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Activities of some Enzymes concerned with Citrate and Glucose Metabolism in Transplanted Rat Hepatomas

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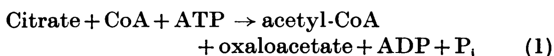
1. Certain enzymes concerned with citrate and glucose metabolism have been measured in two transplanted rat hepatomas, one induced with ethionine (minimal deviation type) and one induced with dimethylaminoazobenzene. In these hepatomas both citrate-cleavage enzyme and NADP-linked isocitrate dehydrogenase in the soluble fraction of the cell were approximately one-third of the values for normal rat liver. These changes have been discussed in relation to the increased citric acid content of tumours and depressed rate of fatty acid synthesis. 2. The glucose–ATP-phosphotransferase activity was below normal liver values in the ethionine-induced tumour but greater than normal in the dimethylaminoazobenzene-induced hepatoma. The apparent K_m values for the glucose–ATP phosphotransferases of these hepatomas were approx. 8×10^{-5}M; no evidence was found for an enzyme with a high K_m for glucose equivalent to liver glucokinase. 3. Of the enzymes of the pentose phosphate pathway, glucose 6-phosphate-dehydrogenase activity was three to five times as great whereas 6-phosphogluconate-dehydrogenase activity was the same or lower than normal liver in the ethionine- and dimethylaminoazobenzene-induced tumours respectively.

Recently interest has been focused on the role of citrate-cleavage enzyme in fatty acid synthesis and on the key part played by citrate concentration in controlling carbohydrate and fat metabolism (Lowenstein, 1964; Srere, 1965). The activity of citrate-cleavage enzyme in tumours is of particular interest in view of the observations by Dickens (1941) that many tumours have a high citric acid content. Further, there is the general finding that tumours have a decreased ability to synthesize fatty acids (Medes, Friedmann & Weinhouse, 1956; Weber, Morris, Love & Ashmore, 1961; Weber, Henry, Wagle & Wagle, 1964) and to form acetoacetate (Dickens & Weil-Malherbe, 1943) and cholesterol (Gore & Popják, 1962), although in certain cases hepatomas appear to have retained some of these synthetic pathways (Begg & Trew, 1956; Siperstein & Fagan, 1964).



$$\text{Citrate}+\text{CoA}+\text{ATP}\rightarrow\text{acetyl-CoA}+\text{oxaloacetate}+\text{ADP}+\text{P}_i \quad (1)$$

Citrate-cleavage enzyme catalysing reaction (1) could clearly play a part in both the supply of acetyl-CoA for these reactions and in controlling the citric acid concentration of the cell.

The tumours used in this present work were transplanted hepatomas derived from primary liver tumours induced with ethionine or dimethylaminoazobenzene. The ethionine tumour was 'minimal deviation' in some respects: thus it contained at least 50% of the normal liver glucose 6-phosphatase, whereas the latter had virtually none of this enzyme (E. Reid, personal communication). In view of this marked difference, measurements were also made of certain enzymes concerned with glucose 6-phosphate metabolism, namely glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and glucose–ATP phosphotransferase. [This general term, employed by Sharma, Sharma, Donnelly, Morris & Weinhouse (1965), is used here to indicate phosphorylation of glucose by ATP irrespective of the K_m value of the enzyme. Elsewhere the enzyme with the low K_m for glucose ($<1\times10^{-4}$M) has been called hexokinase (EC 2.7.1.1) and that with the high K_m glucokinase (EC 2.7.1.2), in accordance with general nomenclature.] The two dehydrogenases of the pentose phosphate pathway are of interest not only in relation to glucose 6-phosphate metabolism but also because they are key enzymes in fatty acid synthesis since they are believed to provide much of the NADPH required in this process (Matthes, Abraham & Chaikoff, 1963; Katz, Rognstad & Kemp, 1965).

METHODS

Materials. Commercial reagents were used with the exception of 6-phosphogluconate dehydrogenase, which was a partially purified preparation from liver and used in the assay of glucose 6-phosphate-dehydrogenase activity as described by Glock & McLean (1953). Nicotinamide nucleotides, CoA and hexokinase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Tumours. Transplantable hepatomas initially induced with DAB* or ethionine were developed at the Chester Beatty Research Institute in the Laboratories of Dr L. A. Elson and Dr E. Reid respectively and were made available for this work through the kindness of Dr E. Reid. Studies on the biochemical properties of these tumours by Dr Reid showed that the ethionine tumour was probably of the minimal deviation type with 50% of the normal rat-liver content of glucose 6-phosphatase whereas the DAB-induced tumour had none of this enzyme. The tumours were transplanted subcutaneously into rats of the hooded Norway strain and were used about 3 weeks later. The ethionine tumours used in these experiments were the thirteenth and sixteenth transplant generation and the DAB-induced tumours the twenty-third generation. The tumours were removed and stored in ice for approx. 1hr. before preparation of homogenates for enzyme determination.

Preparation of tissue extracts. Portions of normal liver and tumour were homogenized, with a Potter homogenizer, in 3vol. of ice-cold medium containing 150mM-KCl–5mM-$MgCl_2$–5mM-EDTA–10mM-mercaptoethanol and adjusted to pH 7·4 with $KHCO_3$. This is similar to the extracting medium used by Sharma, Manjeshwar & Weinhouse (1963). This preparation was centrifuged at 100000*g* (average) for 45min. The supernatant fraction was dialysed against the same extracting medium for 1hr. in the cold to decrease the blank values in certain of the enzyme determinations. This preparation was used for the determination of all the enzymes.

The stability of the NADP-linked dehydrogenases in this extracting medium was tested after storage at $-15°$ for 2 months; only very minor changes occurred during this time, in most cases less than 10% change in activity. The stability of liver and tumour extracts were the same. In the results presented here citrate-cleavage enzyme and glucose–ATP phosphotransferase were measured immediately after dialysis but in certain experiments NADP-linked isocitrate dehydrogenase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were measured after storage for a short period at $-15°$. It is notable that the low-K_m enzyme phosphorylating glucose is stable to storage at $-15°$.

Citrate-cleavage enzyme. The activity of citrate-cleavage enzyme was estimated by the colorimetric procedure described by Kornacker & Lowenstein (1965). A unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1μmole of acetyl-CoA/hr. at 37°.

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The activities of glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate–NADP oxidoreductase; EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate–NADP oxidoreductase; EC 1.1.1.44) were measured by the method of Glock & McLean (1953) with the exception that the amount of NADP was increased by a factor 2. A unit of enzyme is defined as the amount of enzyme catalysing the formation of 1μmole of NADPH/hr. at 25° based on the initial velocity.

Isocitrate dehydrogenase. The activity of NADP-linked isocitrate dehydrogenase in the soluble fraction of the cell [L-isocitrate–NADP oxidoreductase (decarboxylating), EC 1.1.1.42] was measured essentially according to the method of Ochoa (1955) except that the substrate concentration was increased by a factor 2. This increased the period of linearity of the reaction. A unit of enzyme is defined as the amount catalysing the formation of 1μmole of NADPH/hr. at 25°, based on the initial velocity.

Glucose–ATP-phosphotransferase activity. The glucose-phosphorylating activities of the dialysed high-speed supernatant fractions from rat liver and hepatomas were measured essentially according to the method of Sharma *et al.* (1963) but with the following modifications. The amount of purified glucose 6-phosphate dehydrogenase was increased to 0·5i.u. (the preparation used contained 140i.u./mg. of protein; C. F. Boehringer und Soehne G.m.b.H) and excess of 6-phosphogluconate dehydrogenase [partially purified $(NH_4)_2SO_4$ preparation] was added. The glucose concentrations used were 0·5mM and 100mM for the low-K_m and high-K_m enzymes found in liver but a concentration of 10mM was used with the hepatoma extracts since, as shown below, this has a glucose–ATP phosphotransferase with a higher K_m value than that of liver hexokinase. The reaction was started with 0·04ml. of the dialysed high-speed supernatant fraction of liver or hepatoma.

In this assay glucose 6-phosphate is converted into 6-phosphogluconate by excess of purified glucose 6-phosphate dehydrogenase and the NADPH formed is measured spectrophotometrically. The liver supernatant fraction contains the two dehydrogenases of the pentose phosphate pathway so that the 6-phosphogluconate formed will be further metabolized, producing an additional amount of NADPH. This has been allowed for by some authors and various factors have been used to convert the NADPH produced into glucose 6-phosphate formed; for example Sharma *et al.* (1963) has used a factor 1·7 for this conversion.

In this present work a similar method of correction was used to that employed in the routine assay of glucose 6-phosphate dehydrogenase in liver extracts (Glock & McLean, 1953), that is by the addition of excess of 6-phosphogluconate dehydrogenase; each molecule of glucose 6-phosphate then forms two molecules of NADPH by the combined action of the two dehydrogenases.

The $(NH_4)_2SO_4$ preparation of 6-phosphogluconate dehydrogenase was similar to that described by Glock & McLean (1953); this preparation contained no glucokinase or hexokinase activity; 0·3 unit was added to the assay medium, a unit being defined as the amount of enzyme catalysing the formation of 1μmole of NADPH/min. at 25°. This is an amount slightly less than the purified glucose 6-phosphate dehydrogenase added to the assay medium. In the presence of excess of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase the values of μmoles of NADPH produced were divided by 2 to convert into μmoles of glucose 6-phosphate formed. For convenience in comparison of enzyme activities in this present study a unit of glucose–ATP-phosphotransferase activity is defined as the amount of enzyme catalysing the formation of 1μmole of glucose 6-phosphate/hr. at 25°.

* Abbreviation: DAB, dimethylaminoazobenzene.

In studies of K_m values of liver and tumours, $(NH_4)_2SO_4$ fractions (20–50% and 50–75% saturation) were prepared from the dialysed high-speed supernatant extracts. The precipitated proteins were collected by centrifugation and were then dissolved in the KCl–$MgCl_2$–EDTA–mercaptoethanol medium described above and dialysed against this same extracting medium. The reaction velocity was determined from the initial rate of reaction. In Figs. 1–3, $1/v$ is the reciprocal of the extinction change at 340mμ/min.

The rate of reduction of $NADP^+$ in this and the other NADP-linked enzyme reactions was measured with a Unicam SP. 800 double-beam recording spectrophotometer with a constant-temperature cell housing and scale-expansion accessory.

Protein. The protein content of the high-speed supernatant fractions was estimated essentially according to the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

The comparison of the activities of some enzymes concerned with citrate metabolism and glucose oxidation in normal rat liver, in a transplanted hepatoma induced with ethionine (minimal deviation type in some respects) and in a transplanted hepatoma induced with DAB are shown in Table 1.

The activity of the citrate-cleavage enzyme in the transplanted hepatomas is approx. 30% of the value for normal rat liver. This difference is seen when the results are expressed as activity/g. of tissue or on the basis of the protein content in the supernatant fraction after centrifugation at 100000g for 45min. Reid (1962) has examined the various methods of expressing results and comparing values from tumours with normal tissues and has concluded that the activity/g. of tissue forms a suitable basis. The values in Tables 1 and 2 are also given as units/mg. of protein since this is also a widely used basis of expression.

The NADP-linked isocitrate-dehydrogenase activity in the soluble fraction of the cell is also markedly lower in hepatomas when compared with normal liver. This present result is not necessarily in direct contradiction to the evidence showing undiminished rate of isocitrate-dehydrogenase activity in tumour (Wenner, Spirtes & Weinhouse, 1952), since in these latter experiments the whole tissue was used as an acetone-dried preparation whereas the present experiments report only the activity of this enzyme in the soluble fraction.

In contrast with these enzymes, glucose 6-phosphate-dehydrogenase activity increases some three- and five-fold in the hepatomas induced with ethionine and DAB respectively, whereas 6-phosphogluconate-dehydrogenase activity remains almost unchanged in the ethionine tumour but fell to approximately half the normal liver value in the DAB-induced tumour. The relative activity of glucose 6-phosphate dehydrogenase/6-phosphogluconate dehydrogenase changed from 1:2 in the control liver to 1:0·2 in the DAB-induced hepatoma.

Table 1. *Activities of citrate-cleavage enzyme and of some NADP-linked dehydrogenases in normal liver and transplanted rat hepatomas*

Normal animals of the same age and weight as the tumour-bearing rats were used. The results are expressed as units/g. of tissue and units/mg. of protein contained in the supernatant fraction after centrifugation at 100000g for 45min. The units are μmoles of product formed/hr., the product being acetyl-CoA (as acetohydroxamate) for citrate-cleavage enzyme and NADPH for the three dehydrogenases. The temperature was 25° for all enzymes except citrate-cleavage enzyme, which was measured at 37°. The temperature coefficient factor to convert this last-named enzyme activity into units at 25° is 0·42. The results are given as means ±S.E.M. The values for the two transplanted hepatomas induced with DAB are given individually. For details see the Methods section.

		Transplanted hepatomas	
	Liver	Ethionine-induced	DAB-induced
No. of animals ...	10	9	2
Units of enzyme/g. of tissue			
Citrate-cleavage enzyme	98±13	36±4	20, 26
NADP-linked isocitrate dehydrogenase	1110±71	240±21	340, 427
Glucose 6-phosphate dehydrogenase	105±13	312±12	482, 542
6-Phosphogluconate dehydrogenase	201±5	160±13	100, 106
Units of enzyme/mg. of protein			
Citrate-cleavage enzyme	1·05±0·14	0·56±0·05	0·34, 0·43
NADP-linked isocitrate dehydrogenase	11·8±0·76	3·80±0·44	5·75, 7·00
Glucose 6-phosphate dehydrogenase	1·13±0·13	4·86±0·20	8·16, 8·88
6-Phosphogluconate dehydrogenase	2·15±0·06	2·46±0·20	1·68, 1·74
Protein content (mg./g. of tissue) of high-speed supernatant fraction	94±2·7	65±2·6	59, 61

Table 2. *Activity of glucose–ATP phosphotransferases in normal liver and transplanted hepatomas*

Results are expressed as μmoles of glucose 6-phosphate formed/hr./g. of tissue or μmoles of glucose 6-phosphate formed/hr./mg. of protein contained in the supernatant fraction after centrifugation at 100000g for 45min. The temperature was 25°. The enzymes were estimated by following the rate of formation of NADPH at 340mμ in the presence of excess of purified glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase; this yields 2μmoles of NADPH/μmole of glucose 6-phosphate formed, and therefore the extinction readings have been halved to correct the results to μmoles of glucose 6-phosphate formed. The total glucose-phosphorylating activity was determined with a final glucose concentration of 0·1M. The low-K_m enzymes were estimated with 0·5mM and 10mM final glucose concentrations for liver and hepatomas respectively. The high-K_m glucokinase was calculated by difference. The results are given as means ±S.E.M. The values for the two transplanted hepatomas induced with DAB are given individually. For details see the Methods section.

		Transplanted rat hepatomas	
	Liver	Ethionine-induced	DAB-induced
No. of animals ...	9	9	2
μmoles of glucose 6-phosphate formed/g. of tissue/hr.			
Total activity	139±9	63±7	152, 172
Low-K_m (hexokinase)	14±2	63±7	152, 172
High-K_m (glucokinase)	125±8	No activity	No activity
μmoles of glucose 6-phosphate formed/mg. of protein/hr.			
Total activity	1·50±0·10	0·97±0·11	2·6, 2·8
Low-K_m (hexokinase)	0·15±0·02	0·97±0·11	2·6, 2·8
High-K_m (glucokinase)	1·34±0·10	No activity	No activity
Protein content of high-speed supernatant (mg./g. of tissue)	94±2·7	65±2·6	59, 61

These results show that there is not a generalized overall depression in enzymic activity in these transplanted hepatomas and comparison with other values for hepatomas in the literature suggests that the present values fit into a common pattern (see Reid, 1962; Weber *et al.* 1964).

The total rate of glucose phosphorylation, by enzymes in the soluble fraction of the cell in the presence of excess of glucose, is somewhat depressed in the ethionine-induced tumour but is greater than normal in the DAB-induced hepatoma (Table 2). Comparison was made of this activity in the high-speed supernatant fraction in all three tissues; the glucose 6-phosphatase activity of the ethionine tumour should not therefore interfere in this estimation. The total glucose-phosphorylating activity may be somewhat underestimated if part of the phosphorylating activity is associated with the particulate fraction of the cell as it is in brain (Crane & Sols, 1953) and ascites-tumour cells (McComb & Yushok, 1959). It was found in the present work that the substrate concentration which is used for hexokinase assay in normal liver (0·5mM) did not fully saturate the enzyme in the tumour extracts. This led to a study of the K_m values of the enzymes phosphorylating glucose in normal and tumour tissues. The results of these experiments are summarized in Table 3 and Figs. 1, 2 and 3. The apparent K_m values of the dialysed soluble fractions were 1×10^{-5}M for liver and about six- to seven-fold higher for the tumour extracts. It appeared from these studies that there was only one enzyme phosphorylating glucose in the tumour extracts with a relatively low K_m; the enzyme equivalent to glucokinase with a high K_m was absent.

The liver and tumour extracts were fractionated with ammonium sulphate and the K_m values for the 20–50% saturated ammonium sulphate fraction measured in each case. This showed clearly that the K_m for liver and for the hepatomas (ethionine- and DAB-induced) were different (see Figs. 2 and 3): values of 1×10^{-5}M and 8×10^{-5}M were obtained respectively. The small differences between the K_m values for fresh extracts and ammonium sulphate fractions may be accounted for by removal of trace quantities of substrate.

The relative hexokinase/glucokinase activities in liver and the K_m values for the 20–50% saturated ammonium sulphate fraction are in good agreement with other values in the literature (see Table 3). The 20–50% saturated ammonium sulphate fraction of the tumour extracts contained most of the hexokinase activity although higher concentrations

Table 3. *Apparent K_m values for glucose–ATP-phosphotransferase activities of normal rat liver and hepatomas*

Tissue	Preparation	K_m	
(*A*) Present values			
Liver	20–50% satd. $(NH_4)_2SO_4$ (4)	1×10^{-5}M	
Ethionine-induced tumour	(*a*) Dialysed supernatant (4)	$5{\cdot}5\times10^{-5}$M	
	(*b*) 20–50% satd. $(NH_4)_2SO_4$	8×10^{-5}M	
	(*c*) 50–75% satd. $(NH_4)_2SO_4$	8×10^{-5}M	
DAB-induced tumour	(*a*) Dialysed supernatant (2)	$7{\cdot}0\times10^{-5}$M	
	(*b*) 20–50% satd. $(NH_4)_2SO_4$	8×10^{-5}M	
	(*c*) 50–75% satd. $(NH_4)_2SO_4$	No activity	
(*B*) Published values		Low-K_m (hexokinase)	High-K_m (glucokinase)
Liver	(*a*) Unfractionated supernatant		
	Lange & Kohn (1961)	4×10^{-5}M	—
	Walker (1963); Walker & Rao (1963)	$3{\cdot}8\times10^{-5}$M	1×10^{-2}M
	DiPietro, Sharma & Weinhouse (1962)	—	$1\text{–}2\times10^{-2}$M
	(*b*) 20–50% and 60–75% satd.† $(NH_4)_2SO_4$		
	Viñuela, Salas & Sols (1963)	1×10^{-5}M	1×10^{-2}M
	Abraham, Borrebaek & Chaikoff (1964*a*)	5×10^{-5}M	$0{\cdot}8\times10^{-2}$M
	(*c*) DEAE-cellulose fractionation		
	González *et al.* (1964) Peak A	$0{\cdot}6\times10^{-5}$M	$1{\cdot}8\pm0{\cdot}03\times10^{-2}$M
	González *et al.* (1964) B	$4{\cdot}4\times10^{-5}$M	
	González *et al.* (1964) C	$13{\cdot}0\times10^{-5}$M	
Foetal rat liver	Walker & Rao (1963)	4×10^{-5}M	
Tumours	3′-Me-DAB and DAB cholangiocarcinomas		
	Shatton *et al.* (1962)	50×10^{-5}M	$0{\cdot}5\times10^{-2}$M*
	Morris hepatoma 5123A and 7800		
	Elwood, Lin, Cristofalo, Weinhouse & Morris (1963)	10×10^{-5}M	

* High-K_m enzyme found in one tumour composed of mixed cells, hepatocarcinoma and cholangiocarcinoma.
† 20–50% satd. $(NH_4)_2SO_4$ fraction contains low-K_m hexokinase; 60–75% satd. $(NH_4)_2SO_4$ fraction contains high-K_m glucokinase.

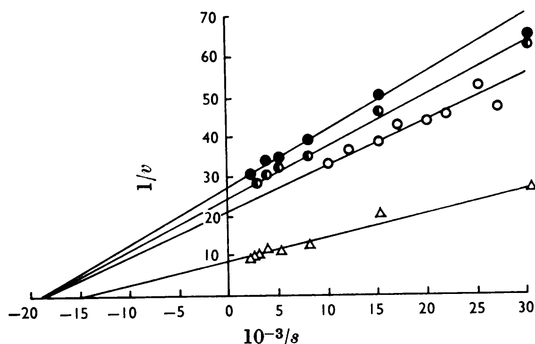


Fig. 1. Effect of variation in glucose concentration on the activity of glucose–ATP phosphotransferase of dialysed high-speed supernatant preparations (100000*g*) of the ethionine-induced tumours and DAB-induced tumour. These extracts were prepared as described in the Methods section. Three separate ethionine-induced tumours (○, ◐, ●) and one DAB-induced tumour (△) were used. The results are plotted according to Lineweaver & Burk (1934).

of ammonium sulphate precipitated additional hexokinase from extracts of the ethionine-induced tumour.

DISCUSSION

The central point of the present work is the activity of citrate-cleavage enzyme in tumours and the relationship of this to the overall pattern of carbohydrate and fat metabolism.

Citrate-cleavage enzyme

In transplantable hepatomas the activity of the enzyme was less than one-third of the value of normal rat liver. This might be expected to have two important consequences: first, to decrease the supply of acetyl-CoA for lipid synthesis, and secondly to increase the citrate concentration in the soluble fraction of the cell. There is indeed considerable evidence that hepatomas have a decreased ability to synthesize fatty acids (see Weber *et al.* 1964). The close parallelism between changes in citrate-cleavage enzyme and changes in the rate of fatty acid synthesis presented here has been noted in a variety of different conditions and tissues, e.g. in the livers of starving and re-fed animals (Kornacker & Lowenstein, 1963, 1965; Abraham, Kopelovich & Chaikoff, 1964*b*), in diabetic and insulin-treated rats (Kornacker &

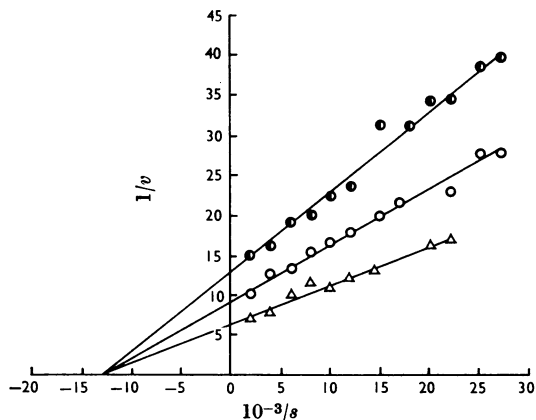


Fig. 2. Effect of variation in glucose concentration on the activity of glucose–ATP phosphotransferase of ethionine-induced tumour and DAB-induced tumour extracts fractionated with $(NH_4)_2SO_4$. The high-speed supernatant fractions from three ethionine-induced tumours were pooled and $(NH_4)_2SO_4$ fractions precipitating between 20–50% and 50–75% saturation were separated (○ and ◐ respectively). The DAB-induced tumour extract was treated similarly; activity was present only in the 20–50% saturated $(NH_4)_2SO_4$ fraction (△). The $(NH_4)_2SO_4$ fractions were prepared from extracts used in the experiments shown in Fig. 1. The results are plotted according to Lineweaver & Burk (1934).

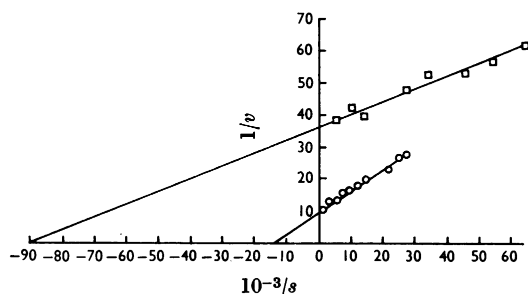


Fig. 3. Comparison of the effect of variation in glucose concentration on the activity of the low-K_m enzyme phosphorylating glucose in liver and ethionine-induced tumours. The fraction of the high-speed supernatant preparation that precipitated between 20 and 50% $(NH_4)_2SO_4$ saturation was used: □, liver; ○, ethionine-induced tumour.

Lowenstein, 1964), in hypophysectomized rats (Abraham *et al.* 1964*b*), in the mammary gland at different stages of lactation (Spencer, Corman & Lowenstein, 1964) and in adipose tissue of normal and diabetic rats (Brown & McLean, 1965). The present results suggest that this may also be true for hepatomas.

Dickens (1941) demonstrated that tumours have, in general, a high content of citric acid and that the citric acid concentration is greater in the rapidly growing peripheral portion of the tumour than in the central region. A decrease in the citrate-cleavage-enzyme activity could be of importance in raising the citrate content of the cell but this represents only one facet of the problem of control of the citrate concentration. The activities of the mitochondrial enzymes must also be considered. The available evidence indicates that both citrate synthetase and isocitrate dehydrogenase are present in tumours in amounts comparable with the activities in normal tissues, whereas, in contrast, aconitase activity is lower than in normal tissues (Wenner *et al.* 1952). Therefore the overall enzyme pattern in the two cell compartments in tumours could well lead to an increase in the citrate concentration, the key enzyme change being the fall in citrate-cleavage enzyme.

There is recent evidence for an additional extramitochondrial pathway of citrate formation from α-oxoglutarate or glutamate (D'Adamo & Haft, 1962, 1965; Madsen, Abraham & Chaikoff, 1964; Lardy, Shrago, Young & Paetkan, 1964; Srere, 1965). The results of isotopic experiments indicate the conversion of glutamate into citrate with subsequent enzymic cleavage of the citrate. The enzymes aconitase and NADP-linked isocitrate dehydrogenase are present in the soluble fraction of the cell where this pathway operates in the reverse direction to that normally occurring in the tricarboxylic acid cycle (see Srere, 1965). Part of this route of citrate formation has been measured in transplanted hepatomas in the present experiments. The observation that the soluble NADP-linked isocitrate dehydrogenase is low in the hepatomas compared with liver suggests that this route plays a more minor role in this tissue. It is noteworthy, in relation to the citrate concentration, that the relative activities of citrate-cleavage enzyme and NADP-linked isocitrate dehydrogenase remain favourable to citrate formation (Table 1).

An important implication of the accumulation of citrate in the cell is that citrate is an inhibitor of phosphofructokinase and could thus have a powerful effect on the control of the glycolytic pathway at this rate-limiting step. There is considerable evidence that metabolic control can be exerted at this stage in many tissues both by citrate accumulation (Parmeggiani & Bowman, 1963; Passonneau & Lowry, 1964) and by ATP and inorganic phosphate accumulation (Park *et al.* 1961; Newsholme & Randle, 1961; Passonneau & Lowry, 1964; Wu, 1964). There is evidence that the phosphofructokinase of Novikoff hepatomas is inhibited under aerobic conditions consistent with an inhibition by ATP (Wu, 1964). The extent of control by citrate

in tumours having a high glycolytic rate would be of interest, particularly since certain feedback control mechanisms are lost in tumours. An example in lipid metabolism is the loss in hepatomas of the cholesterol feedback mechanism at the point of conversion of β-hydroxy-β-methylglutaric acid into mevalonic acid (Siperstein & Fagan, 1964).

Glucose phosphorylation

There is now considerable evidence that glucokinase activity is greatly decreased in or absent from many hepatomas (Shatton, Donnelly & Weinhouse, 1962; Sharma *et al.* 1965). These authors have shown that in pre-cancerous liver there is a progressive rise in hexokinase to a value some five or six times as high as normal and a fivefold lowering in glucokinase. In a series of ten transplanted tumours eight had low or negligible glucokinase activity. The present results, with two types of transplanted hepatomas, fit the general pattern described by these authors. The changes in K_m of the glucose–ATP phosphotransferase involve not only the loss of the high-K_m glucokinase but also a change in K_m of the enzyme with glucose at low concentrations to a value some five to eight times the average for normal rat-liver hexokinase (Fig. 3).

The work of González, Ureta, Sánchez & Niemeyer (1964) showing multiple molecular forms of glucose–ATP phosphotransferase is of interest here since these authors were able to distinguish three isoenzymes with low K_m values. It is possible that the proportions of these isoenzymes are altered in hepatomas and studies with fractionation of these tumour extracts on DEAE-cellulose similar to those of Gonzalez *et al.* (1964) would be of value here. There is evidence for alterations in the lactate-dehydrogenase isoenzyme pattern in a variety of human tumours compared with adjacent normal tissue (Kaplan & Goodfriend, 1964) and Weber *et al.* (1964) have also shown that the K_m of phosphoglucomutase is higher in hepatomas than in normal liver. Similar alterations in enzymes controlling glucose phosphorylation may occur.

Pentose phosphate pathway

Weber *et al.* (1964) have examined a number of biochemical parameters in a range of transplanted hepatomas of different growth rates and have found that, in general, glucose 6-phosphate-dehydrogenase activity is increased. There is an increase in the activity of glucose 6-phosphate dehydrogenase as early as 2–4 weeks after feeding with a diet containing carcinogenic azo-dyes (Nodes & Reid, 1964; McLean, Reid & Gurney, 1964). The two transplanted tumours studied here followed this general pattern with a three- to five-fold increase in glucose 6-phosphate-dehydrogenase activity. By contrast, 6-phosphogluconate-dehydrogenase activity either remained unchanged or fell in the ethionine- and DAB-induced tumours respectively.

The very high glucose 6-phosphate-dehydrogenase activity and the marked alternations in the relative activities of the two dehydrogenases of the pentose phosphate pathway do not appear to fit readily the overall pattern of metabolism of the hepatomas. First, there is, in general, a decline in the total NADP content of most tumours and hepatomas, often to extremely low concentrations (Glock & McLean, 1957; Nodes & Reid, 1964; Greenbaum, Clark & McLean, 1965). Further, the reductive synthetic utilization of NADP for fatty acid synthesis is decreased and the dehydrogenases of the pentose phosphate pathway are thought to be tightly geared to this process (see Dickens, 1959). Thus the functional significance of this increased glucose 6-phosphate-dehydrogenase activity remains in question.

Many authors have speculated on the nature of the stimulus causing increased formation of glucose 6-phosphate dehydrogenase in such conditions as re-feeding after starvation. Glucose 6-phosphate, in particular, has been considered as a possible inducer, but no firm conclusion has been reached about this particular role of glucose 6-phosphate (Steiner & Williams, 1959; Potter & Ono, 1961; Bottomley, Pitot, Potter & Morris, 1963; Tepperman & Tepperman, 1963; Pitot, Peraino, Pries & Kennan, 1964). It seemed possible that the high glucose 6-phosphate-dehydrogenase activity of hepatomas might be related to an increased rate of formation of glucose 6-phosphate and some evidence for this is summarized in Table 4.

The glucose 6-phosphate of liver undergoes marked variation according to dietary conditions (Steiner & Williams, 1959). The alterations in the concentration of glucose 6-phosphate can be largely accounted for by the presence of the adaptive enzyme glucokinase. Time-sequence studies also show that glucokinase increases in activity before glucose 6-phosphate dehydrogenase (Potter & Ono, 1961; Pitot *et al.* 1964). Many hepatomas appear to lose the adaptable glucokinase activity found in liver and to have a high concentration of glucose–ATP phosphotransferase with a high affinity for glucose, conditions that would permit a rapid and constant rate of formation of glucose 6-phosphate. Another factor which could contribute to the increased availability of glucose 6-phosphate is the relatively low activity of glucose 6-phosphatase in these hepatomas (E. Reid, personal communication). Walker, Parry & Vernon (1965) have suggested that the activity of glucose 6-phosphatase rather than glucokinase might be the principal factor controlling the liver glucose 6-phosphate

Table 4. *Factors concerned in metabolism of glucose 6-phosphate in normal liver and hepatomas*

References: 1, Tepperman & Tepperman (1963); 2, Potter & Ono (1961); 3, Bottomley *et al.* (1963); 4, Pitot *et al.* (1964); 5, Shatton *et al.* (1962); 6, Sharma *et al.* (1965).

	Normal liver	Hepatoma	Reference
Glucose 6-phosphate dehydrogenase	100%	Increased up to 500%	1, 2, 3, 4
Glucose phosphorylation			
Hexokinase (low K_m)	Low	High	5, 6
Glucokinase (high K_m)	High	Low or absent	6
Re-feeding after starvation	Increased glucokinase followed by increased glucose 6-phosphate dehydrogenase	No change in activity	2, 3
Availability of glucose as substrate	Variable via portal circulation	Relatively constant in transplanted hepatomas via peripheral circulation	

concentration. There appears to be a relationship between conditions giving a high rate of formation of glucose 6-phosphate and a high glucose 6-phosphate-dehydrogenase activity.

It is thus postulated that glucose 6-phosphate may, in fact, be a regulatory factor for glucose 6-phosphate dehydrogenase.

Measurements of the glucose 6-phosphate content of liver and tumours are only partially in agreement with the role of glucose 6-phosphate in controlling glucose 6-phosphate dehydrogenase. Tumours, in general, appear to contain a very similar concentration of glucose 6-phosphate to that found in normal tissues (LePage, 1948). It is possible that the rate of formation of glucose 6-phosphate is the more critical factor, particularly since a glucose 6-phosphate-induced increase in glucose 6-phosphate-dehydrogenase activity would tend to maintain the concentration of the substrate at the normal value. Product inhibition of hexokinase by glucose 6-phosphate could also be of importance (Weil-Malherbe & Bone, 1951). The experiments of Steiner & Williams (1959) and of Tepperman & Tepperman (1963) are of particular importance here. The first authors, by measuring glucose 6-phosphate concentrations in the liver within a short time (20 min.) after administration of glucose, were able to show rapid changes which were not apparent when longer time-intervals were used.

There is evidence that glucose 6-phosphate dehydrogenase may be under various forms of metabolic control. One example is the inhibition of this enzyme by long-chain acyl-CoA derivatives (Eger-Neufeldt, Teinzer, Weiss & Wieland, 1965). Here, again, there is an interplay of factors between carbohydrate and fat metabolism; the decreased fatty acid synthesis of the tumour, perhaps in part mediated by changes in the citrate-cleavage enzyme, may release glucose 6-phosphate dehydrogenase from this inhibition.

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