behaviour of Morris hepatoma 16 cannot be readily explained by an isoenzyme or alloenzyme difference.

The results reported in this paper further support the contention that glycerol 3-phosphate dehydrogenase activity in liver is related to its essential role in a pathway of glucose synthesis from glycerol.

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