Biochem. J. (1969) **115**, 1009
Printed in Great Britain

The Pentose Phosphate Pathway of Glucose Metabolism

ENZYME PROFILES AND TRANSIENT AND STEADY-STATE CONTENT OF INTERMEDIATES OF ALTERNATIVE PATHWAYS OF GLUCOSE METABOLISM IN KREBS ASCITES CELLS

BY K. A. GUMAA AND PATRICIA McLEAN
Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London W1P 5PR

(*Received* 20 *June* 1969)

1. The pentose phosphate pathway in Krebs ascites cells was investigated for regulatory reactions. For comparison, the glycolytic pathway was studied simultaneously. 2. Activities of the pentose phosphate pathway enzymes were low in contrast with those of the enzymes of glycolysis. The K_m values of glucose 6-phosphate dehydrogenase for both substrate and cofactor were about four times the reported upper limit for the enzyme from normal tissues. Fructose 1,6-diphosphate and NADPH competitively inhibited 6-phosphogluconate dehydrogenase. 3. About 28% of the hexokinase activity was in the particulate fraction of the cells. The soluble enzyme was inhibited by fructose 1,6-diphosphate and ribose 5-phosphate, but not by 3-phosphoglycerate. The behaviour of the partially purified soluble enzyme *in vitro* in a system simulating the concentrations of ATP, glucose 6-phosphate and P_i found *in vivo* is reported. 4. Kinetics of metabolite accumulation during the transient state after the addition of glucose to the cells indicated two phases of glucose phosphorylation, an initial rapid phase followed abruptly by a slow phase extending into the steady state. 5. Of the pentose phosphate pathway intermediates, accumulation of 6-phosphogluconate, sedoheptulose 7-phosphate and fructose 6-phosphate paralleled the accumulation of glucose 6-phosphate. Erythrose 4-phosphate reached the steady-state concentration by 2min., whereas the pentose phosphates accumulated linearly. 6. The mass-action ratios of the pentose phosphate pathway reactions were calculated. The transketolase reaction was at equilibrium by 30sec. and then progressively shifted away from equilibrium towards the steady-state ratio. The glucose 6-phosphate dehydrogenase was far from equilibrium at all times. 7. Investigation of the flux of [^{14}C]glucose carbon confirmed the existence of an operative pentose phosphate pathway in ascites cells, contributing 1% of the total flux in control cells and 10% in cells treated with phenazine methosulphate. 8. The pentose phosphate formed by way of the direct oxidative route and estimated from the $^{14}CO_2$ yields represented 20% of the total accumulated pentose phosphate, the other 80% being formed by the non-oxidative reactions of the pentose phosphate pathway. 9. The pentose phosphate pathway appears to function as two separate pathways, both operating towards pentose phosphate formation. Control of the two pathways is discussed.

The alternative pathways of glucose metabolism, the glycolytic pathway and the pentose phosphate pathway, are closely integrated in that there are common intermediates, glucose 6-phosphate, fructose 6-phosphate and glyceraldehyde 3-phosphate, and that the intermediates of one pathway can act as inhibitors of the other route of glucose metabolism. For instance, sedoheptulose 7-phosphate, erythrose 4-phosphate and 6-phosphogluconate are all inhibitors of phosphoglucose isomerase (Venkataraman & Racker, 1961; Parr, 1956), and fructose 1,6- diphosphate is an inhibitor of 6-phosphogluconate dehydrogenase (Carter & Parr, 1967). In ascites cells there is possibly a further point of interrelationship since there is evidence that lactate dehydrogenase may be concerned in the reoxidation of NADPH (Wenner, 1959) and thus the availability of pyruvate may have a controlling effect on the oxidative reactions of the pentose phosphate pathway. Therefore any study of the control of the pentose phosphate pathway must, of necessity, involve a parallel consideration of changes occurring in the glycolytic route.

Interrelationships between pathways of glucose metabolism may be revealed if conditions are chosen in which the metabolite pattern is in a state

of transition rather than solely in the steady-state condition. For this reason measurements were made of the metabolites of the pentose phosphate pathway and glycolytic route during the initial period after the addition of glucose to washed ascites cells *in vitro*; rapid changes in the rate of glucose 6-phosphate accumulation occur after the glucose addition. Three phases of glucose 6-phosphate accumulation have been distinguished: an initially rapid rate of glucose uptake and phosphorylation followed by a short period of inhibition, and finally a low steady rate that is only 10–20% of the initial rate (Maitra & Chance, 1965; Rose, 1965; Wu, 1965*a*,*b*; Sauer, 1968). In the present study measurements of metabolites have been made at all three stages.

The assay of enzyme profiles has yielded valuable information about enzymes of regulatory significance in a number of tissues, and in the present experiments the activities of the oxidative and non-oxidative enzymes of the pentose phosphate pathway were determined and compared with those of the glycolytic pathway. Some kinetic properties of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and hexokinase are described, with particular reference to the influence of sugar phosphates.

An important question that arises is the extent of the operation of the oxidative and non-oxidative reactions of the pentose phosphate pathway in ribose 5-phosphate formation. There is evidence, from labelling patterns of ribose in nucleic acids, that both routes participate in HeLa carcinoma cells (Hiatt, 1957). In the present experiments the rate of oxidation of specifically labelled glucose by Krebs ascites cells was measured and the rate of the oxidative formation of pentose phosphate was determined. When these results were considered in relation to pentose phosphate accumulation they suggested a possible means whereby the approximate relative contributions of the two routes of ribose 5-phosphate formation might be assessed. The difficulties inherent in this method are discussed. Part of this work has appeared in a preliminary form (Gumaa, McLean & Bennette, 1968; Gumaa & McLean, 1969).

MATERIALS AND METHODS

All substrates and purified ancillary enzymes were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, with the following exceptions. Transaldolase was a gift from Dr B. L. Horecker. Transketolase was prepared by the method of Simpson (1960) with the modifications described by Gumaa *et al.* (1968). The mixture of ribose 5-phosphate isomerase and ribulose 5-phosphate epimerase was prepared by the method of Ashwell & Hickman (1957). Ribose 5-phosphate isomerase was purified as described by Novello & McLean (1968). The mixture of orotidine 5′-phosphate pyrophosphorylase and orotidine 5′-phosphate decarboxylase was prepared by the method of Flaks (1963). Erythrose 4-phosphate was prepared by the method of Ballou (1963). 5-Phosphoribosyl pyrophosphate was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.; orotic acid was from Calbiochem, Los Angeles, Calif., U.S.A. Specifically labelled [^{14}C]-glucoses were purchased from The Radiochemical Centre, Amersham, Bucks. [3,4-$^{14}C_2$]Glucose and [*carboxy*-^{14}C]-orotic acid were from New England Nuclear Corp., Boston, Mass., U.S.A.

Preparation of cells and incubation procedures. Krebs 2 ascites-tumour cells were maintained by weekly inoculation into mice and were used 7–9 days after inoculation. Cells were collected into a heparinized beaker and washed free of erythrocytes by repeated suspension in 0·154 M-NaCl containing 1 unit of heparin/ml. and centrifuging at 60*g* at 3°. When essentially freed of erythrocytes, the ascites cells were packed by centrifugation at 2000*g* for 10 min., weighed and suspended in Krebs–Ringer phosphate buffer (Umbreit, Burris & Stauffer, 1949) without Ca^{2+}, to make a suspension of 1 part of cells to 9 parts of medium (w/v).

For steady-state metabolites, at zero time and after 10 min. incubation with glucose, the tumour-cell suspensions were incubated in 50 ml. conical flasks fitted with centre wells and rubber caps. Flasks were gassed with 100% O_2 for 10 min. while shaking in a water bath at 37°. For the determination of the zero-time metabolite content, the ascites cells and medium were then transferred into tubes containing ice-cold $HClO_4$. For the metabolite content after 10 min. incubation with glucose, glucose was injected into flasks through the rubber caps to make a final concentration of 12·5 mM and the flasks were incubated for a further 10 min. at 37° with shaking. The reactions were then stopped by injecting $HClO_4$ into the flasks, homogenizing the flask contents in the cold and centrifuging down the protein precipitates. Extracts were neutralized to pH 6·7–6·8 with 6 M-K_2CO_3 in 5 mM-triethanolamine and after standing in ice for 30 min. the $KClO_4$ precipitates were removed by centrifugation at 3°. When ascites cells were incubated with glucose for periods of 1–10 min. this was carried out in conical flasks as described above, with deproteinization at the appropriate times. When shorter periods of 3–60 sec. were studied the incubations were carried out in wide glass tubes with O_2 bubbling through the cell suspension. Glucose was added at the starting of a stop-watch and the time when $HClO_4$ was added was recorded. Syringes were used for the additions of glucose and acid since preliminary studies with dyes indicated that this procedure gave very rapid mixing. When 5-phosphoribosyl pyrophosphate contents were to be measured, deproteinization of the flask contents was achieved by heating for 1 min. in a boiling-water bath, homogenizing the contents and centrifuging down the protein precipitates at 20000*g* for 15 min. at 3°.

Preparation of cell fractions. The packed ascites cells, free from erythrocytes, were suspended in 0·5 vol (w/v) of 0·154 M-NaCl, and 6 vol. of water was added. The mixture was stirred slowly at 0° for 10 min. and homogenized in a Potter–Elvehjem homogenizer fitted with a Teflon plunger by using strong pressure on the downward strokes. Homogenization was achieved with 20 strokes and was immediately followed by the addition of 2·5 vol. (v/v) of 1 M-sucrose in 20 mM-triethanolamine–HCl buffer, pH 7·6, and mixed. The homogenate was centrifuged at 1600*g* for 10 min. at 3° and

the pellet discarded. The supernatant was centrifuged at 12000*g* for 10min. and the pellet washed with 0·25M-sucrose in 5mM-triethanolamine–HCl buffer, pH7·6 ('washing medium'), by suspending and centrifuging three times. The final pellet was suspended in fresh washing medium and designated the '12000*g* particles'. The first 12000*g* supernatant was centrifuged at 100000*g* for 45min. at 3°, and the 100000*g* supernatant as well as the 12000*g* particles were dialysed against 100vol. of fresh washing medium for 1–2hr. at 3° to remove substrates.

Metabolite assays. In general, metabolites were assayed by the standard methods (see Bergmeyer, 1965). The term pentose phosphates, as used in the present study, denotes the sum of ribose 5-phosphate, xylulose 5-phosphate, ribulose 5-phosphate and the ribose 5-phosphate released on acid hydrolysis of 5-phosphoribosyl pyrophosphate. The assay of pentose phosphates was in a system containing 37mM-glycylglycine–KOH buffer, pH7·6, 0·22mM-NADH, triose phosphate isomerase (4·8 units), α-glycerophosphate dehydrogenase (1 unit), aldolase (0·5 unit), ribose 5-phosphate isomerase–ribulose 5-phosphate epimerase mixture (1 unit) and transketolase (0·3 unit). Aldolase was included in this system to remove fructose 1,6-diphosphate before the addition of transketolase, which was not completely free from this enzyme. Sedoheptulose 7-phosphate and erythrose 4-phosphate were measured by the transaldolase method as described by Racker (1965). Citrate was assayed by the citrate lyase method described by Moellering & Gruber (1966). 5-Phosphoribosyl pyrophosphate was determined by measuring the release of $^{14}CO_2$ from [*carboxy*-^{14}C]orotic acid in a system similar to that described by Flaks (1963) for the assay of orotidine 5′-phosphate pyrophosphorylase activity. Glucose was measured by the hexokinase method.

Enzyme assays. Hexokinase (glucose–ATP phosphotransferase, EC 2.7.1.1) was assayed in a final volume of 1·35ml. in the following system: 40mM-tris–HCl buffer, pH7·4, 7·4mM-$MgCl_2$, 3·7mM-ATP, 0·32mM-$NADP^+$ and 10mM-glucose. In view of the high activity of hexokinase in these ascites cells, high dilutions of the enzyme for its assay were used, and, since interference by the endogenous 6-phosphogluconate dehydrogenase was negligible, the system was supplemented with only 1·4 units of glucose 6-phosphate dehydrogenase. In this assay system a unit of hexokinase activity is defined as that amount catalysing the formation of 1μmole of glucose 6-phosphate (or NADPH)/min. at 25°. 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 (decarboxylating), EC 1.1.1.44] were assayed by the methods of Glock & McLean (1953). Ribulose 5-phosphate 3-epimerase (D-ribulose 5-phosphate 3-epimerase, EC 5.1.3.1), ribose 5-phosphate isomerase (D-ribose 5-phosphate ketol-isomerase, EC 5.3.1.6), transketolase (sedoheptulose 7-phosphate–D-glyceraldehyde 3-phosphate glycolaldehydetransferase, EC 2.2.1.1) and transaldolase (sedoheptulose 7-phosphate–D-glyceraldehyde 3-phosphate dihydroxyacetonetransferase, EC 2.2.1.2) were assayed as described by Novello & McLean (1968). Phosphoglucose isomerase (D-glucose 6-phosphate ketol-isomerase, EC 5.3.1.9) was assayed by the method of Noltmann (1966). The activities of the glycolytic enzymes were assayed by methods essentially similar to those described by Wu & Racker (1959).

Kinetic studies. The inhibition of hexokinase by fructose 1,6-diphosphate was studied in two different assay systems. The first involved coupling the reaction to glucose 6-phosphate dehydrogenase and following the reduction of $NADP^+$, blanks for fructose 1,6-diphosphatase activity being assayed simultaneously; the second method was by following the rate of ADP formation by coupling the system to pyruvate kinase, lactate dehydrogenase and phosphoenolpyruvate and following the oxidation of NADH, allowance being made for the adenosine triphosphatase activity of the tumour-cell extracts.

The kinetic parameters of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were investigated over the substrate and cofactor concentration range 1μM–1mM in the presence and absence of inhibitors. The inhibitor (fructose 1,6-diphosphate) concentrations used were 1·85 and 2·70mM respectively, and NADPH initial concentrations were 0·11–0·44mM. The kinetics of phosphoglucose isomerase were investigated over the substrate-concentration range 0·04–1·5mM in the presence and absence of erythrose 4-phosphate at 2·6 and 5·2μM. The amount of enzyme used was chosen such that interference from transaldolase was negligible.

All enzyme assays were followed by coupling the reactions to systems oxidizing NADH or reducing $NADP^+$ and following the rates in a Unicam SP.800 recording spectrophotometer with a constant-temperature cell housing and a scale-expansion accessory. In each case a unit of enzyme activity is defined as the amount catalysing the formation of 1μmole of product/min. at 25°.

[^{14}C]*Glucose carbon flow.* Ascites cells were incubated as described above with the modification that the centre wells of flasks contained 1ml. of a 1M-Hyamine hydroxide solution in methanol. After the initial 10min. equilibration period, the specifically labelled [^{14}C]glucose was injected through the rubber caps such that the final glucose concentration was 12·5mM and 0·5μc/flask. Incubations were terminated after 10min. by injecting HCl through the caps, followed 1min. later by Na_2CO_3; 1hr. of further incubation was allowed for the $^{14}CO_2$ to be absorbed into the Hyamine. The Hyamine was later transferred to counting vials with 2ml. of methanol, toluene-based scintillation mixture added and the $^{14}CO_2$ determined by counting in a Packard liquid-scintillation spectrometer. [^{14}C]Lactate was isolated from the contents of the main compartments of flasks by the $CuSO_4$–$Ca(OH)_2$ method described by Katz, Landau & Bartsch (1966). Because this treatment removes about 99% of the glucose, flasks containing the complete system except for ascites cells were treated similarly and the residual glucose was determined by liquid-scintillation counting. The mean of four such determinations was 1% and this factor was used for correcting the [^{14}C]lactate counts.

RESULTS

Activities of enzymes of the pentose phosphate pathway and glycolysis. In a preliminary study, all the enzymes of the pentose phosphate pathway were found to be in the 100000*g* supernatant fraction, except for some latent activities in the particulate fraction (Zaheer, Tewari & Krishnan, 1967). Similarly, in the Ehrlich ascites cells, Wu & Racker (1959) found that, with the exception of

Table 1. *Activities of enzymes of the pentose phosphate pathway compared with enzymes of the glycolytic pathway in Krebs ascites-tumour cells*

The results are expressed as means ± S.E.M. of six determinations, and each determination was made on a separate cell population. The protein content of the high-speed supernatant fraction that was used for the enzyme determinations was 62·5 ± 0·3 mg./g. of cells. All enzymes were assayed at 25° in the forward reaction, i.e. the direction of converting glucose into lactate, except where otherwise stated. The backward reaction is that in the direction of converting lactate into glucose. Triose phosphate isomerase was measured in the direction glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. Values for the soluble and mitochrondrial fraction hexokinase are given. The 12000*g* particles from 1g. of cells contained 16·5 ± 0·8 mg. of protein.

	Activity	
	(units/g. of cells)	(milliunits/mg. of protein)
Hexokinase		
Soluble fraction	10·3 ± 0·2	137 ± 2
12000*g* particles	2·85 ± 0·1	173 ± 5
Enzymes of the pentose phosphate pathway		
Glucose 6-phosphate dehydrogenase	1·47 ± 0·09	19·5 ± 1·3
6-Phosphogluconate dehydrogenase	1·25 ± 0·07	16·8 ± 1·1
Ribose 5-phosphate isomerase	7·25 ± 0·58	97·4 ± 7·8
Ribulose 5-phosphate epimerase	15·9 ± 0·9	214 ± 12
Transketolase	0·94 ± 0·05	12·6 ± 0·8
Transaldolase	1·63 ± 0·04	21·8 ± 0·7
Enzymes of the glycolytic pathway		
Phosphoglucose isomerase		
Forward reaction	53·6 ± 7·9	760 ± 130
Backward reaction	53·6 ± 5·3	760 ± 80
Phosphofructokinase	10·3 ± 1·0	165 ± 16
Fructose diphosphatase	0·23 ± 0·01	3·7 ± 0·2
Aldolase	2·47 ± 0·18	39·5 ± 2·9
Triose phosphate isomerase		
Backward reaction	230 ± 23	3680 ± 37
Glycerol 1-phosphate dehydrogenase	0·29 ± 0·02	4·50 ± 0·34
Glyceraldehyde 3-phosphate dehydrogenase		
Forward reaction	80·8 ± 2·3	1290 ± 38
Backward reaction	141 ± 25	2260 ± 395
Phosphoglycerate kinase	216 ± 8	3450 ± 131
Phosphoglycerate phosphomutase	26 ± 2·0	415 ± 35
Pyruvate kinase	69 ± 4	1100 ± 68
Enolase	29 ± 0·7	460 ± 13
Lactate dehydrogenase		
Forward reaction	117 ± 21	1880 ± 343
Backward reaction	3·25 ± 0·26	52 ± 4

hexokinase, the presence of glycolytic enzyme activities in the particulate fraction could be accounted for by the trapping of soluble enzymes. For these reasons all enzymes have been assayed in the 100000*g* supernatant fraction with the exception of hexokinase, which was also assayed in the washed 12000*g* particles.

The activities of the enzymes of the pentose phosphate pathway were, in general, low compared with those of glycolytic enzymes, with activities ranging from about 1·0 to 1·5 units/g. wet wt. of cells (Table 1). However, ribose 5-phosphate isomerase and ribulose 5-phosphate epimerase had activities about seven- and 15-fold higher respectively. Of the enzymes of glycolysis, glycerol 1-phosphate dehydrogenase had the lowest activity, with aldolase about an order of magnitude more active than it but yet fourfold less active than phosphofructokinase and hexokinase. The other glycolytic enzymes had activities ranging from two to 20 times that of phosphofructokinase, with triose phosphate isomerase having the highest. These activities are in fair agreement with the values reported by Wu & Racker (1959) for the Ehrlich ascites-tumour cells, which, however, had lower hexokinase and phosphofructokinase activities and higher aldolase activity, with phosphoglycerate kinase having the highest specific activity.

Transient and steady-state content of metabolites. The results shown in Figs. 1(*a*), 1(*b*) and 1(*c*) summarize the transient changes found in the contents of the intermediates of the pentose phosphate pathway and certain intermediates of the glycolytic pathway after the addition of glucose to washed ascites cells *in vitro* under aerobic conditions. The zero-time endogenous content of intermediates of these two pathways of glucose metabolism and the steady-state content, measured after incubation for 10min. with 12·5mM-glucose, are given in Table 2.

Glucose 6-phosphate accumulation is very rapid during the first 30sec. after the addition of glucose. This is followed by a period of inhibition and then by a lower steady rate of accumulation at an apparent rate of about 5–10% of the initial value. The steady-state content is reached after approx. 5min. This pattern of change is similar to that reported by Sauer (1968) for Ehrlich–Lettre ascites-tumour cells. The 6-phosphogluconate content of the cells follows a course closely parallel to that of glucose 6-phosphate (see Fig. 1*a*), but in contrast with this the total pentose phosphate increases almost linearly throughout the 10min. incubation period, a point of considerable importance in the assessment of the relative roles of the oxidative and non-oxidative reactions of this pathway in the formation of pentose phosphate.

The results for the intermediates related to the

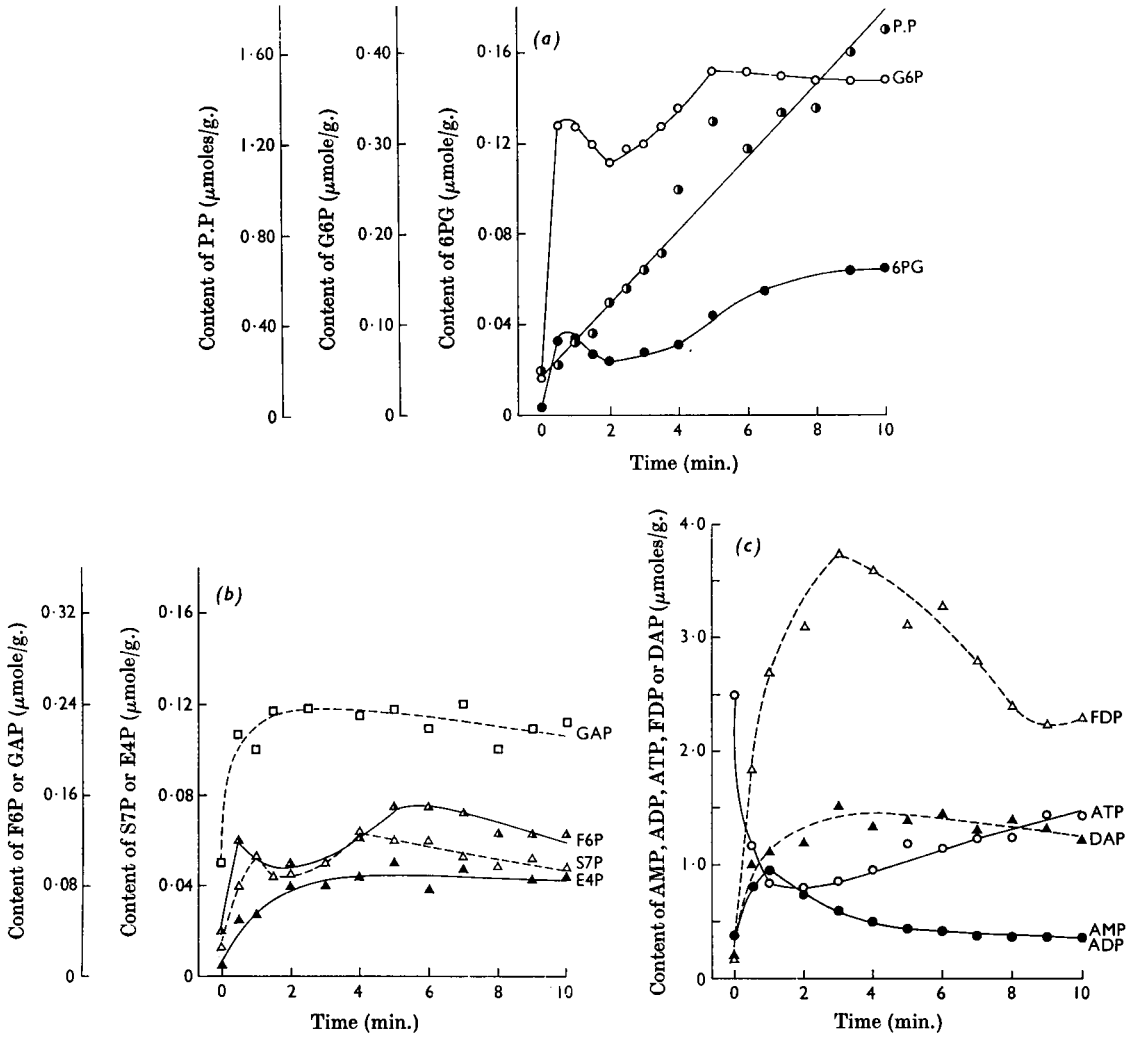


Fig. 1. Content of intermediates of the pentose phosphate pathway and glycolytic pathway at different times after the addition of glucose to ascites-tumour cells. The incubation procedures and assay of metabolites were as described in the Materials and Methods section. The initial glucose concentration was 12·5 mM, and the glucose was added after withdrawal of the zero-time sample. The results are given as μmoles of substrate/g. of packed cells. (*a*) Intermediates of the oxidative part of the pentose phosphate pathway. (*b*) Intermediates of the non-oxidative part of the pentose phosphate pathway. (*c*) Some glycolytic intermediates and adenine nucleotides. The abbreviations used are: G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; P.P, pentose phosphate; GAP, glyceraldehyde 3-phosphate; F6P, fructose 6-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; FDP, fructose 1,6-diphosphate, DAP, dihydroxyacetone phosphate.

non-oxidative reactions of the cycle are shown in Fig. 1(*b*). Sedoheptulose 7-phosphate content shows a pattern of change very similar to those of fructose 6-phosphate and glucose 6-phosphate, although there is a lag in the onset of the period of inhibition of accumulation, the linear rate of accumulation extending over the first 1 min. There does not appear to be any direct relationship between pentose phosphate and sedoheptulose 7-phosphate accumulation as might have been expected if the cycle were operating mainly in the direction of resynthesis of hexose monophosphate from pentose phosphate, suggesting that fructose 6-phosphate may be the more important precursor. Erythrose

Table 2. *Endogenous and steady-state content of metabolites in Krebs ascites-tumour cells incubated with glucose*

Endogenous values were measured in washed aerated Krebs ascites cells and steady-state values were after aerobic incubation for 10 min. with 12·5 mM-glucose. Reactions were stopped with ice-cold $HClO_4$ and metabolites assayed as described in the Materials and Methods section. The range of endogenous values are given (three or four values for each metabolite) and the means ± S.E.M. of six values for steady-state metabolites.

	Content (μmoles/g. of cells)		
Metabolite	Endogenous	Endogenous range	10 min. steady state
Glucose 6-phosphate	0·038	0·023–0·043	0·444 ±0·014
6-Phosphogluconate	0·008	0·005–0·011	0·102 ±0·005
Pentose phosphates	0·200	0·100–0·300	2·103 ±0·078
Sedoheptulose 7-phosphate	0·019	0·009 0·035	0·045 ±0·005
Erythrose 4-phosphate	0·005	0·002–0·008	0·036 ±0·003
5-Phosphoribosyl pyrophosphate	Too low to measure		1·000*
Fructose 6-phosphate	0·033	0·023–0·043	0·161 ±0·009
Fructose 1,6-diphosphate	0·161	0·136–0·218	2·151 ±0·083
Dihydroxyacetone phosphate	0·214	0·174–0·250	1·033 ±0·105
Glyceraldehyde 3-phosphate	0·107	0·065–0·208	0·137 ±0·013
α-Glycerophosphate	0·019	0·017–0·022	0·143 ±0·009
3-Phosphoglycerate	0·075	0·052–0·098	0·188 ±0·014
2-Phosphoglycerate	0·036	0·022–0·054	0·031 ±0·003
Phosphoenolpyruvate	0·087	0·071–0·113	0·109 ±0·005
Pyruvate	0·234	0·185–0·261	0·449 ±0·042
Lactate	3·250	2·610–4·070	15·261 ±0·331
Malate	0·065	0·061–0·070	0·216 ±0·013
Citrate	0·177	0·152–0·200	0·246 ±0·011
ATP	2·393	2·020–2·714	1·725 ±0·094
ADP	0·469	0·313–0·554	0·543 ±0·019
AMP	0·456	0·391–0·592	0·388 ±0·010
NAD^+			0·2000 ±0·0140
NADH			0·0414 ±0·0014
$NADP^+$			0·0091 ±0·0006
NADPH			0·0069 ±0·0001

* Two results only in this group.

4-phosphate content changes in a manner distinct from that of other intermediates of the pentose phosphate pathway, increasing during the first 2 min. to reach the steady-state value, which is thereafter maintained. The content of glyceraldehyde 3-phosphate, which is also a reactant in the transketolase and transaldolase reactions, shows a very rapid initial rise, reaching the steady-state value at about 1–2 min. after addition of glucose.

The accumulation of pentose phosphate is an outstanding feature of the metabolite pattern (see Table 2), and is 20 times as great as the 6-phosphogluconate accumulation and 50 times as great as sedoheptulose 7-phosphate or erythrose 4-phosphate accumulation. The conversion of pentose phosphate into phosphoribosyl pyrophosphate was also investigated: during the 10 min. incubation period approx. 1 μmole of phosphoribosyl pyrophosphate was formed, which is approx. 50% of the total pentose phosphate present.

The transient-state changes in the contents of fructose diphosphate, dihydroxyacetone phosphate and adenine nucleotides are shown in Fig. 1(*c*). A very large amount of fructose diphosphate accumulates in ascites cells, as much as 4 μmoles/g. of cells after the addition of glucose, falling to a steady-state value of 2 μmoles/g. of cells at 10 min. These values are broadly in agreement with those found by Hess (1963) and Wu (1965*b*). The reciprocal relationship between fructose diphosphate and ATP contents is clearly shown in Fig. 1(*c*), a pattern of change closely similar to that reported by Maitra & Chance (1965).

The steady-state contents of metabolites in Ehrlich ascites cells reported by Hess (1963) are in general somewhat higher than those given in Table 2. In comparison with the present results the values given by Hess (1963) are tenfold higher for malate and α-glycerophosphate, fivefold higher for glucose 6-phosphate and fructose 6-phosphate and threefold higher for 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate and the sum of the

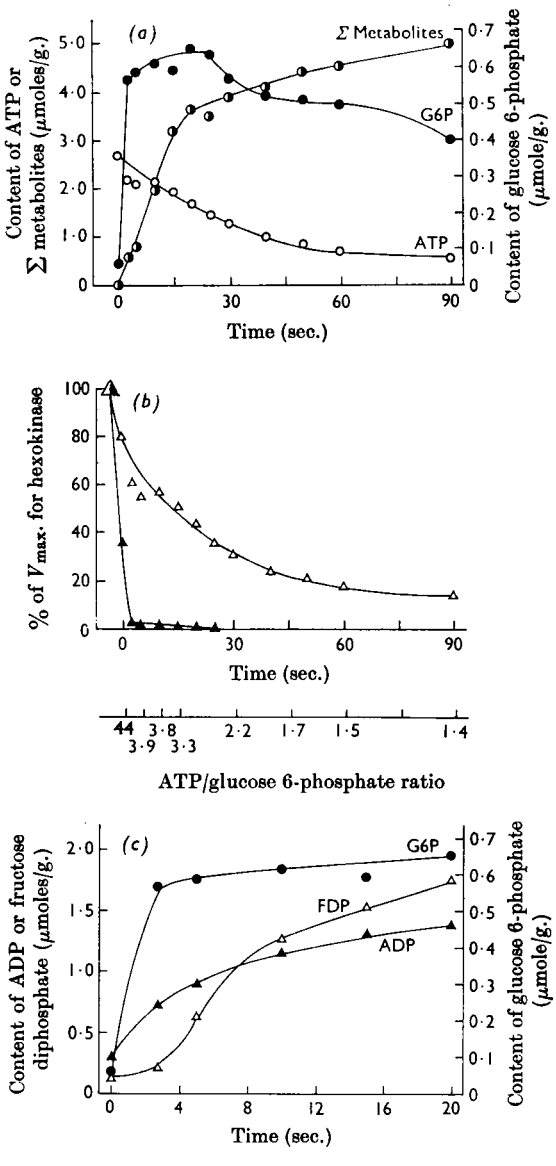


Fig. 2. Interrelationship between glucose 6-phosphate (G6P), fructose diphosphate (FDP), ATP, ADP and the total rate of glucose phosphorylation (Σ metabolites) in intact cells and in simulated systems during the transient state, the first 90sec., after the addition of glucose to Krebs ascites-tumour cells. (*a*) Changes in the contents of glucose 6-phosphate, ATP and total rate of phosphorylation. The cells were incubated aerobically with glucose (initial concn. 12·5mM) and the results are given as μ moles/g. of packed cells. The metabolites were summated from measurements of all the intermediates of the glycolytic pathway between glucose and lactate (with the exception of 1,3-diphosphoglycerate) together with intermediates of the pentose phosphate pathway. (*b*) Activity of partially purified hexokinase in simulated systems. The hexokinase activity in the presence of ATP concentrations found in the cell 0–90sec. after the addition of glucose is shown by △; the activity in the presence of ATP plus glucose 6-phosphate concentrations and 3mM-P_i at the same time-intervals is shown by ▲. The time-scale and the ATP and glucose 6-phosphate concentrations are similar to those shown in (*a*) above. The ATP/glucose 6-phosphate ratio is given for the appropriate time-intervals. The results are expressed as a percentage of the maximum velocity measured in the presence of optimum substrate concentrations in the absence of glucose 6-phosphate. (*c*) Changes in the contents of glucose 6-phosphate, fructose diphosphate and ADP during the first 20sec. after the addition of glucose to ascites cells. The conditions were as in (*a*) above.

adenine nucleotides; in contrast, the dihydroxyacetone phosphate content is only one-fifth of the present value, and further the two triose phosphates do not appear to be in equilibrium. In addition to differences in the ascites cells a lower temperature (22°) and shorter period of incubation (5min.) was used by Hess (1963).

One of the most striking features of the metabolite pattern was the extremely rapid rate of glucose 6-phosphate accumulation followed by a very sharp onset of inhibition. Analysis of the rate of glucose 6-phosphate accumulation at short time-intervals varying from 3sec. to 90sec. after the addition of glucose indicated that as much as 0·5μmole of glucose 6-phosphate could be formed/g. of cells in the first 3sec. of the addition of glucose (Fig. 2*a*), equivalent to a rate of glucose phosphorylation of 10μmoles/min./g. of cells at 37°. Rose (1965) equated the rate of hexokinase activity in ascites cells with the total formation of anionic material that is held on a Dowex 1 (acetate form) column, assuming that the losses by incorporation into polysaccharides or oxidation to carbon dioxide are negligible. The glucose 6-phosphate accumulating in the cell is a resultant of the difference in the rates of formation and utilization, and in the present experiment it was decided to measure the rate of glucose 6-phosphate formation as opposed to that of its accumulation. This was done by a method in principle similar to that employed by Rose (1965), Coe (1966) and Lee & Coe (1967), namely that all the metabolites between glucose and lactate at any one time were summed and the total sum of the metabolites (Σ metabolites) was corrected for the amount present at zero time before the addition of glucose. As shown in Fig. 2(*a*) the rate of glucose phosphorylation was linear for the first 15sec., after which the rate decreased sharply. The initial rate of glucose phosphorylation was 12·5μmoles/min./g. of cells at 37°. The total hexokinase activity, including all cell fractions, was 14·3 ± 0·3 units/g. of cells under optimum conditions, corresponding to a rate of phosphorylation of about 28μmoles of

glucose/min./g. of cells at 37° (based on a Q_{10} value of just under 2 to correct for the temperature difference between spectrophotometric assay at 25° and the incubation of cells with glucose at 37°; see England & Randle, 1967). Thus hexokinase appears to be operating at approx. 45% of $V_{max.}$ during this initial phase, a value that is somewhat higher than that (30%) given by Rose (1965) for hexokinase of sarcoma 37 ascites cells.

The extremely rapid initial accumulation of glucose 6-phosphate is probably due, at least in part, to the control of phosphofructokinase by the relative ATP:ADP:AMP content of the cell. It was found that there was practically no accumulation of fructose diphosphate during the first 3sec. of the incubation, the increment in fructose diphosphate content being only 0·06μmole. Thereafter there was a rapid rise in fructose diphosphate content: the increment in the next period of 3–5sec. was 0·4μmole and between 5 and 10sec. was 0·6μmole (see Fig. 3c). The rapid formation of fructose diphosphate and other metabolites of the glycolytic pathway accounts for the observation that glucose 6-phosphate accumulation is inhibited at 3sec. after the addition of glucose whereas the rate of glucose phosphorylation as calculated from total metabolite accumulated is linear for up to 15sec.

To study the factors responsible for the marked inhibition of glucose phosphorylation after 30sec. in the present system, with which glucose 6-phosphate, the ATP/glucose 6-phosphate quotient and ADP, AMP and P_i have been implicated in ascites cells (Rose, 1965; Kosow & Rose, 1968; Sauer, 1968), simulated systems were studied. In these the activity of hexokinase was measured in the presence of concentrations of ATP, glucose 6-phosphate and P_i that would be present in the cell at different times after addition of glucose. The results of these experiments (Fig. 2b) show that the decrease in ATP alone could account, in part, for the decline in hexokinase activity, for the concentration of ATP present in the cell at 15sec. after the addition of glucose would have caused a fall to approx. 50% of $V_{max.}$, whereas at 1min. the ATP concentration would support a rate of only 20% of $V_{max.}$. However, this alone does not account for the sharp change in slope at 15–20sec. The lower curve on Fig. 2(b) shows the effect of ATP plus glucose 6-phosphate, present in concentrations similar to those at the appropriate times after glucose addition. At zero time the ATP/glucose 6-phosphate ratio is 45 and the velocity of the hexokinase reaction is approx. 37% of the $V_{max.}$ observed in the absence of added glucose 6-phosphate. This is in good agreement with the value for the initial rate of glucose phosphorylation in intact cells estimated in Fig. 2(a), which was found to be about 45% of the theoretical $V_{max.}$ of the hexokinase measured *in vitro*. However, in the presence of the concentrations of ATP and glucose 6-phosphate similar to those found 3sec. after addition of glucose, the rate of glucose phosphorylation decreased to only about 3% of the maximum velocity even though 3mM-P_i was present which should cause some relief of inhibition, (see Rose, 1965; Sauer, 1968). It may be noted that Lowry & Passonneau (1964) did not find a marked relief of glucose 6-phosphate inhibition of brain hexokinase by P_i. As the glucose 6-phosphate concentration is increased and that of ATP is decreased in parallel with the values shown in Fig. 2(a), so the inhibition of hexokinase becomes almost complete. Thus there seem to be other factors of importance in counteracting the inhibition by glucose 6-phosphate since, as shown in Fig. 2(a), the rate of phosphorylation is not inhibited until 15–20sec. after the addition of glucose, a situation not found in the simulated system. One important factor may be that in this simulated system soluble hexokinase was used and not the particulate form. It is known that the particulate form is less susceptible to inhibition by glucose 6-phosphate (Li & Ch'ien, 1966; Kosow & Rose, 1968; Sauer, 1968).

Three factors seemed to merit further investigation: first, the relationship between the ATP concentration and the rate of glucose phosphorylation as shown in the intact cells; secondly, the relationship between the rate of phosphorylation and the ATP/glucose 6-phosphate ratio (values for the ratio of 3 have been cited as causing a 90–95% inhibition of hexokinase in the presence of 1mM-ATP and 3mM-P_i; Sauer, 1968); and, finally, the possibility that two phases occur with a second form of control coming into play 15sec. after the addition of glucose, which might be related to the accumulation of some other metabolite. With regard to this third point, either fructose diphosphate or pentose phosphate might be considered as possible feedback inhibitors of hexokinase, since they both accumulate and are present in fairly high concentrations. This point was investigated and is reported below.

The relationship between the rate of glucose phosphorylation and the ATP content of the cell is shown in Fig. 3. During the initial period up to 10sec. after the addition of glucose the rate of phosphorylation is independent of the ATP concentration, between 10 and 60sec. the rate of phosphorylation is approximately proportional to the ATP concentration, and at longer time-intervals the plot of the rate of phosphorylation becomes essentially parallel to the abscissa and appears relatively independent of the ATP concentration, the altered relationship starting at about 1–2min. after the addition of glucose. The results in Fig. 4 show the relationship between ATP and the glucose 6-phosphate content of the cell and show that again

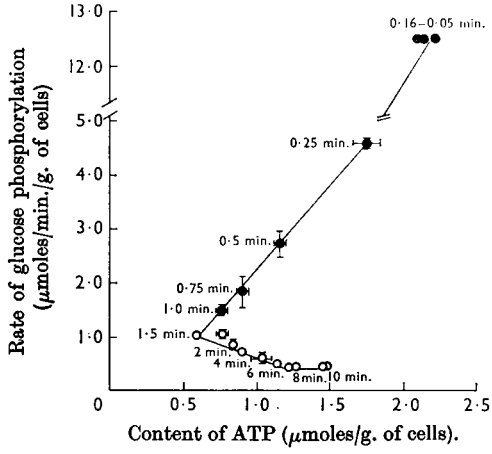


Fig. 3. Relationship between the rate of glucose phosphorylation and the ATP content of the Krebs ascites-tumour cells at different times after the addition of glucose. The rate of glucose phosphorylation was determined by constructing tangents to curves for the change in content of the metabolites with time (see, for example, Fig. 2*a*). The rate of glucose phosphorylation was then plotted as ordinate against the ATP content of the cell at that time as abscissa. The points represent the mean values for two to five experiments together with the S.E.M. shown in two directions, one for each parameter; where no S.E.M. value is given the value is too small to show. The values for time-intervals up to 0·25 min. and after 6 min. are the means of two experiments only. The time-intervals at which the experimental points were determined are shown on the graph as minutes or fractions of a minute; for clarity alternate symbols are given for time-intervals above 1 min. Experimental points up to 1 min. are shown as ● and those from 1·5 to 10 min. as ○.

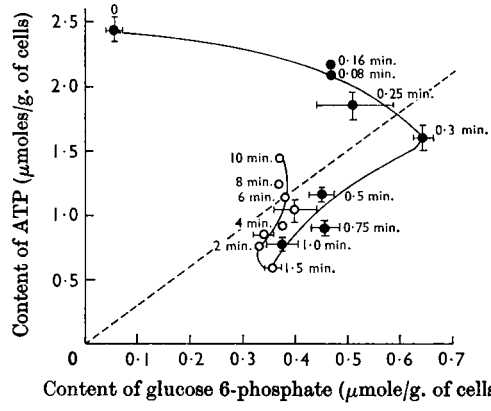


Fig. 4. Interrelationship between ATP and glucose 6-phosphate content of the Krebs ascites-tumour cells at different time-intervals after the addition of glucose. The points represent the mean values of two to five experiments together with the S.E.M. shown in two directions, one for each metabolite. Where no S.E.M. is given the value is too small to show. The values for the time-intervals up to 0·25 min. and after 6 min. are the means of two experiments only. The time-intervals at which the experimental points were determined are shown on the graph as minutes or fractions of a minute; for clarity alternate symbols are given for time-intervals above 1 min. Experimental points up to 1 min. are shown as ● and those from 1·5 to 10 min. as ○.

there are two distinct phases, an initial period up to 15 sec. after the addition of glucose in which the two are not directly related, followed by a period when the glucose 6-phosphate content of the cell appears to be directly proportional to the ATP content. The ATP/glucose 6-phosphate ratio during this second phase is approx. 3 and remains essentially constant, in agreement with the results of Wu (1965*a*,*b*), Maitra & Chance (1965) and Sauer (1968).

Kinetic properties of hexokinase and some enzymes of the pentose phosphate pathway. The persistence of hexokinase activity in the 12000*g* particulate fraction after repeated washing is consistent with a bound form of the enzyme (McComb & Yushok, 1959; Rose & Warms, 1967). That this binding is specific is supported by the observation that the hexokinase typeII/type I ratio differs widely for the soluble and particulate forms of the enzyme (Table 3), a finding further substantiated by the kinetic properties of the two fractions. The apparent Michaelis constants for glucose and ATP suggest a predominance of type II hexokinase in the supernatant fraction and favours the presence of equal proportions of the two isoenzymes in the particulate fraction. Similarly, by comparison with purified isoenzymes (Grossbard & Schimke 1966), the inhibitor constants of glucose 6-phosphate for the soluble enzyme are in accord with a predominance of type II isoenzyme.

In view of the massive accumulation of certain metabolic intermediates during the initial phase of glucose catabolism with subsequent decrease in the rate of glucose phosphorylation, the possibility of feedback inhibition of the hexokinase reaction by sugar phosphates other than the product of its own reaction was tested. The inhibition of hexokinase by 3-phosphoglycerate, first reported by Z. Dische (1940; quoted by Stadtman, 1966), could not be reproduced with the Krebs ascites-cell hexokinase at concentrations of 3-phosphoglycerate similar to those found during the steady state. Ribose 5-phosphate at 5 mM concentration resulted in a 25% inhibition of the hexokinase reaction, whereas fructose 1,6-diphosphate at 2 mM concentration caused 23% and 90% inhibition when the Mg^{2+} concentrations in the assay system were 15 mM and 7·5 mM respectively. The Mg^{2+} content of the

Table 3. *Some properties of the soluble and particulate hexokinase of Krebs ascites-tumour cells*

'Soluble hexokinase' refers to the hexokinase in the supernatant fraction after centrifugation at 100000*g* for 45min. 'Particulate hexokinase' refers to the fraction collected after centrifugation at 12000*g* for 10min., after removal of the debris and nuclei by centrifugation at 600*g* for 10min. Heat treatment for 1hr. at 45° was used to differentiated type I and type II hexokinase (see the Materials and Methods section). Kinetic constants were determined on extracts without heat treatment. The K_i for glucose 6-phosphate was determined with a partially purified hexokinase preparation taken up to the DEAE-cellulose-column chromatographic step of the method of Grossbard & Schimke (1966). The effect of fructose diphosphate on hexokinase was measured by two methods (by assay of hexokinase after glucose 6-phosphate formation and also by ADP formation); corrections were made for fructose diphosphatase activity (for details see the Materials and Methods section).

		Hexokinase	
		Soluble	Particulate
Hexokinase activity (units/g. of cells)			
Total activity		10·30±0·22	2·85±0·10
Type I (heat-stable form)		0·45±0·03	1·06±0·04
Type II (heat-labile form)		9·85±0·19	1·79±0·11
Kinetic constants			
K_m for glucose (3·7mM-ATP)		$1·4\times10^{-4}$M	$0·8\times10^{-4}$M
K_m for ATP (10mM-glucose)		$6·2\times10^{-4}$M	$4·4\times10^{-4}$M
K_i for glucose 6-phosphate			
versus glucose		$1·6\times10^{-4}$M	
versus ATP		$7·0\times10^{-5}$M	
Percentage inhibition by sugar phosphate			
Fructose diphosphate (2mM) (3·7mM-ATP, 7·4mM-$MgCl_2$)		90%	
Fructose diphosphate (2mM) (3·7mM-ATP, 15mM-$MgCl_2$)		23%	
Ribose 5-phosphate (5mM)		25%	
3-Phosphoglycerate (0·3mM)		0	
Published kinetic constants			
K_m for glucose	Type I	$4·7\times10^{-5}$M	—
	Type II	$2·5\times10^{-4}$M	$1·5\times10^{-4}$M
K_m for ATP	Type I	$4·2\times10^{-4}$M	—
	Type II	$7·6\times10^{-4}$M	10×10^{-4}M
K_i for glucose 6-phosphate			
versus glucose	Type I	$2·1\times10^{-4}$M	
	Type II	$1·6\times10^{-4}$M	
versus ATP	Type I	$2·6\times10^{-5}$M	
	Type II	$2·1\times10^{-5}$M	
Reference		Grossbard & Schimke (1966)	Kosow & Rose (1968)

washed Krebs ascites cells determined by emission flame spectrophotometry was found to be 7 μmoles/g., a concentration fairly close to that employed in the assay system in which fructose 1,6-diphosphate caused 90% inhibition.

The extent of operation of the fructose 1,6-diphosphate inhibition *in vivo* will be dependent on the Mg^{2+} concentration in the direct environment of hexokinase and may be subject to modifications by the Mg^{2+} in the incubation medium.

Glucose 6-phosphate dehydrogenase (Table 4) had apparent Michaelis constants for both substrate and cofactor about five to ten times the values reported for the enzyme from normal tissues. On the other hand, 6-phosphogluconate dehydrogenase showed K_m values for 6-phosphogluconate and $NADP^+$ very similar to those for normal tissues and was competitively inhibited, with respect to 6-phosphogluconate, by fructose diphosphate (Carter & Parr, 1967). NADPH, which is a competitive inhibitor of glucose 6-phosphate dehydrogenase with K_i $2·7\times10^{-5}$M with respect to $NADP^+$ (Glaser & Brown, 1955), also inhibited 6-phosphogluconate dehydrogenase, but the inhibitor constant was higher than the coenzyme concentration encountered in these tumour cells (see Table 2). Phosphoglucose isomerase exhibited kinetic constants very similar to those of the enzyme from normal tissues, and was inhibited by erythrose 4-phosphate, as was reported for the rabbit muscle enzyme (Grazi, de Flora & Pontremoli, 1960).

Identification of 'non-equilibrium' reactions. The

Table 4. *Some properties of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of Krebs ascites-tumour cells*

References: 1, Glock & McLean (1953); 2, Levy (1963); 3, Noltmann & Kuby (1963); 4, McLean (1958); 5, Carter & Parr (1967); 6, Reithel (1966); 7, Grazi *et al.* (1960).

	Krebs ascites-tumour cells	Published values for normal tissues	References
Glucose 6-phosphate dehydrogenase			
K_m for glucose 6-phosphate	$13{\cdot}3\times10^{-5}$ M	$1{\cdot}3$–$3{\cdot}9\times10^{-5}$ M	1, 2, 3
K_m for NADP+	$5{\cdot}7\times10^{-5}$ M	$0{\cdot}4$–$1{\cdot}3\times10^{-5}$ M	1, 2, 3
6-Phosphogluconate dehydrogenase			
K_m for 6-phosphogluconate, pH 7·6	$1{\cdot}0\times10^{-5}$ M	1×10^{-5} M	1, 3, 4
K_m for NADP+, pH 7·6	$1{\cdot}1\times10^{-5}$ M	$2{\cdot}8\times10^{-5}$ M	1
K_i for fructose diphosphate	$7{\cdot}2\times10^{-5}$ M	Competitive inhibitor‡	5
K_i for NADPH	15×10^{-5} M		
Phosphoglucose isomerase			
K_m for glucose 6-phosphate	50×10^{-5} M	12×10^{-5} M	6
K_m for fructose 6-phosphate	17×10^{-5} M	7–8×10^{-5} M	6, 7
K_i for erythrose 4-phosphate			
versus glucose 6-phosphate	$0{\cdot}75\times10^{-5}$ M*	Similar to value for fructose 6-phosphate §	7
versus fructose 6-phosphate	$0{\cdot}07\times10^{-5}$ M†	$0{\cdot}07$–$0{\cdot}10\times10^{-5}$ M	7

* Mixed-type inhibition.

† Competitive inhibition.

‡ Carter & Parr (1967) demonstrated 25% inhibition when fructose diphosphate was present in equimolar concentration with 6-phosphogluconate; the inhibition was competitive.

§ Quoted by Grazi *et al.* (1960) as similar to the value found with fructose 6-phosphate as substrate; the inhibition was competitive.

following assumptions were made in calculating the mass-action ratios of the reactions of the pentose phosphate and glycolytic pathways shown in Table 5. The $NADP^+/NADPH$ concentration ratio is 1:240 (Krebs, 1967) and the $NAD^+/NADH$ concentration ratio in the cytoplasmic compartment is 1100:1, calculated from the equilibrium constant of the lactate dehydrogenase reaction (Krebs, 1967); the $[CO_2]$ is 1·16mM (Krebs & Veech, 1969); and the ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase catalyse equilibrium reactions with ribose 5-phosphate:ribulose 5-phosphate:xylulose 5-phosphate maintained at the molar proportions 35:27:38 respectively (Ashwell & Hickman, 1957). No correction for the contribution of phosphoribosyl pyrophosphate to the total pentose phosphate was attempted, since this does not greatly alter the calculated mass-action ratios.

The apparent equilibrium constants of the two dehydrogenases of the pentose phosphate pathway were calculated from $\Delta G = -RT\ln K$ by using the free-energy values presented by Dickens, Glock & McLean (1959). The apparent equilibrium constant of the glucose 6-phosphate dehydrogenase–lactonase couple is regarded as an approximate value, since no accurate measurements of the free energy of the lactonase reaction are available (Glaser & Brown, 1955). The apparent equilibrium constant for the 6-phosphogluconate dehydrogenase reaction calculated from the free-energy value agrees closely with the constant estimated by Horecker & Smyrniotis (1952). Of the reactions of the pentose phosphate pathway, only that catalysed by the glucose 6-phosphate dehydrogenase–lactonase couple was greatly displaced from equilibrium and this by a factor of about 500. The reactions catalysed by 6-phosphogluconate dehydrogenase and by the non-oxidative enzymes, namely transketolase and transaldolase, were almost at equilibrium, although the transketolase reaction in the direction of sedoheptulose 7-phosphate formation was displaced from equilibrium by a factor of 100 (Table 5). The results in Fig. 5 demonstrate the changes in the mass-action ratios of reactions of the pentose phosphate pathway during the transient state of metabolism. The mass-action ratio of phosphofructokinase, an established allosteric regulatory enzyme, is included for comparison. The glucose 6-phosphate dehydrogenase reaction was maintained far from equilibrium at all times, in contrast with the transketolase reaction, which approached equilibrium in 30sec. and thereafter progressively departed from it. The transaldolase and 6-phosphogluconate dehydrogenase reactions

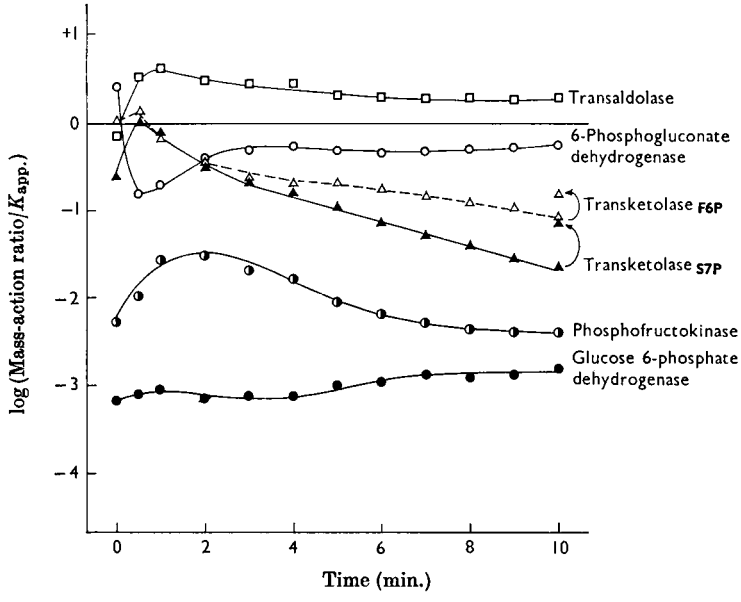


Fig. 5. Changes in the mass-action ratio of enzymes of the pentose phosphate pathway and of phosphofructokinase during transient and steady-state conditions after the addition of glucose to ascites-tumour cells. The mass-action ratios were determined from measurements of metabolites at short time-intervals after the addition of glucose (final concn. 10mM) to ascites cells. The value of log (mass-action ratio/$K_{app.}$) has been plotted as ordinate against time after the addition of glucose. The substrate concentrations are expressed as μmoles/g. of cells. The pentose phosphate pathway enzymes are considered in the direction towards oxidative decarboxylation of glucose 6-phosphate and resynthesis of hexose monophosphate from pentose phosphate. For details of the factors used in the calculation of the mass-action ratio and $K_{app.}$, see Table 5. The two transketolases are represented by the subscript S7P for the reaction yielding sedoheptulose 7-phosphate and F6P for the reaction yielding fructose 6-phosphate (see Table 5). The correction in the value log (mass-action ratio/$K_{app.}$) due to the accumulation of phosphoribosyl pyrophosphate is shown by the second triangle and upward arrow at 10min., the only time when phosphoribosyl pyrophosphate content was specifically measured. The correction is probably very small at shorter time-intervals (see Henderson & Khoo, 1965).

were close to equilibrium at all times except during the resting phase before the addition of glucose to the washed ascites cells.

The regulatory nature of ribose phosphate pyrophosphokinase (ATP–D-ribose 5-phosphate pyrophosphotransferase, EC 2.7.6.1) is confirmed by the present finding that the mass-action ratio is 100-fold lower than the $K_{app.}$ (see Table 5) (Switzer, 1969; Klungsøyr, Hagemen, Fall & Atkinson, 1968).

Of the glycolytic pathway enzymes, phosphoglucose isomerase, aldolase, triose phosphate isomerase, phosphoglycerate mutase and enolase catalysed equilibrium reactions. Glycerol 1-phosphate dehydrogenase and the glyceraldehyde 3-phosphate dehydrogenase–phosphoglycerate kinase couple catalysed reactions displaced from equilibrium by a factor of 100, whereas the lactate dehydrogenase reaction was at equilibrium (Table 5). That the glycerol 1-phosphate dehydrogenase catalysed a 'non-equilibrium' reaction is consistent with the observed low $V_{max.}$ of the enzyme in the Krebs ascites cells, a situation closely related to that encountered in brain (Lowry & Passonneau, 1964) and in HeLa cells (Wu, 1959). Phosphofructokinase, pyruvate kinase and hexokinase catalysed reactions greatly displaced from equilibrium, consistent with their role as regulatory enzymes. The inclusion of hexokinase in the group of regulatory enzymes is based on the assumption that the glucose in the incubation medium was freely available for the hexokinase reaction (Hess, 1963). The mass-action ratio of the adenylate kinase reaction was 5·0 initially, rapidly reaching a value close to equilibrium of 0·44 by 40sec., only to be displaced again, as the steady state was approached, to a value of 2·27.

Incorporation of ^{14}C *from specifically labelled glucose into carbon dioxide and lactate.* The results shown in Table 6 summarize experiments in which

Table 5. *Comparison between mass-action ratios and apparent equilibrium constants for reactions of the pentose phosphate pathway and glycolysis in ascites-tumour cells*

Mass-action ratios were calculated from the concentration of intermediates given in Table 2; the substrate concentrations are expressed as μmoles/g. of cells throughout. The general reaction considered was A+B=C+D; the pentose phosphate pathway enzymes are considered in the direction towards oxidative decarboxylation of glucose 6-phosphate and the resynthesis of hexose monophosphate from pentose phosphates; the glycolytic enzymes are considered in the direction of lactate formation. The mass-action ratio was calculated by using the expression [C][D]/[A][B]. Factors assumed in the mass-action ratios are: (*a*) NADPH/$NADP^+$ ratio 240 (Krebs, 1967); (*b*) $[CO_2]$ value 1·16 mM (Krebs & Veech, 1969); (*c*) ribulose 5-phosphate, xylulose 5-phosphate and ribose 5-phosphate concentrations were calculated from the total pentose phosphate present by using the equilibrium constants for ribose 5-phosphate isomerase and ribulose 5-phosphate epimerase given by Ashwell & Hickman (1957); (*d*) in accordance with Hess (1963), the glucose concentration was assumed to be similar to the concentration in the medium, i.e. 12·5 mM. Apparent equilibrium constants were taken from these references: 1, Dickens *et al.* (1959) calculated from $\Delta G = -RT\ln K'$; 2, Dickens *et al.* (1959); 3, Horecker & Smyrniotis (1952); 4, Datta & Racker (1961); 5, Venkataraman & Racker (1961); 6, Switzer (1969); 7, calculated by Rolleston & Newsholme (1967) by using $\Delta G = -RT\ln K'$ from Krebs & Kornberg (1957); 8, Hess (1963); 9, Reithel (1966); 10, Krebs (1967); 11, Bowen & Kerwin (1954); the $K_{app.}$ is dependent on the Mg^{2+} concentration.

	Apparent equilibrium constants	Mass-action ratios observed	Published mass-action ratios for ascites cells (Hess, 1963)
Pentose phosphate pathway enzymes			
Glucose 6-phosphate dehydrogenase+lactonase	$2·74 \times 10^4$ (1)	$0·55 \times 10^2$ (*a*)	
6-Phosphogluconate dehydrogenase	2·3–5·0 (2, 3)	1·55 (*a*, *b*, *c*)	
Transketolase [Sedoheptulose 7-phosphate][glyceraldehyde 3-phosphate] / [Ribose 5-phosphate][xylulose 5-phosphate]	1·0 (4)	$0·6 \times 10^{-2}$ (*c*)	
Transketolase [Fructose 6-phosphate][glyceraldehyde 3-phosphate] / [Erythrose 4-phosphate][xylulose 5-phosphate]	10·0 (4)	0·75 (*c*)	
Transaldolase [Fructose 6-phosphate][erythrose 4-phosphate] / [Sedoheptulose 7-phosphate][glyceraldehyde 3-phosphate]	1·05 (5)	1·65	
Ribose phosphate pyrophosphokinase	28·6 (6)	0·28	
Glycolytic-pathway enzymes			
Hexokinase	$3·9–5·5 \times 10^3$ (7, 8)	$1·1 \times 10^{-2}$ (*d*)	$1·9 \times 10^{-3}$
Phosphoglucose isomerase	0·32–0·47 (8, 9)	0·36	0·23
Phosphofructokinase	$0·91–1·2 \times 10^3$ (7, 8)	4·2	0·63
Aldolase	$6·8 \times 10^{-5}$ (8)	$6·6 \times 10^{-5}$	$2·87 \times 10^{-5}$
Triose phosphate isomerase [Dihydroxyacetone phosphate] / [Glyceraldehyde 3-phosphate]	22 (8)	7·5	0·65
Glycerol 3-phosphate dehydrogenase	$1·78 \times 10^4$ (10)	154	—
Glyceraldehyde 3-phosphate dehydrogenase+phosphoglycerate kinase	$1·83–3·1 \times 10^2$ (7, 8)	0·44	17·8
Phosphoglycerate mutase	0·18–0·166 (7, 8)	0·164	2·3
Enolase	1·4–2·8 (8, 7)	3·5	1·6
Pyruvate kinase	$1·58–2·0 \times 10^4$ (8, 7)	13·1	2·8
Lactate dehydrogenase	$1·11 \times 10^4$ (10)	$3·8 \times 10^4$	
Adenylate kinase	0·33–1·06 (11)		

ascites cells were incubated with specifically labelled glucose and the incorporation of ^{14}C into carbon dioxide and lactate was measured. The disappearance of glucose was measured in order to calculate the specific yields of $^{14}CO_2$ and [^{14}C]lactate by the method of Katz *et al.* (1966).

The ascites cells incubated with glucose alone showed a greater yield of $^{14}CO_2$ from C-1 ('1-$^{14}CO_2$') than from C-6 ('6-$^{14}CO_2$') of glucose, in keeping with the operation of the pentose phosphate pathway, and a low rate of oxidation through the tricarboxylic acid cycle. The amount of oxidation of C-2 of glucose is almost identical with that of C-6, suggesting that recycling via the pentose phosphate pathway is relatively low. The amount of oxidation of C-3 and C-4 of glucose is considerably higher than that of C-6 or even that of C-1 of glucose. In a tissue in which the predominant pathway of metabolism

Table 6. *Flow of glucose carbon in the alternative pathways of glucose metabolism in Krebs ascites-tumour cells*

Incubation procedures are given in the Materials and Methods section. Glucose carbon flow was calculated from the ratio (radioactivity in product/specific radioactivity of glucose). Results are the means of four experiments and are expressed as μg.atoms of glucose carbon/g. of cells. Glucose utilization was calculated by subtracting the amount of glucose in the incubation medium at the end of the experiment from the initial concentration. Methods for calculating the percentage contribution of the pentose phosphate pathway (PC) to glucose metabolism were:

(*a*) $PC = \frac{[1\text{-}^{14}CO_2]-[6\text{-}^{14}CO_2]}{\text{glucose utilized}} \times 100$;

(*b*) $PC = \frac{S}{3-2S} \times 100$, where $S = \frac{\text{G-1-}CO_2 - \text{G-6-}CO_2}{1-\text{G-6-}CO_2}$ and G-CO_2 is the specific yield of CO_2;

(*c*) $PC = \frac{S'}{3-2S'} \times 100$ where $S' = \text{G-1-}CO_2 - (\text{G-6-}CO_2.\gamma)$ and $\gamma = \frac{^{14}C \text{ from } [1\text{-}^{14}C]\text{glucose into lactate}}{^{14}C \text{ from } [6\text{-}^{14}C]\text{glucose into lactate}}$;

(*d*) $PC = \frac{1-\gamma}{1+2\gamma} \times 100$;

(*e*) $PC = \frac{^{14}C \text{ from } [1\text{-}^{14}C]\text{glucose into lactate}}{^{14}C \text{ from } [2\text{-}^{14}C]\text{glucose into lactate}}$, from the plot by Katz & Wood (1960).

References: (*a*), Beck (1958); (*b*), (*c*), (*d*), Katz *et al.* (1966); (*e*), Katz & Wood (1960).

	Product isolated	[^{14}C]Glucose [1-^{14}C]	[2-^{14}C]	[3,4-^{14}C]	[6-^{14}C]	[U-^{14}C]	Glucose utilized	C-1/C-6	Calculated percentage contribution of pentose phosphate pathway by method (*a*)	(*b*)	(*c*)	(*d*)	(*e*)
Contro	$^{14}CO_2$	0·460	0·054	0·466	0·054	0·212	12·0	8·52	3·4	1·2	1·2	10·6	12·0
	[^{14}C]Lactate	6·93	9·30	8·70	9·40	7·20		0·74					
Phenazine metho-sulphate	$^{14}CO_2$	7·60	1·06	0·75	0·62	1·98	25·0	12·2	27·9	11·4	11·8	18·0	24·0
	[^{14}C]Lactate	4·05	6·06	7·59	6·73	4·90		0·60					

is via the glycolytic route, as shown by the high lactate formation and low rate of C-6 oxidation, this would indicate that there is an appreciable rate of decarboxylation of pyruvate, and the acetyl-CoA so formed is partly oxidized by the tricarboxylic acid cycle and the excess may be incorporated into lipid or may possibly accumulate as acetate, as shown to occur in ascites cells by Hepp, Prüsse, Weiss & Wieland (1966). The relative rates of formation of $^{14}CO_2$ from the different carbon atoms of glucose are in good agreement with those reported by Wenner (1959) for Ehrlich ascites cells.

In the presence of the artificial electron acceptor phenazine methosulphate there is a considerable shift in the pattern of glucose oxidation; the $^{14}CO_2$ formation from [1-^{14}C]glucose, [2-^{14}C]glucose and [6-^{14}C]glucose all increased 20-fold whereas that from [3,4-^{14}C]glucose increased only twofold (Table 6). Qualitatively this indicates a large increase in oxidation of glucose by way of the pentose phosphate pathway and by way of the tricarboxylic acid cycle, with a more moderate increase in the rate of decarboxylation of pyruvate to acetyl-CoA. There was some evidence for recycling of pentose phosphate back to hexose monophosphate in the presence of phenazine methosulphate, as suggested by the greater yield of $^{14}CO_2$ from [2-^{14}C]glucose than from [6-^{14}C]-glucose.

The pattern of incorporation of glucose carbon into lactate is also broadly in agreement with the operation of the pentose phosphate pathway, as shown by the higher rate of incorporation into lactate of C-6 of glucose than C-1 of glucose.

The present results are in good agreement with the experiments of Wenner, Hackney & Moliterno (1958), who studied the effects of Methylene Blue and phenazine methosulphate on the oxidation of specifically labelled glucose by ascites-tumour cells.

Calculation of the contribution of the pentose phosphate pathway. The contribution of the pentose phosphate pathway to glucose metabolism was estimated from the $^{14}CO_2$ yields from specifically labelled glucose by three different methods, that of Beck (1958) and those of Katz *et al.* (1966); these are summarized in Table 6. The calculation (*a*) is based on the formula proposed by Beck (1958) and gives the highest estimate for the pentose phosphate pathway contribution, probably because this does not take into account the effect of recycling to glucose 6-phosphate. In the calculations based on the formula of Katz *et al.* (1966), methods (*b*) and (*c*), the specific yields of $^{14}CO_2$ were used. The specific yield has been defined as the fraction or

percentage of utilized ^{14}C recovered in the product (Katz & Wood, 1963). Method (*b*) in Table 6 is dependent only on specific yields of $^{14}CO_2$ from C-1- and C-6-labelled glucose, and triose phosphate equilibration is assumed (Katz *et al.* 1966); this gives a value of 1·2% for the contribution of the pentose phosphate pathway. Method (*c*) in Table 6 is based on specific yields of $^{14}CO_2$ and lactate ratios [fatty acid ratios were used in the equation derived by Katz *et al.* (1966) for adipose tissue] and is valid in the absence of triose phosphate equilibration. This also gives a value of 1·2% for the contribution of the pentose phosphate pathway, in excellent agreement with method (*b*). This supports the view that there is rapid equilibration of triose phosphates in ascites cells, in keeping with the high triose phosphate isomerase activity found in these cells (see Table 1).

In the presence of phenazine methosulphate the contribution of the pentose phosphate pathway is greatly increased, by about tenfold; again good agreement is obtained between methods (*b*) and (*c*).

The contribution of the pentose phosphate pathway may also be estimated by measurement of the incorporation of specifically labelled glucose into a triose phosphate derivative, in the present case lactate, by the methods given by Katz *et al.* (1966) and by Katz & Wood (1960). In method (*d*) in Table 6 triose phosphate equilibration is not assumed (Katz *et al.* 1966), whereas in method (*e*) triose phosphate equilibration is assumed (see Fig. 8 in Katz & Wood, 1960). It is clear that, although methods (*d*) and (*e*) agree relatively well with each other, they give considerably higher estimates of the contribution of the pentose phosphate pathway than those based on specific yields of $^{14}CO_2$. This discrepancy is more marked when the ascites cells are incubated with glucose alone than when the cells are incubated with glucose and phenazine methosulphate. Here it must be remembered that the model was initially used by Katz *et al.* (1966) for studies on adipose-tissue metabolism, where the basal contribution of the pentose phosphate pathway is much greater than in ascites cells, and direct transfer of the use of the model from one tissue to another may not be justified. This point is further examined when the results pertaining to the direction of flow of carbon through the non-oxidative reactions are considered (see the Discussion section). The effect of transaldolase on estimation of pathway contributions to glucose metabolism has been investigated by Landau & Bartsch (1966).

Katz *et al.* (1966) have stated that calculations of the contribution of the pentose phosphate cycle, from specific yields of $^{14}CO_2$, are less sensitive to errors arising from neglecting incomplete isomerization of triose phosphate than are calculations involving a triose phosphate derivative. Further, if the carbon dioxide yields from C-1 of glucose are twice or more than those from C-6 then a fair estimate of the contribution of the pentose phosphate cycle will be obtained even if equilibration of triose phosphate is limited. Thus in the present context methods (*b*) and (*c*) are considered to give the best estimate of the contribution of the pentose phosphate pathway.

Assessment of the relative contribution of the oxidative and non-oxidative routes of pentose phosphate formation. It was found, in studies of transient and steady-state concentrations of metabolites, that pentose phosphate accumulated in a linear manner during a 10min. incubation period with glucose; there was a net accumulation of 1·9μmoles of pentose phosphate. The rate of formation of pentose phosphate by the oxidative reactions of the pentose phosphate pathway can, as a first approximation, be equated to the difference between the conversions of [1-^{14}C]glucose into $^{14}CO_2$ and [6-^{14}C]glucose into $^{14}CO_2$. In cells incubated with glucose alone this difference amounted to 0·4μmole of glucose decarboxylated to yield pentose phosphate. As shown in Table 6 this may be an overestimate of the contribution of the oxidative pentose phosphate pathway (cf. methods *a*, *b* and *c*). Thus there is a large amount of pentose phosphate that cannot be accounted for by the oxidative route of formation, namely 1·9 − 0·4μmoles, i.e. 1·5μmoles, of pentose phosphate. Thus approx. 20% of the accumulated pentose phosphate can be accounted for by the oxidative route and 80% by non-oxidative formation involving transketolase and transaldolase, a proportion in reasonable agreement with that calculated by Hiatt (1957) based on the labelling pattern in ribose of RNA of HeLa cells incubated with glucose labelled at C-1 or C-2.

The present method, although clearly an oversimplification of the system, does allow some estimate to be made of the flow of glucose carbon atoms into pentose phosphate by the oxidative and non-oxidative segments of the pathway.

This model is based on several assumptions. The first is that the accumulated pentose phosphate represents the total amount synthesized. Pentose phosphate could be drained away into several products, but, of these, sedoheptulose 7-phosphate and erythrose 4-phosphate accumulation is very low (0·05μmole) relative to values for pentose phosphate and does not represent a major error. Ribose 5-phosphate may be converted into phosphoribosyl pyrophosphate and incorporated into nucleotides and RNA. In the present experiments there is an extensive accumulation of phosphoribosyl pyrophosphate, approx. 50% of the total pentose phosphate; however, this would be measured

as pentose phosphate since it undergoes very rapid hydrolysis in cold perchloric acid. The incorporation into RNA was not determined in the present experiments. The recycling of pentose phosphate back to hexose monophosphate appears to be very small, since the rate of oxidation of C-2 of glucose was almost identical with that of C-6 of glucose. Thus, as a first approximation, it has been assumed that the linear rate of pentose accumulation is representative of the rate of formation or synthesis of pentose phosphate.

The good agreement between the two entirely different methods, that of Hiatt (1957) and the present studies, of estimating the relative formation of pentose phosphate in ascites-tumour cells by the oxidative and non-oxidative routes also lends considerable support to the present experiments.

DISCUSSION

Krebs & Kornberg (1957) suggested that, from a knowledge of the sequence of reactions in a metabolic pathway, it would be possible to predict which enzymes would be likely to play a regulatory role. Possible candidates are those enzymes catalysing reactions immediately after a branch point on the pathway, enzymes catalysing two separate reactions that comprise a forward and a backward reaction of one step in a pathway and those requiring nucleotides or inorganic ions as cofactors. Investigation of such regulatory systems for the pathways of glycolysis and gluconeogenesis in liver and kidney has been reviewed (Newsholme & Gevers, 1967; Scrutton & Utter, 1968). Application of these characteristics to the reactions of the pentose phosphate pathway as presented by Racker (1957), Horecker & Hiatt (1958) and Horecker (1962) lead to the prediction that glucose 6-phosphate dehydrogenase, transketolase and phosphoribosyl pyrophosphokinase could be of regulatory significance; but, before consideration of the control mechanisms operating at these reactions, attention must be given to some features of control of hexokinase in ascites cells.

Hexokinase. The accumulation of glucose 6-phosphate has a powerful inhibitory effect on hexokinase, as first shown by Weil-Malherbe & Bone (1951) and recently studied extensively in ascites cells by Rose (1965), Kosow & Rose (1968) and Sauer (1968). An ATP/glucose 6-phosphate ratio of 3 has been cited as causing a 90–95% inhibition of hexokinase in the presence of 1mM-ATP and with 3mM-P_i (Sauer, 1968).

In the present experiments the rate of glucose phosphorylation, i.e. hexokinase activity of the intact cell, was equated with the $\sum$ metabolites rather than with glucose 6-phosphate accumulation, and studies were made of the interrelationship between the rate of glucose phosphorylation and the changes in content of ATP, glucose 6-phosphate and other metabolites, notably fructose diphosphate and ribose 5-phosphate. These measurements were made at very short time-intervals after the addition of glucose (3–90sec.) and reveal some further aspects of the sequence of control mechanisms of hexokinase.

It was found that the rate of glucose phosphorylation continued in a rapid and linear manner up to 15sec. after addition of glucose, despite the fact that at 3sec. the glucose 6-phosphate content had risen to almost the steady-state value and the ATP/glucose 6-phosphate ratio had fallen to a value between 3 and 4. Thus the feedback control mechanism does not appear to be operating at this time. A possible explanation for this may be found in the work of Kosow & Rose (1968), who showed with ascites cells that there was a difference between the free and bound forms of hexokinase in the type of inhibition produced by anhydroglucitol 6-phosphate with respect to ATP and a greater sensitivity of the unbound enzyme to inhibition by this compound at low ATP concentrations. Anhydroglucitol 6-phosphate behaves similarly to glucose 6-phosphate as an inhibitor of hexokinase. Thus it may be postulated that binding of hexokinase may prevent feedback inhibition occurring during this initial phase. The simulated system shown in Fig. 2(*b*) also supports this, since the soluble hexokinase of Krebs ascites cells was almost completely inhibited by ATP–glucose 6-phosphate mixtures similar to those found in the cell at 5sec. after addition of glucose. The rate of glucose phosphorylation in the intact cell is approx. 50% of the $V_{max.}$ of hexokinase in the cell extract; however, only 20% appears to be bound to the 12000*g* fraction, so that binding of hexokinase would not seem to provide the complete answer. However, it is questionable how far binding in broken-cell suspensions represents the binding of hexokinase in the intact cell; moreover, a much higher proportion of the total hexokinase has been reported in the bound form in ascites cells by McComb & Yushok (1959) and Rose (1965).

The question that now arises is that, if the glucose 6-phosphate content and the relative amounts of ATP and glucose 6-phosphate are not inhibitory in the first 15sec., why is there a sudden onset of inhibition at 15–20sec. with a decrease in the rate of glucose phosphorylation to only 5–10% of the $V_{max.}$ of extracted hexokinase? Two possible explanations may be suggested. First, there may be release of bound hexokinase by the accumulated glucose 6-phosphate (Rose & Warms, 1967), with a consequent onset of inhibition since the free form is more sensitive than the bound form to inhibition

by glucose 6-phosphate. The studies by Rose & Warms (1967) on release of hexokinase from mitochondria by glucose 6-phosphate show that approx. 60% was released by 0·2mM-glucose 6-phosphate. The cell content is 0·5mM 3sec. after the addition of glucose in the present experiments. Rose & Warms (1967) also studied the time-course of release of hexokinase from mitochondria by 0·1mM-glucose 6-phosphate and found a half-time of 18sec. at 35°. All these factors suggest that a change in the binding of hexokinase may occur at the critical period of 15–20sec. after the addition of glucose, followed by a sharp onset of a feedback inhibition.

The differences in the relative distribution of type I and type II hexokinase between the mitochondria and the soluble fraction are also noteworthy in the present experiments and possibly suggest a further refinement in the control mechanisms.

Secondly, it seemed possible that the accumulation of some other metabolite might play an important role in the inhibition of hexokinase. Examination of metabolites increasing rapidly over the appropriate time-interval, i.e. about 15sec., shows that fructose diphosphate might be a candidate for such a role. This metabolite increases in a sigmoid fashion (see Fig. 2*c*) and reaches relatively very high concentrations at 15sec., approx. 1·5μmoles/g. of cells. It was found that 2mM-fructose diphosphate inhibited soluble hexokinase by 90% in the presence of 7mM-magnesium chloride, so that the accumulation of this metabolite could well be an important contributory factor to hexokinase inhibition after 15sec. incubation with glucose. The inhibition of hexokinase by fructose diphosphate may well be a special feature of ascites cells, since other tissues contain considerably lower steady-state concentrations of this metabolite, i.e.: liver, 0·01–0·05μmole/g. (Bücher, Krejci, Rüssmann, Schnitger & Wesemann, 1964); brain, 0·1μmole/g. (Lowry, Passonneau, Hasselberger & Schutz, 1964); perfused rat heart, 0·032μmole/g. (Newsholme & Randle, 1961).

Pentose phosphate accumulates to a marked extent in ascites cells during longer periods of incubation; ribose 5-phosphate at 5mM, which is twice the concentration found after incubation for 10min., caused only a 28% inhibition of hexokinase. As early as 1940 Z. Dische (cited by Stadtman, 1966) described the inhibition of hexokinase by 3-phosphoglycerate and postulated a feedback control mechanism. However, with the low concentration of 3-phosphoglycerate found in ascites cells at steady-state conditions virtually no inhibition was found.

Both Lowry & Passonneau (1964) from studies with brain and Kosow & Rose (1968) from studies of ascites cells reached the conclusion that inhibition by glucose 6-phosphate, although probably the major mechanism, does not completely account for the control of the hexokinase reaction. The present approach is very similar to that used by Coe (1966) and Lee, Strunk & Coe (1967), who concluded that control of the initial steps of glycolysis is attributable to ADP activation of phosphofructokinase and limitation of hexokinase activity by product inhibition.

Oxidative reactions of the pentose phosphate pathway. Glucose 6-phosphate dehydrogenase occupies a key position at a branch point in metabolism and this may be considered as a site at which control mechanisms might operate. Determination of the overall mass-action ratio of the two enzymes glucose 6-phosphate dehydrogenase and lactonase show that this is displaced from the equilibrium position by a factor of 500 and thus may be considered as a possible control point. This is in contrast with 6-phosphogluconate dehydrogenase, which, from mass-action-ratio determinations, appears to be close to equilibrium (Table 3).

There is clear evidence that one factor limiting the oxidative reactions of the pentose phosphate pathway is the rate of reoxidation of NADPH. In ascites-tumour cells the addition of the artificial electron acceptor phenazine methosulphate stimulates the oxidative part of the pentose phosphate pathway very markedly. There is a 20-fold increase in the formation of $^{14}CO_2$ from [1-^{14}C]glucose (Wenner *et al.* 1958), and calculation of the percentage contribution of the pentose phosphate pathway by the method of Katz *et al.* (1966) shows that this increased tenfold in the presence of phenazine methosulphate (Table 6). Examination of the $NADP^+$ plus NADPH content of the ascites cells shows that this is very low, only about 16μM, with an equal distribution between oxidized and reduced forms. This is close to the K_m for $NADP^+$ of 6-phosphogluconate dehydrogenase. However, glucose 6-phosphate dehydrogenase has a fivefold higher apparent K_m value for $NADP^+$ and therefore is more likely to be rate-limited by the rate of reoxidation of NADPH, a second reason in its favour as a control point.

In the presence of phenazine methosulphate the rate of decarboxylation of glucose by way of the pentose phosphate pathway is given by the difference ([1-^{14}C]glucose oxidized to $^{14}CO_2$) − ([6-^{14}C]-glucose oxidized to $^{14}CO_2$) and this is approx. 7μmoles/10min./g. of cells at 37°. The maximum velocity of the overall system of glucose 6-phosphate dehydrogenase plus 6-phosphogluconate dehydrogenase is approx. 25μmoles/10min./g. of cells, corrected to 37°, i.e. the oxidative reactions are effectively working at 28% of $V_{max.}$. The limitation here may still be the availability of $NADP^+$, since the content of the cell appears to have an upper

limit of approx. 16 μM, which is only about one-third of the K_m value of glucose 6-phosphate dehydrogenase for $NADP^+$. The reaction rate of this enzyme would therefore be limited to approx. 16% of $V_{max.}$ These values are in reasonable agreement when the difficulties of measuring very small amounts of NADPH are taken into consideration. This is a difficulty arising from the high nucleic acid content of the cells and the high viscosity of extracts obtained after treatment with 0·1M-sodium hydroxide, which will tend to give a low value for NADPH content. Another aspect to be considered is that there may be factors modifying the apparent K_m of glucose 6-phosphate dehydrogenase for $NADP^+$, the value of which in ascites cells is unusually high. Preincubation of glucose 6-phosphate dehydrogenase may be necessary before determination of the K_m, since this is known to be required in the combination of subunits of this enzyme (Kirkman & Hendrickson, 1961; Cohen & Rosemeyer, 1969).

Substrate limitation does not seem to play such an important role as coenzyme limitation, since the steady-state content of glucose 6-phosphate is well above the K_m of glucose 6-phosphate dehydrogenase, despite the unusually high K_m value for glucose 6-phosphate of the enzyme from Krebs ascites-tumour cells compared with other normal tissues.

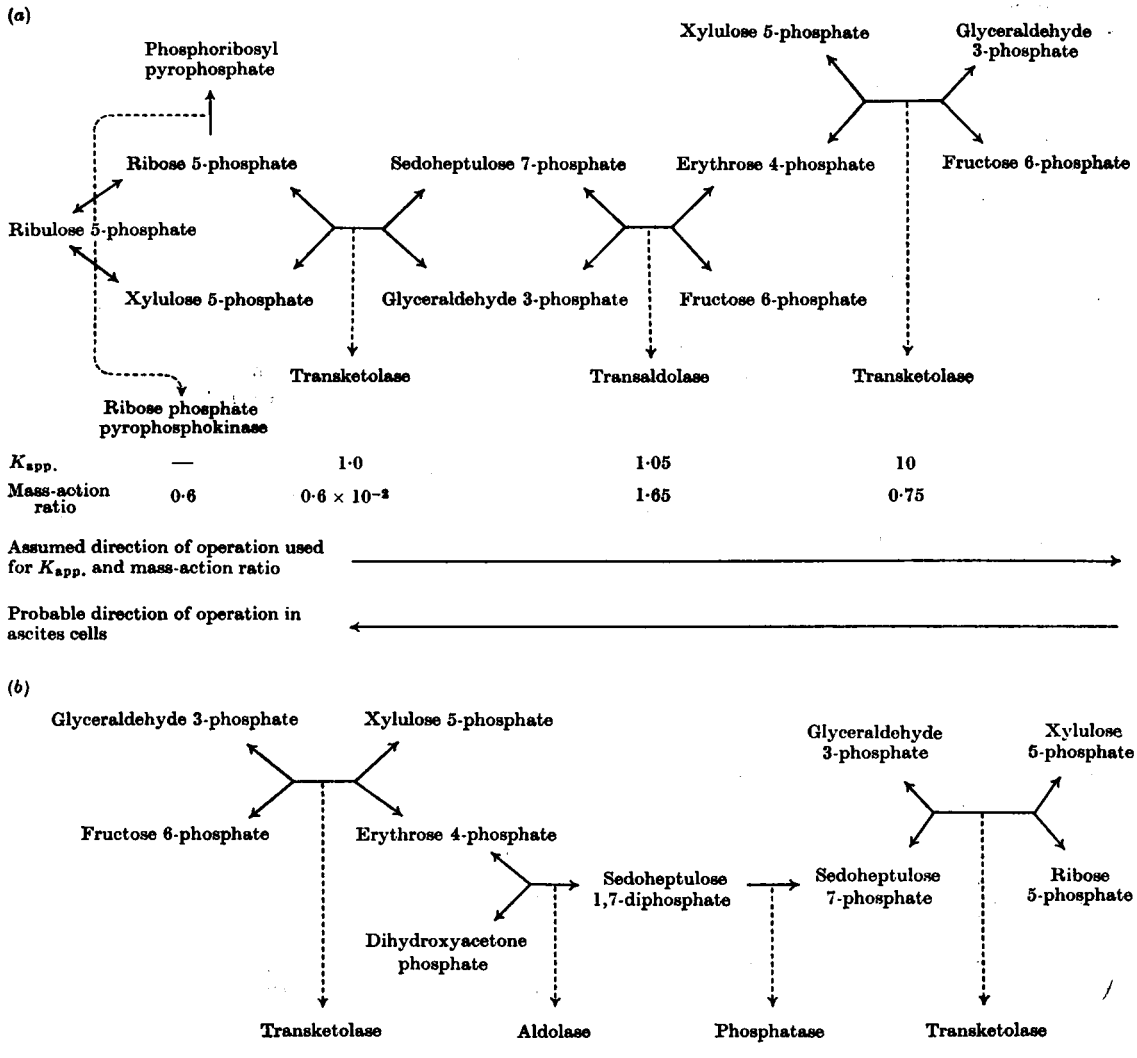
6-Phosphogluconate is also present in about tenfold excess over the K_m of 6-phosphogluconate dehydrogenase. Both enzymes appear to be subject to inhibition by NADPH (see Noltmann & Kuby, 1963, and Table 4). For 6-phosphogluconate dehydrogenase the K_i is 10–20-fold greater than the measured cellular content of NADPH.

Other small-molecular-weight effectors may also be important in the control of the oxidative reactions of the pentose phosphate pathway; for example, glucose 6-phosphate dehydrogenase is markedly inhibited by long-chain acyl-CoA derivatives (Eger-Neufeldt, Teinzer, Weiss & Wieland, 1965). Carter & Parr (1967) first described the competitive inhibition of 6-phosphogluconate dehydrogenase by fructose diphosphate. The high steady-state concentration of fructose diphosphate (2mM) in ascites cells suggested the possibility that this might be an important control factor, for this metabolite was present in a concentration some 20 times that of 6-phosphogluconate and 30 times the K_i for fructose diphosphate, which was $7·2 \times 10^{-5}$M. This suggested that other factors must come into play to modify the inhibitory action of this metabolite.

The inhibitory action of fructose diphosphate on hexokinase and 6-phosphogluconate dehydrogenase and the activation by fructose diphosphate of phosphofructokinase, pyruvate kinase (see Scrutton & Utter, 1968, p. 279) and fatty acid synthesis (Plate, Joshi, Sedgewick & Wakil, 1968) suggests that this metabolite may occupy a key position in the integration of pathways of metabolism.

The foregoing evidence is by no means conclusive as to which of the enzymes of the oxidative part of the pentose phosphate pathway is the key in the control of this sequence. Kauffman, Brown, Passonneau & Lowry (1968), from a study of metabolites in brain under conditions of increased and decreased flux through the pentose phosphate pathway, found that the evidence pointed to a control point at 6-phosphogluconate dehydrogenase.

Non-oxidative reactions of the pentose phosphate pathway. Of the non-oxidative reactions, transketolase, with ribose 5-phosphate and xylulose 5-phosphate as substrates, is the rate-limiting reaction, transaldolase having a twofold higher activity. The mass-action ratios show that, at steady-state conditions, transketolase with ribose 5-phosphate and xylulose 5-phosphate as substrate is displaced from equilibrium by a factor of 150. With xylulose 5-phosphate and erythrose 4-phosphate as substrates the reaction is displaced from equilibrium by a factor of 10, whereas transaldolase is close to equilibrium. On this basis the transketolase reaction appears to be the enzyme most likely to be under metabolic control. The effective direction of the operation of the non-oxidative reactions of the pentose phosphate pathway is a point of some importance in the control of this pathway of metabolism. The direction of these reactions from the apparent equilibrium constants appears to be in favour of the formation of fructose 6-phosphate from xylulose 5-phosphate and erythrose 4-phosphate, since this transketolase reaction has a $K_{app.}$ value of 10 (see Scheme 1*a*). However, there is considerable evidence from the present experiments suggesting that these reactions operate effectively in the direction of pentose phosphate formation from hexose monophosphate. First there is a large accumulation of pentose phosphate and of phosphoribosyl pyrophosphate, this being much greater than could be formed by the oxidative reactions of the pentose phosphate pathway as shown by the oxidative decarboxylation of C-1 of glucose. These experiments suggest that 80% of the accumulated pentose phosphate is formed non-oxidatively. Secondly, the mass-action ratios also suggest that the direction is in favour of pentose phosphate formation (see Scheme 1*a*). The formation of relatively large amounts of phosphoribosyl pyrophosphate (1 μmole/10min./g. of cells) will also, by removal of ribose 5-phosphate, tend to shift the equilibrium in favour of pentose phosphate formation and away from sedoheptulose 7-phosphate production. Thirdly, as shown in transient-state concentrations of metabolites, there is a marked parallelism between fructose 6-phosphate and sedoheptulose 7-phosphate formation, whereas no



Scheme 1. Direction of operation of non-oxidative reactions of the pentose phosphate pathway. References: Krebs & Kornberg (1957); Horecker (1962); Sable (1966).

obvious relationship appeared to exist between pentose phosphate and sedoheptulose 7-phosphate formation; this suggested that fructose 6-phosphate was the more likely precursor of sedoheptulose 7-phosphate. Since both the oxidative and non-oxidation reactions contribute to pentose phosphate formation, precursor–product relationships are difficult to demonstrate between sedoheptulose 7-phosphate and ribose 5-phosphate.

The present work on ascites cells is in accordance with the view put forward by Horecker (1962) that the pentose phosphate pathway in animals does not operate as a cycle but rather as two parallel mechanisms for the conversion of hexose monophosphate into pentose phosphate. The present calculation of the relative contribution of the oxidative and non-oxidative routes of pentose phosphate formation in ascites cells is in good agreement with the results of Hiatt (1957) on the pathways of ribose synthesis in HeLa cells in tissue culture. Hiatt (1957) used glucose specifically labelled at C-1 or C-2 and determined the distribution of ^{14}C in ribose of RNA by degradation; this method showed a predominance of the non-oxidative route.

If the widely accepted model shown in Scheme 1(*a*) is correct then the situation appears to exist in which an identical pathway is used for pentose phosphate synthesis and utilization, and control of

this process would then largely depend on the requirement for ribose 5-phosphate for phosphoribosyl pyrophosphate formation, the rate of formation of which is very high in ascites cells (Henderson & Khoo, 1965). In tissues such as adipose tissue and lactating rat mammary gland, where there is a rapid utilization of NADPH for lipid synthesis, the rate of pentose phosphate formation by the oxidative route is correspondingly increased and the non-oxidative route is therefore shifted in favour of conversion of pentose phosphate into hexose monophosphate, as clearly shown by the high rate of oxidation of C-2 of glucose (which becomes C-1 on recycling) relative to C-6 of glucose (Abraham, Hirsch & Chaikoff, 1954; Abraham, Cady & Chaikoff, 1960; McLean, 1964; Walters & McLean, 1968).

A further possibility, allowing greater flexibility of control, may be that the synthetic and degradative reactions may have one enzyme not in common to the two routes, yielding a situation more analogous to that found in the glycolytic pathway (see Scheme 1*b*). It has been postulated that aldolase, sedoheptulose 1,7-diphosphate and sedoheptulose diphosphatase may be involved in the conversion of hexose monophosphate into pentose phosphate (Krebs & Kornberg, 1957; Racker & Schroeder, 1958; Pontremoli & Grazi, 1960; Horecker, 1962; Sable, 1966).

The operation of the pentose phosphate pathway as two independent routes to pentose phosphate must influence to some degree the calculation of the relative contribution of the pentose phosphate pathway to glucose metabolism. The model used by Katz & Wood (1960) and Katz *et al.* (1966) was based on recycling of the pentose phosphate to hexose monophosphate, and is applicable to adipose tissue or mammary gland but probably not to ascites cells. It may be that the more simplified model based on differences between $^{14}CO_2$ production from [1-^{14}C]glucose and [6-^{14}C]glucose (method *a* in Table 6) may well give a more realistic value for the contribution of the oxidative segment of the pentose phosphate pathway to glucose metabolism. The effect of phenazine methosulphate on the contribution of the oxidative pentose phosphate pathway to glucose metabolism shows clearly the critical importance of the rate of reoxidation of NADPH in the overall control of the whole pathway.

The authors are indebted to Mr B. C. Teo and Mr K. R. Greenslade for skilled technical assistance, to Dr J. G. Bennette and Mr J. Moore for kindly providing the ascites-tumour cells and to Mr R. L. Warren for kindly performing the magnesium determinations. Acknowledgement is also made to the Medical Research Council for a grant to purchase a Unicam SP.800 recording spectrophotometer. K.A.G. was supported by the University of Khartoum. This work was in part supported by a grant to The Middlesex Hospital Medical School from the British Empire Cancer Campaign.

REFERENCES

Abraham, S., Cady, P. & Chaikoff, I. L. (1960). *Endocrinology*, **66**, 280.
Abraham, S., Hirsch, P. F. & Chaikoff, I. L. (1954). *J. biol. Chem.* **211**, 31.
Ashwell, G. & Hickman, J. (1957). *J. biol. Chem.* **226**, 65.
Beck, W. S. (1958). *J. biol. Chem.* **232**, 271.
Ballou, C. E. (1963). In *Methods in Enzymology*, vol. 6, p. 479. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press Inc.
Bergmeyer, H.-U. (Ed.) (1965). *Methods of Enzymatic Analysis*, 2nd ed. Berlin: Verlag Chemie; New York and London: Academic Press Inc.
Bowen, W. J. & Kerwin, T. D. (1954). *Arch. Biochem. Biophys.* **42**, 149.
Bücher, Th., Krejci, K., Rüssmann, W., Schnitger, H. & Wesemann, W. (1964). *Rapid Mixing and Sampling Techniques in Biochemistry*, p. 225. New York: Academic Press Inc.
Carter, N. D. & Parr, C. W. (1967). *Abstr. 4th Meet. Fed. Europ. biochem. Soc., Oslo*, p. 58 (Abstr. 230).
Coe, E. L. (1966). *Biochim. biophys. Acta*, **118**, 495.
Cohen, P. & Rosemeyer, M. A. (1969). *Europ. J. Biochem.* **8**, 8.
Datta, A. G. & Racker, E. (1961). *J. biol. Chem.* **236**, 617.
Dickens, F., Glock, G. E. & McLean, P. (1959). In *Ciba Found. Symp.: Regulation of Cell Metabolism*, p. 150. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.
Eger-Neufeldt, I., Teinzer, A., Weiss, L. & Wieland, O. (1965). *Biochem. biophys. Res. Commun.* **19**, 43.
England, P. J. & Randle, P. J. (1967). *Biochem. J.* **105**, 907.
Flaks, J. A. (1963). In *Methods in Enzymology*, vol. 6, p. 148. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press Inc.
Glaser, L. & Brown, D. H. (1955). *J. biol. Chem.* **216**, 67.
Glock, G. E. & McLean, P. (1953). *Biochem. J.* **55**, 400.
Grazi, E., deFlora, A. & Pontremoli, S. (1960). *Biochem. biophys. Res. Commun.* **2**, 121.
Grossbard, L. & Schimke, R. T. (1966). *J. biol. Chem.* **241**, 3546.
Gumaa, K. A. & McLean, P. (1969). *Biochem. biophys. Res. Commun.* **35**, 86.
Gumaa, K. A., McLean, P. & Bennette, J. G. (1968). *FEBS Lett.* **1**, 125.
Henderson, J. F. & Khoo, M. K. Y. (1965). *J. biol. Chem.* **240**, 2349.
Hepp, D., Prüsse, E., Weiss, H. & Wieland, O. (1966). In *Advances in Enzyme Regulation*, vol. 4, p. 89. Ed. by Weber, G. London: Pergamon Press Ltd.
Hess, B. (1963). In *Control Mechanisms in Respiration and Fermentation*, p. 333. Ed. by Wright, B. New York: Ronald Press Co.
Hiatt, H. H. (1957). *J. clin. Invest.* **36**, 1408.
Horecker, B. L. (1962). In *Ciba Lect. Microbial Biochemistry: Pentose Metabolism in Bacteria*, pp. 30, 873. New York and London: John Wiley and Sons Inc.
Horecker, B. L. & Hiatt, H. H. (1958). *New Engl. J. Med.* **258**, 177.

Horecker, B. L. & Smyrniotis, P. Z. (1952). *J. biol. Chem.* **196**, 135.
Katz, J., Landau, B. R. & Bartsch, G. E. (1966). *J. biol. Chem.* **241**, 727.
Katz, J. & Wood, H. G. (1960). *J. biol. Chem.* **235**, 2165.
Katz, J. & Wood, H. G. (1963). *J. biol. Chem.* **238**, 517.
Kauffman, F. C., Brown, J. G., Passonneau, J. V. & Lowry, O. H. (1968). *Fed. Proc.* **27**, 463.
Kirkman, H. N. & Hendrickson, E. M. (1961). *J. biol. Chem.* **237**, 2371.
Klungsøyr, L., Hagemen, J. H., Fall, L. & Atkinson, D. E. (1968). *Biochemistry*, **7**, 4035.
Kosow, D. P. & Rose, I. A. (1968). *J. biol. Chem.* **243**, 3623.
Krebs, H. A. (1967). In *Advances in Enzyme Regulation*, vol. 5, p. 409. Ed. by Weber, G. London: Pergamon Press Ltd.
Krebs, H. A. & Kornberg, H. L. (1957). *Ergebn. Physiol.* **49**, 212.
Krebs, H. A. & Veech, R. L. (1969). In *The Energy Level and Metabolic Control in Mitochondria*, p. 329. Ed. by Papa, S., Tager, J. M., Quagliariello, E. & Slater, E. C. Bari: Adriatica Editrice.
Landau, B. R. & Bartsch, G. E. (1960). *J. biol. Chem.* **241**, 741.
Lee, I.-Y. & Coe, E. L. (1967). *Biochim. biophys. Acta*, **131**, 441.
Lee, I.-Y., Strunk, R. C. & Coe, E. L. (1967). *J. biol. Chem.* **242**, 2021.
Levy, R. H. (1963). *J. biol. Chem.* **238**, 775.
Li, W.-Y. & Ch'ien, J. L. (1965). *Acta Biol. exp. sin.* **10**, 112; cited in *Chem. Abstr.* (1966) **65**, 7498a.
Lowry, O. H. & Passonneau, J. V. (1964). *J. biol. Chem.* **239**, 31.
Lowry, O. H., Passonneau, J. V., Hasselberger, F. X. & Schultz, D. W. (1964). *J. biol. Chem.* **239**, 18.
McComb, R. B. & Yushok, W. D. (1959). *Biochim. biophys. Acta*, **34**, 515.
McLean, P. (1958). *Biochim. biophys. Acta*, **30**, 303.
McLean, P. (1964). *Biochem. J.* **90**, 271.
Maitra, P. K. & Chance, B. (1965). In *Control of Energy Metabolism*, p. 157. Ed. by Chance, B., Estabrook, R. W. & Williamson, J. R. New York: Academic Press Inc.
Moellering, H. & Gruber, W. (1966). *Analyt. Biochem.* **17**, 369.
Newsholme, E. A. & Gevers, W. (1967). *Vitam. & Horm.* **25**, 1.
Newsholme, E. A. & Randle, P. J. (1961). *Biochem. J.* **80**, 655.
Noltmann, E. A. (1966). In *Methods in Enzymology*, vol. 9. p. 557. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press Inc.
Noltmann, E. A. & Kuby, S. A. (1963). In *The Enzymes*, vol. 7, p. 223. Ed. by Boyer, P. D., Lardy, H. A. & Myrbäck, K. New York: Academic Press Inc.
Novello, F. & McLean, P. (1968). *Biochem. J.* **107**, 775.
Parr, C. W. (1956). *Nature, Lond.*, **178**, 1401.
Plate, C. A., Joshi, V. C., Sedgewick, B. & Wakil, S. J. (1968). *J. biol. Chem.* **243**, 5439.
Pontremoli, S. & Grazi, E. (1960). *Bull. Soc. Chim. biol., Paris*, **42**, 753.
Racker, E. (1957). *Harvey Lect.* **51**, 143.
Racker, E. (1965). In *Methods of Enzymatic Analysis*, 2nd ed., p. 107. Ed. by Bergmeyer, H.-U. Berlin: Verlag Chemie; New York and London: Academic Press Inc.
Racker, E. & Schroeder, E. A. R. (1958). *Arch. Biochem. Biophys.* **74**, 326.
Reithel, F. J. (1966). In *Methods in Enzymology*, vol. 9, p. 565. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press Inc.
Rolleston, F. S. & Newsholme, E. A. (1967). *Biochem. J.* **104**, 524.
Rose, I. A. (1965). In *Control of Energy Metabolism*, p. 177. Ed. by Chance, B., Estabrook, R. W. & Williamson, J. R. New York: Academic Press Inc.
Rose, I. A. & Warms, J. V. B. (1967). *J. biol. Chem.* **242**, 1635.
Sable, H. Z. (1966). *Advanc. Enzymol.* **28**, 391.
Sauer, L. A. (1968). *J. biol. Chem.* **243**, 2429.
Scrutton, M. C. & Utter, M. F. (1968). *Annu. Rev. Biochem.* **37**, 249.
Simpson, F. J. (1960). *Canad. J. Biochem. Physiol.* **38**, 115.
Stadtman, E. R. (1966). *Advanc. Enzymol.* **28**, 41.
Switzer, R. L. (1969). *J. biol. Chem.* **244**, 2854.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, 2nd ed., p. 119. Minneapolis: Burgess Publishing Co.
Venkataraman, R. & Racker, E. (1961). *J. biol. Chem.* **236**, 1876.
Walters, E. & McLean, P. (1967). *Biochem. J.* **105**, 615.
Walters, E. & McLean, P. (1968). *Biochem. J.* **109**, 407.
Weil-Malherbe, H. & Bone, A. D. (1951). *Biochem. J.* **49**, 339.
Wenner, C. E. (1959). *J. biol. Chem.* **234**, 2472.
Wenner, C. E., Hackney, J. H. & Moliterno, F. (1958). *Cancer Res.* **18**, 1105.
Williamson, J. R. (1965). In *Control of Energy Metabolism*, p. 333. Ed. by Chance, B., Estabrook, R. W. & Williamson, J. R. New York and London: Academic Press Inc.
Wu, R. (1959). *J. biol. Chem.* **234**, 2806.
Wu, R. (1965*a*). *Biochem. biophys. Res. Commun.* **18**, 402.
Wu, R. (1965*b*). In *Control of Energy Metabolism*, p. 187. Ed. by Chance, B., Estabrook, R. W. & Williamson, J. R. New York and London: Academic Press Inc.
Wu, R. & Racker, E. (1959). *J. biol. Chem.* **234**, 1029.
Zaheer, N., Tewari, K. K. & Krishnan, P. S. (1967). *Arch. Biochem. Biophys.* **120**, 23.