A POSSIBLE INTERRELATIONSHIP BETWEEN BINDING OF HEXOKINASE AND THE SITE OF ATP FORMATION IN KREBS ASCITES CELLS

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The overall utilisation of ATP and the partitioning of ATP regeneration between the mitochondrial and cytoplasmic compartments of ascites cells were calculated from the metabolite profiles over a period of 3 - 30 sec. after the addition of glucose. Both compartments contribute about equally to the regeneration of ATP, the glycolytic lagging by 5 sec. pending the "filling up" of glycolytic intermediates between 3-phosphoglycerate and lactate. Regeneration of ATP by adenylate kinase appears to be linear over these time intervals. ATP regeneration slows down appreciably between 15 and 20 sec., coinciding with the release and partial inhibition of mitochondrial hexokinase by glucose-6-phosphate with consequent limitation of the ADP supply. Other possible modulators of mitochondrial phosphorylation are discussed.

The known sequence of events following addition of glucose to Ehrlich ascites tumour cells is that there is an immediate increase in respiratory rate from an initial rate of 0.48 to 0.97 μmoles O_2/min/g cells for a period of about 20 - 40 sec. after addition of glucose; thereafter the rate falls to 0.21 μmoles/g cells/min (1,2). Following the addition of glucose ADP rises, ATP falls and there is a state 4 → 3 transition due to increased ADP (2). Chance and Hess (1) also noted that the mitochondrial NADH is affected by the rise of ADP level before the reduction of NAD^+ in the cytoplasm takes place; this transient oxidation of NADH also lasted about 20 sec. They suggested that there is a direct shuttle of ADP from the hexokinase system to the respiratory chain of the mitochondria.

The parallel observations that a large proportion of hexokinase is bound to mitochondria in ascites tumour cells (3,4,5) and the evidence from studies of the properties of the bound form of hexokinase that this was less susceptible to inhibition by glucose-6-phosphate (G6P) (6-9) and could be

eluted from mitochondria by G6P with a half time of 18 sec. at 35° both in vitro (5) and in the intact cell (10), suggested that this coincidence of time sequence might be of some significance. The short term control of mitochondrial ADP in ascites cells following addition of glucose might therefore be related to the binding of hexokinase as suggested by Chance and Hess (1) and discussed by Rose and Warms (5).

In the present experiments measurements have been made of changes in the concentration of intermediates of the glycolytic pathway and of adenine nucleotides from 3 to 30 sec. following addition of glucose to Krebs ascites cells. These have been used to calculate the ATP utilised in the phosphorylation of glucose and fructose-6-phosphate (F6P) and this has been compared with the observed fall in the ATP content of the cell; from the difference the ATP regenerated can be estimated. The ATP regenerated has been partitioned among three systems, the glycolytic formation (from the pyruvate and lactate production), the formation by adenylate kinase (from the changes in AMP), the remainder being the contribution of the mitochondrial fraction.

METHODS

The treatment of the Krebs ascites cells and determination of metabolites were as described previously (10,11,12). The washed cells were incubated aerobically at 37° in Ca^{++}-free Krebs-Ringer phosphate medium with an initial concentration of 12.5 mM glucose. The utilisation of ATP and partitioning of ATP regeneration between the cytoplasmic and mitochondrial compartments were calculated from the metabolite concentrations shown in Table 1. These were calculated as shown in Table 2.

RESULTS

The general pattern which emerges is that, following glucose addition to Krebs ascites cells, there is a rapid and linear rate of ATP utilisation for the first 15 sec. which declines sharply after 20 sec. The ATP regeneration largely parallels this curve, divergence being observed after 15 sec. (Fig. 1). Initially the glycolytic pathway makes no contribution to

TABLE 1

METABOLITE PROFILES IN KREBS ASCITES CELLS
MEASURED AT SHORT TIME INTERVALS (0 - 30 sec) AFTER ADDITION OF GLUCOSE

	Metabolite content (μmoles/g cells)							
Time (sec)	0	3	5	10	15	20	25	30
G6P	0.061	0.574	0.470	0.470	0.592	0.653	0.626	0.574
F6P	0.070	0.139	0.139	0.157	0.157	0.183	0.157	0.157
FDP	0.150	0.220	0.630	1.26	1.44	1.76	1.96	2.24
DAP	0.070	0.210	0.313	0.313	0.730	0.974	0.974	1.08
αGP	0.009	0.011	0.015	0.017	0.006	0.013	0.015	0.011
GAP	0.210	0.210	0.104	0.140	0.280	0.244	0.174	0.190
3PG	0.052	0.078	0.087	0.078	0.078	0.044	0.030	0.026
2PG	0.035	0.004	0.017	0.026	0.044	0.017	0.026	0.017
PEP	0.078	0.070	0.044	0.044	0.078	0.078	0.035	0.044
PYR	0.235	0.200	0.183	0.174	0.226	0.191	0.165	0.157
LAC	2.75	2.75	2.75	3.48	4.10	4.13	3.92	4.13
PP	0.087	0.220	0.087	0.175	0.175	0.306	0.220	0.260
6PG	0.011	0.017	0.013	0.017	0.037	0.026	0.034	0.033
S7P	0.009	0.009	0.017	0.022	0.022	0.026	0.026	0.026
ATP	2.71	2.23	2.12	2.16	1.95	1.74	1.46	1.29
ADP	0.313	0.730	0.905	1.15	1.32	1.39	1.39	1.46
AMP	0.470	0.520	0.574	0.680	0.800	0.905	1.11	1.16

Abbreviations for pentose phosphate pathway metabolites: PP, sum of ribose 5-phosphate, ribulose 5-phosphate and xylulose 5-phosphate; 6PG, 6-phosphogluconate; S7P, sedoheptulose 7-phosphate.

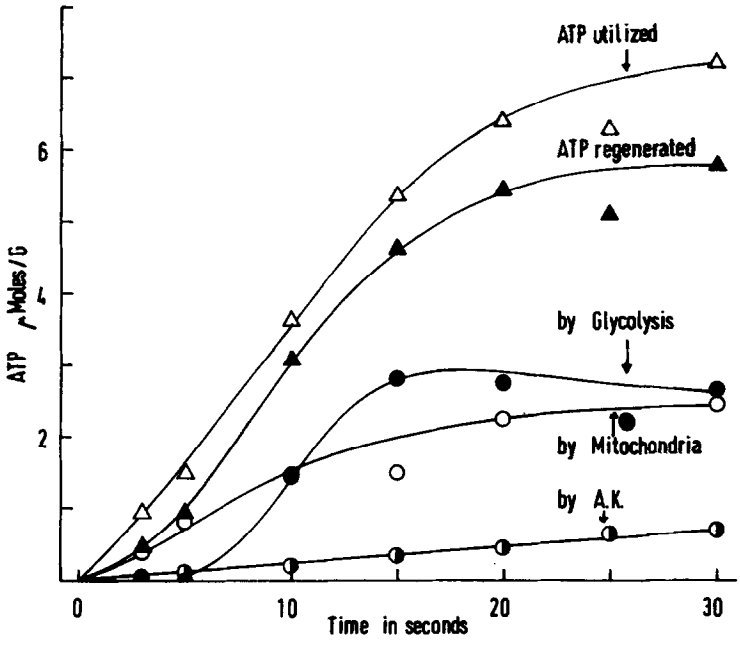

FIGURE 1 The relative contributions of the glycolytic, the mitochondrial systems and adenylate kinase to ATP regeneration at different time intervals after addition of glucose to Krebs ascites cells.

The results are given as μmoles ATP/g cells corrected for the zero time value and the calculations upon which they are based are given in Table 2.

△—△ ATP utilised in glucose metabolism, see Table 2, B.

▲—▲ ATP, total regenerated, see Table 2, C.

●—● ATP regenerated by glycolysis, see Table 2, D.

◑—◑ ATP regenerated by adenylate kinase (AK), see Table 2, E.

○—○ ATP regenerated by mitochondria, see Table 2, F.

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the formation of ATP; this is clearly seen in Table 1, where the fructose diphosphate (FDP) remains virtually unchanged at 3 sec. and no increase in lactate is found at either 3 or 5 sec. after the addition of glucose. At this point in time the regeneration of ATP can be almost entirely ascribed to mitochondrial oxidative phosphorylation (see Table 2). At 10 - 15 sec. after glucose addition the relative contributions of the glycolytic pathway and

TABLE 2

CALCULATIONS OF ATP UTILISATION AND ATP REGENERATION IN THE CYTOPLASMIC AND MITOCHONDRIAL FRACTIONS IN KREBS ASCITES CELLS AT DIFFERENT TIMES FOLLOWING THE ADDITION OF GLUCOSE

	ATP (μmoles/g cells)						
Time (sec)	3	5	10	15	20	25	30
A. ATP decrease	-0,49	-0,59	-0,56	-0,76	-0,97	-1,25	-1,43
B. ATP utilised	0,95	1,52	3,65	5,41	6,43	6,33	7,21
C. ATP regenerated	0,46	0,93	3,09	4,65	5,46	5,08	5,78
D. ATP regenerated by glycolysis	0	0	1,43	2,83	2,76	2,23	2,64
E. ATP regenerated by adenylate kinase	0,05	0,10	0,21	0,33	0,44	0,64	0,69
F. ATP regenerated by mitochondria	0,41	0,83	1,45	1,49	2,26	2,21	2,45

The results are given as the difference between the value at each individual time interval and the zero time value. The ATP partitioning was calculated as follows:

A. The overall nett decrease of ATP was determined by direct measurement of the cellular content.

B. The total ATP utilised was calculated from the sum of the increases in G6P (one equivalent of ATP), FDP (two equivalents of ATP), any increases in metabolites between triose phosphate and lactate (one equivalent of ATP each) and any increases in intermediates of the pentose phosphate pathway (one equivalent of ATP each).

C. The ATP regenerated is the difference between B and A.

D. The ATP regenerated by glycolysis was calculated from the sum of the intermediates 3-phosphoglycerate, 2-phosphoglycerate and phosphoenol-pyruvate (one equivalent of ATP each) plus pyruvate and lactate (two equivalents of ATP each).

E. The ATP regenerated by adenylate kinase was calculated from the AMP value assuming that this reaction was the main source of AMP.

F. The ATP formed by the mitochondria was the ATP regenerated not accounted for by glycolysis and adenylate kinase, i.e. B-(D+E). This may also be calculated from the known oxygen uptake of ascites cells at different time intervals after addition of glucose.

mitochondrial systems are almost equal and there is a burst of lactate production (see Table 1), the mitochondrial contribution to ATP formation

remaining linear at this time. At 20 - 30 sec. after glucose addition both the rate of ATP utilisation and ATP regeneration have decreased, there is little further accumulation of lactate and the slow rate of ATP regeneration at this time interval may be largely ascribed to mitochondrial phosphorylation and adenylate kinase. The overall relative contribution of the two compartments throughout the 30 sec. period is an equal contribution from the glycolytic pathway and the mitochondrial phosphorylation sequence, the adenylate kinase system possibly accounting for about 10% of the total ATP regenerated. The mitochondrial contribution to ATP regeneration has here been calculated indirectly, however these figures are in excellent agreement with direct measurements of the oxygen uptake of ascites cells at different periods following glucose addition. Maitra and Chance (2) reported that the oxygen uptake of Ehrlich ascites cells in the initial period (20 - 40 sec.) following glucose addition is 0.97 µmoles/g cells/min at 22 - 25°. Assuming a P/O ratio of 3 (see Wenner, ref. 13) and a Q_{10} value of 2 for the temperature difference, this would correspond to an ATP production of about 6 µmoles/g cells/min at 37°. In the present experiment it was calculated that the rate of ATP production by mitochondria during the first 20 sec. is equivalent to 6.6 µmoles ATP/g cells/min at 37° (Table 2). After the initial rapid period, the rate of oxygen uptake falls to 0.21 µmoles/g cells/min at 22 - 25° (2), corresponding (using the same assumptions) to an ATP production of 1.2 µmoles ATP/min at 37°. From the results in Table 2 it may be calculated that the rate of ATP regeneration by mitochondria 20 - 30 sec. after glucose addition is also 1.2 µmoles. This agreement gives support to the calculations of partitioning of ATP rephosphorylation between the soluble and mitochondrial fractions from changes in the metabolite levels. An amount of ATP equivalent to the cellular content is utilised and regenerated within 10 sec. of addition of glucose.

DISCUSSION

The changing pattern of the contribution of different compartments to

ATP phosphorylation may be examined in relation to the intracellular localisation of hexokinase and factors operating in the control of glycolysis.

There is direct evidence *in vitro* (5), and indirect evidence in intact cells (10), for the release of mitochondrial bound hexokinase by G6P. Three lines of evidence suggest release of mitochondrial hexokinase within 20 sec. of addition of glucose to ascites cells: firstly, glucose phosphorylation occurs rapidly and linearly for 15 sec. in ascites cells in the presence of apparently inhibitory concentrations of G6P (0.5 mM), suggesting that hexokinase was at this stage not susceptible to inhibition by G6P, i.e. that it was bound to mitochondria; secondly, there is a dramatic onset of inhibition from 15 - 20 sec. with little modification in the G6P or ADP content of the cell, suggesting that if G6P inhibition is now manifest the hexokinase must be in a different relationship to the inhibitor, i.e. in the free form in the cytoplasm; thirdly, the *in vitro* studies of Rose and Warms (5) give a half time of release of the enzyme from mitochondria by G6P which corresponds well with the time of onset of inhibition of glucose phosphorylation.

Considering first the changes in the rate of mitochondrial regeneration of ATP, it may be seen in the present experiments that all the regeneration of ATP occurred in the mitochondrial compartment during the first 5 sec. after addition of glucose; this is particularly evident at 3 sec. where there is a high rate of G6P formation but as yet very little formation of FDP and no net formation of lactate. The rate of mitochondrial phosphorylation of ATP remains linear for 10 sec., declining at 20 - 30 sec. to one-fifth of the initial rate. Two points emerge: firstly, the evidence suggests direct utilisation of the ADP generated by the hexokinase reaction by mitochondria during the initial period following glucose addition; secondly, the ADP coupling or shuttle system slows down considerably at a time, about 20 sec., when evidence points to the release of mitochondrially bound hexokinase into the cytoplasm and inhibition of this enzyme by G6P.

The control of ascites cell glycolysis has been widely studied and ADP, Pi and G6P have all been implicated (1,2,13-17). When the present changes in the glycolytic contribution are examined it is apparent that there is a burst of glycolytic ATP production between 5 and 15 sec., preceded and followed by slower rates. It was observed (Table 1) that FDP accumulation follows a sigmoid curve in the initial stages, suggesting the necessity for accumulation of an allosteric effector of phosphofructokinase (PFK) before rapid FDP formation can take place. Increasing concentrations of F6P have been shown to produce a sigmoid curve in PFK activity in the presence of inhibitory concentrations of ATP (18). The rapid rate of lactate production lags about 2 sec. behind the onset of a high rate of FDP formation; a similar finding was previously reported by Maitra and Chance (2). In oscillating systems it is generally found that pyruvate formation lags behind FDP formation, the two being 90° out of phase (19,20).

Thus at the short time interval when there is a rapid and linear rate of glucose phosphorylation and when the initial lag in FDP and lactate formation have been overcome, that is 5 - 15 sec. after glucose addition, the ADP rephosphorylation occurs equally in both cell compartments, and it is suggested that ADP from the hexokinase reaction is related to the mitochondrial compartment and ADP from the PFK reaction is related to the cytoplasmic compartment. In these particular experiments the onset of inhibition at 20 sec. may possibly be ascribed to release of hexokinase from mitochondria and inhibition by G6P.

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