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REGULATION OF CELLULAR ENERGY METABOLISM

THE CRABTREE EFFECT

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Summary

The Crabtree effect (inhibition of respiration by glycolysis) is observed in cells with approximately equal glycolytic and respiratory capacities for ATP synthesis. Addition of glucose to aerobic suspensions of glucose-starved cells (Sarcoma 180 ascites tumor cells) causes a burst of respiration and lactate production due to ATP utilization for glucose phosphorylation by hexokinase and phosphofructokinase. This burst of activity is followed by inhibition of both respiration and glycolysis, the former to below the value before glucose addition (Crabtree effect). Both the respiratory rate and the glycolytic flux appear to be regulated by the cytosolic $[ATP]/[ADP][P_i]$ albeit by completely different mechanisms. Respiration is regulated by the free energy of hydrolysis of ATP, such that the rate increases as the $[ATP]/[ADP][P_i]$ decreases and decreases as the $[ATP]/[ADP][P_i]$ increases. The regulatory enzymes of glycolysis are activated by ADP (AMP) and P_i and inhibited by ATP. Thus both respiration and glycolysis increase or decrease as the $[ATP]/[ADP][P_i]$ decreases or increases. The parallel regulation of both ATP-producing pathways by this common metabolite ratio is consistent with the cytoplasmic $[ATP]/[ADP][P_i]$ being an important determinant of homeostatic regulation of cellular energy metabolism.

Introduction

Cellular ATP is derived from two sources, glycolysis and oxidative phosphorylation. The second of these is characteristic of aerobic organisms and provides 17 times as much ATP/mol of glucose as does anaerobic glycolysis;

it is the main energy-producing mechanism in most eukaryotes. The two pathways are located in different cellular compartments but they both carry out net phosphorylation of cytosolic ADP by P_i to form ATP.

The mutual relations between glycolysis and oxidative phosphorylation are reflected in the so-called Pasteur and Crabtree effects. The first of those is exhibited by most tissues and is defined as inhibition of glycolysis under aerobic conditions. The second, which will be discussed here is an inhibition of respiration which occurs upon stimulation of glycolysis. In contrast to the Pasteur effect, the Crabtree effect [1—6] is observed only in a rather limited number of cell types such as yeast and tumor cells which are known to possess high glycolytic activities. The occurrence of the two phenomena suggests that respiration and glycolysis are regulated by common variables.

The present work is intended to re-examine the regulatory parameters which are responsible for the occurrence of the Crabtree effect. Although the time courses of the changes of various metabolites and glycolytic intermediates which are initiated by glucose addition were extensively investigated in the past [1—15] and are in general agreement with the changes reported here a uniform explanation has not yet emerged and no consensus has been reached as to the etiology of the Crabtree effect. The results presented in this paper provide a coherent picture of mutual relations between glycolysis and oxidative phosphorylation and indicate that the Crabtree effect can be explained on the basis of changes which occur in the cellular energetic parameters.

Materials and Methods

Cell preparations. Sarcoma 180 ascites tumor cells (a line obtained from the Cell Center of the University of Pennsylvania) were grown for 6—8 days in the peritoneal cavity of Balb/c male mice. The cells were harvested, diluted approx. 2-fold in Krebs-Henseleit saline containing 10 mM Tris-HCl, pH 7.4, and centrifuged for 2 min at 100—200 $\times g$ in a table-top International Clinical Centrifuge. The supernatant (which sometimes contains traces of blood) was carefully removed by suction and the white pellet was washed once in the Krebs-Henseleit buffer. Pellets containing visible quantities of erythrocytes were discarded. The pellet was suspended in the Krebs-Henseleit saline containing appropriate concentration of inorganic phosphate at a 1 : 15 dilution of the packed cell volume. (The Krebs-Henseleit buffer from which the phosphate was omitted is referred to throughout the text as the buffer containing zero P_i.) The dry weights of the cells and of the medium were determined for each preparation.

Incubation. Cell suspensions were gently shaken at 22°C in open Erlenmeyer flasks to provide proper aeration and prevent sedimentation. Incubations were started by the addition of 4 mM hexose (time 'zero'). Aliquots were withdrawn at chosen time intervals and either quenched in cold $HClO_4$ (4% final concentration) for assays of various metabolites or centrifuged through a layer of silicone oil into the 4% $HClO_4$ solution for the determination of intracellular inorganic phosphate. The $HClO_4$ extracts were neutralized with 3 M K_2CO_3/0.5 M triethanolamine mixture and the precipitated $KClO_4$ was removed by centrifugation. The entire experiment was monitored by the behavior of the

respiratory rate which was measured simultaneously in a separate vessel equipped with an oxygen cathode.

Determination of intracellular water. The intracellular water and trapped water volumes were determined using ^3H_2O [^{14}C]poly(ethylene glycol) (M_r 4000). Cell suspensions (4—6 mg dry weight/ml) were supplemented with the above reagents and centrifuged through a layer of silicone oil in a Beckman 152 microfuge. The ^{3}H and ^{14}C activities in the supernatant and pellet fractions were measured and used to calculate the poly(ethylene glycol)-inaccessible water space (intracellular water) and poly(ethylene glycol)-accessible water space (trapped volume). The intracellular water was found to be 3.7 ml/g dry weight and the trapped space was 20% of the total water volume of the pellet.

Measurements of the concentrations of ATP, ADP, P_i and glycolytic intermediates. ATP and glucose 6-phosphate (Glc-6-*P*) concentrations were determined by the method of Lamprecht and Trautschold [16], the ADP concentration by the procedure of Adams [17] and the P_i concentration according to Hess and Derr [18]. The P_i measured in the $HClO_4$ fraction was corrected for P_i in the extracellular medium which accompanied the cells through the silicone oil (trapped volume). The cell separation and quenching was considered to occur at the time the centrifuge was started.

The concentration of fructose 1,6-bisphosphate (Fru-1,6-P_2) was determined by the method of Michal and Beutler [19], and lactate by the procedure of Gawehn and Bergmeyer [20].

Measurement of the redox state of the cytochromes. A dual-wavelength spectrophotometer designed and built in the shops of the Johnson Foundation was used to measure the redox state of cytochrome *c* in cell suspensions using the absorbance change at 550 nm minus 540 nm. In order to prevent cell sedimentation the suspension was mixed continuously with a vibrating stirrer. Spectrophotometric readings were made during the time course of experiments identical to those described above. At 3—5 min after the addition of glucose, i.e. at the time when the respiratory rate became stable antimycin A was added to determine the fully oxidized level of cytochrome *c* followed by 1 mM KCN to measure the fully reduced state of this cytochrome (for details see Figs. 3—5).

Spectra were measured using a scanning dual-wavelength spectrophotometer designed in the shops of the Johnson Research Foundation and interfaced to a PDP 1110 digital computer. The computer was used to obtain the indicated spectra by digital processing of the photomultiplier output.

Reagents. All enzymes, coenzymes and metabolites were purchases from Sigma Chemical Company, St. Louis, MO.

Pyruvate kinase (type II); lactate dehydrogenase (type III); hexokinase (type IV); glucose-6-phosphate dehydrogenase (type VII); nicotinamide adenine dinucleotide phosphate from yeast; nicotinamide adenine dinucleotide from yeast (grade III); phospho*enol*pyruvate; reduced nicotinamide adenine dinucleotide from yeast (grade III).

All other reagents were analytical reagent grade.

Results

The effect of glucose on the respiratory rate

The effect of glucose addition on respiration of ascites tumor cells suspended in a Krebs-Henseleit saline containing zero phosphate is shown in Fig. 1. A biphasic response of a transient stimulation followed by an inhibition of respiration is observed. The phase of the increased respiration was usually of a very short duration, of the order of 15—30 s, and of variable intensity, i.e. cell preparations exhibited a 20—50% increase in respiration. The same general pattern was observed at different concentrations of extracellular phosphate although the respiratory rate before glucose addition was higher in the media contaning more phosphate (the rate was 0.65 μmol O_2/min per mg wet wt. at zero P_i, 0.70 μmol/min per mg wet wt. at 13 mM P_i and 0.85 μmol/min per mg wet wt. at 26 mM P_i). The extent of stimulation of respiration which followed the addition of glucose did not appear to correlate with the concentration of extracellular phosphate but was somewhat variable among cell preparations.

The subsequent inhibition of respiration was observed at all concentrations of extracellular P_i and was usually greater in media containing no or small concentrations of phosphate. At longer time intervals, in most experiments, respiration became 'deinhibited' and in some case reached almost the original level.

Changes in concentrations of metabolites

Metabolic changes which occurred after the addition of glucose were analyzed in cells suspended in Krebs-Henseleit saline containing no phosphate and in the same buffer supplemented with either 1, 13, or 26 mM phosphate. The changes observed in the absence of phosphate in the medium were very similar to those seen in the presence of 1 mM P_i and the changes measured in the presence of 13 mM phosphate and 26 mM phosphate also showed great similarities. We have therefore limited our presentation to the results obtained in media with zero P_i and 1 mM P_i as representative of low intracellular P_i concentration and to the data obtained in the presence of 13 mM phosphate

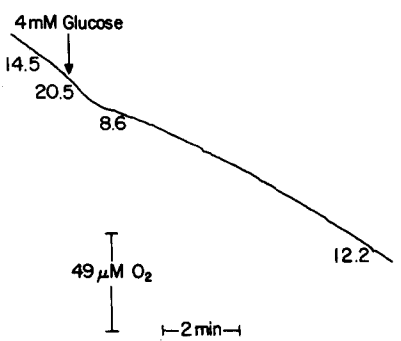

Fig. 1. The changes in respiratory rate following the addition of 4 mM glucose to ascites tumor cells suspended in a Krebs-Henseleit solution containing zero P_i concentration. Rates of respiration are indicated in figure in units of μM O_2/min. Cells were suspended at a concentration of 22.0 mg wet weight/ml.

for high extracellular P_i concentration. The values are summarized in Tables I-III. Fig. 2 gives a graphical representation of the changes in concentrations of various metabolites, or their ratios, which follow the addition of glucose. The results were presented for a single experiment with cells suspended in 13 mM P_i and offer a possibility of detailed analysis of temporal relationships in changes of various parameters.

The effect of glucose on the concentrations of Glc-6-P and Fru-1,6-P_2. The initial intracellular concentrations of the two phosphorylated sugars were low (less than 0.1 mM) and subsequent changes induced by glucose addition followed the same pattern irrespective of the concentration of P_i in the suspending medium (Tables I—III). The concentration of Fru-1,6-P_2 increased rapidly during the first 20 s, reached a maximum level at about 40 s and began to decline slowly during the next 3—10 min. Glc-6-*P* rose at a slower rate, attained its maximum concentration in about 90 s and slowly declined thereafter. Maximum intracellular concentrations of Fru-1,6-P_2 were somewhat

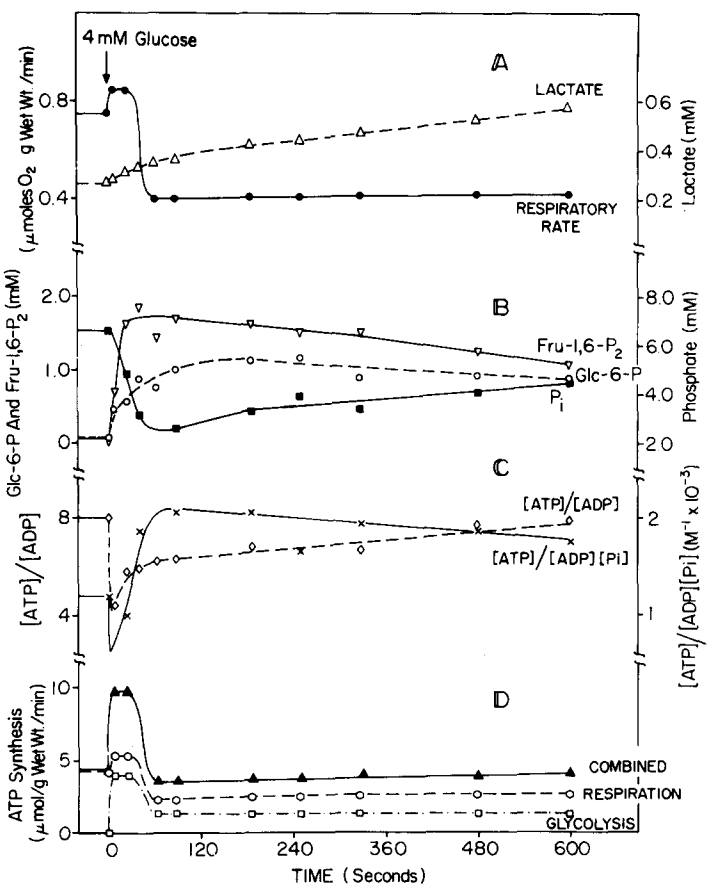

Fig. 2. Changes in the measured metabolic parameters as a function of time following addition of glucose to an ascites tumor cell suspension containing 13 mM P_i. The cells were suspended at approx. 21 mg wet weight/ml in a Krebs-Henseleit solution containing 13 mM P_i. The measured values are presented as mean values (see Tables I and II). The rate of ATP synthesis was calculated as described in Discussion.

TABLE I

CHANGES IN CONCENTRATIONS OF PHOSPHORYLATED GLYCOLYTIC INTERMEDIATES AND GLYCOLYTIC END PRODUCT FOLLOWING THE ADDITION OF 4 mM GLUCOSE TO SARCOMA 180 CELLS SUSPENDED IN A MEDIUM WITH ZERO P_i CONCENTRATION

The cells were suspended in a Krebs-Henseleit solution from which the phosphate has been omitted at 21.4 mg wet weight/ml. Values are mean ± S.E. for four experiments for Glc-6-*P* and Fru-1,6-P_2 concentrations, three experiments for respiratory rate and two experiments for lactate concentration.

Time (s)	Respiratory rate (μmol O_2/min per mg wet wt.)	Intracellular (mM)		[Lactate] (extracellular mM)	Intracellular (mM)		[P_i]	[ATP]/[ADP]	[ATP]/[ADP][P_i] (M^{-1})
		[Glc-6-*P*]	[Fru-1,6-P_2]		[ATP]	[ADP]			
Control	0.65 ± 0.10	0.08 ± 0.04	0.08 ± 0.02	0.46 ± 0.03	5.78 ± 0.46	0.54 ± 0.15	3.62 ± 0.44	10.70	2957
8— 12	0.95 ± 0.02	0.38 ± 0.18	0.35 ± 0.10	0.46 ± 0.05	5.34 ± 0.71	1.02 ± 0.06	3.00 *	5.24	1745
17— 22	0.95 ± 0.02	0.26 ± 0.12	0.97 ± 0.34	—	5.35 ± 0.50	0.81 ± 0.16	2.29 ± 0.35	6.60	2884
27	0.95 ± 0.02	0.49 ± 0.01	1.10 ± 0.04	0.49 ± 0.04	5.70 ± 0.75	0.82 ± 0.16	—	6.95	—
35— 38	turning off	0.40 ± 0.24	1.76 ± 0.21	—	5.66 ± 0.52	0.68 ± 0.07	—	8.32	—
50— 56	turning off	0.46 ± 0.22	1.47 ± 0.25	0.54 ± 0.01	5.06 ± 0.14	0.69 ± 0.11	—	7.33	—
61— 64	0.29 ± 0.06	0.81 ± 0.19	1.57 ± 0.15	0.53 ± 0.03	5.06 ± 0.12	0.78 ± 0.24	—	6.49	—
81— 91	0.29 ± 0.06	1.06 ± 0.44	1.63 ± 0.25	0.55 ± 0.04	5.80 ± 0.32	0.73 ± 0.16	1.09 ± 0.29	7.94	7289
124	0.29 ± 0.06	1.09 ± 0.08	1.27 ± 0.10	—	5.55 ± 0.64	0.88 ± 0.16	1.04 ± 0.22	6.31	6064
151	0.29 ± 0.06	1.19 ± 0.50	1.71 ± 0.33	0.54 ± 0.02	5.50 ± 0.32	0.74 ± 0.23	—	7.43	—
180—186	0.29 ± 0.06	1.31 ± 0.15	1.14 ± 0.15	0.55 ± 0.03	6.41 ± 0.49	0.97 ± 0.25	—	6.61	—
244	turning on	0.74 ± 0.19	1.51 ± 0.40	0.61 ± 0.04	5.12 ± 0.32	0.71 ± 0.25	1.11 ± 0.51	7.21	6497

* This value was approximated from a graph of P_i concentration vs. time since it was experimentally impossible to obtain at such a short time interval.

TABLE II

CHANGES IN CONCENTRATIONS OF PHOSPHORYLATED GLYCOLYTIC INTERMEDIATES AND GLYCOLYTIC END PRODUCT FOLLOWING THE ADDITION OF 4 mM GLUCOSE TO SARCOMA 180 CELLS SUSPENDED IN THE PRESENCE OF 1 mM P_i

The cells were suspended in a Krebs-Henseleit solution containing 1 mM P_i at a wet weight concentration of 21.2 ± 1.4 mg/ml. Values are mean ± S.E. for three experiments in all cases excluding the Fru-1,6-P_2 concentration for which two experiments were used.

Time (s)	Respiratory rate (μmol O_2/min per mg wet wt.)	Intracellular (mM)		[Lactate] (extracellular; mM)	Intracellular (mM)			[ATP]/[ADP]	[ATP]/[ADP][P_i] (M^{-1})
		[Glc-6-P]	[Fru-1,6-P_2]		[ATP]	[ADP]	[P_i]		
Control	0.74 ± 0.08	0.07 ± 0.03	0.06 ± 0.04	0.24 ± 0.01	4.17 ± 0.72	0.40 ± 0.05	4.50 ± 0.32	10.42	2317
11— 13	1.14 ± 0.03	—	—	—	3.86 ± 0.68	0.77 ± 0.05	3.70 *	5.01	1354
22— 28	1.14 ± 0.03	0.48 ± 0.24	1.75 ± 0.25	0.24 ± 0.02	3.82 ± 0.49	0.61 ± 0.03	3.43 ± 0.16	4.96	1446
35— 43	1.14 ± 0.03	0.72 ± 0.28	1.84 ± 0.27	0.28 ± 0.01	4.05 ± 0.57	0.57 ± 0.07	2.56 ± 0.20	7.10	2775
59— 64	0.35 ± 0.07	0.89 ± 0.22	1.79 ± 0.32	0.29 ± 0.01	3.76 ± 0.19	0.54 ± 0.08	2.21 ± 0.10	6.96	3151
75— 78	0.35 ± 0.07	1.23 ± 0.22	1.65 ± 0.37	0.30 ± 0.02	4.36 ± 0.52	0.54 ± 0.08	2.21 ± 0.13	8.07	3653
90— 96	0.41 ± 0.04	—	—	—	3.66 ± 0.34	0.55 ± 0.07	2.23 ± 0.02	6.66	2983
115—117	0.41 ± 0.04	1.35 ± 0.29	1.49 ± 0.45	0.35 ± 0.02	4.10 ± 0.62	0.46 ± 0.03	2.21 ± 0.10	8.91	4033
150—160	0.45 ± 0.04	1.10 ± 0.20	1.47 ± 0.43	0.36 ± 0.01	4.13 ± 0.60	0.58 ± 0.15	2.20 ± 0.11	7.12	3237
188—199	0.45 ± 0.04	—	—	—	3.87 ± 0.21	0.51 ± 0.09	2.23 ± 0.13	7.59	3403
211—228	0.45 ± 0.04	1.04 ± 0.27	1.35 ± 0.41	0.38 ± 0.01	3.60 ± 0.31	0.60 ± 0.10	2.23 ± 0.09	6.00	2691
281—285	0.45 ± 0.04	—	—	—	3.73 ± 0.44	0.55 ± 0.08	2.33 ± 0.11	6.78	2911
454—488	0.50 ± 0.04	0.97 ± 0.60	1.13 ± 0.45	0.49 ± 0.03	3.90 ± 0.50	0.49 ± 0.11	2.48 ± 0.21	7.96	3209

* This value was approximated from a graph for the P_i concentration vs. time since it was experimentally impossible to obtain at such a short time interval.

TABLE III

CHANGES IN CONCENTRATIONS OF PHOSPHORYLATED GLYCOLYTIC INTERMEDIATES AND LACTATE FOLLOWING THE ADDITION OF 4 mM GLUCOSE TO ASCITES TUMOR CELL SUSPENSIONS IN THE PRESENCE OF 13 mM P_i

The cells were suspended at 21.0 ± 1.0 mg wet weight/ml in a Krebs-Henseleit solution containing 13 mM P_i. Values are mean ± S.E. for four experiments.

Time (s)	Respiratory rate (μmol O_2/min per mg wet wt.)	Intracellular (mM)		[Lactate] (extracellular; mM)	Intracellular (mM)			[ATP]/[ADP]	[ATP]/[ADP][P_i] (M^{-1})
		[Glc-6-*P*]	[Fru-1,6-P_2]		[ATP]	[ADP]	[P_i]		
Control	0.70 ± 0.60	0.08 ± 0.05	0.10 ± 0.04	0.27 ± 0.02	4.30 ± 0.22	0.54 ± 0.05	6.64 ± 0.98	7.96	1199
8— 10	0.87 ± 0.12	0.44 ± 0.04	0.72 ± 0.26	0.28 ± 0.01	4.05 ± 0.28	0.92 ± 0.08	6.00 *	4.40	734
24	0.89 ± 0.12	0.56 ± 0.17	1.62 ± 0.17	0.31 ± 0.01	4.36 ± 0.47	0.75 ± 0.09	4.85 ± 0.87	5.81	1001
37— 42	turning off	0.87 ± 0.14	1.86 ± 0.29	0.33 ± 0.01	4.48 ± 0.28	0.76 ± 0.09	3.17 ± 0.25	5.90	1860
59— 69	0.40 ± 0.02	0.77 ± 0.12	1.42 ± 0.12	0.35 ± 0.02	4.25 ± 0.26	0.68 ± 0.06	—	6.25	—
79— 95	0.40 ± 0.02	0.99 ± 0.11	1.74 ± 0.33	0.36 ± 0.01	4.29 ± 0.23	0.79 ± 0.06	2.64 ± 0.33	6.31	2057
177—193	0.42 ± 0.04	1.13 ± 0.09	1.62 ± 0.26	0.42 ± 0.02	4.35 ± 0.06	0.64 ± 0.05	3.29 ± 0.80	6.80	2066
245—252	0.42 ± 0.04	1.15 ± 0.08	1.51 ± 0.28	0.44 ± 0.01	4.23 ± 0.25	0.65 ± 0.08	3.95 ± 0.70	6.51	1648
321—329	0.43 ± 0.03	0.90 ± 0.08	1.51 ± 0.32	0.47 ± 0.01	4.16 ± 0.23	0.62 ± 0.07	3.45 ± 0.73	6.71	1945
473—481	0.44 ± 0.03	0.91 ± 0.08	1.23 ± 0.25	0.53 ± 0.01	4.14 ± 0.12	0.54 ± 0.07	4.12 ± 0.45	7.67	1861
596—600	0.44 ± 0.03	0.88 ± 0.20	1.06 ± 0.17	0.58 ± 0.02	3.78 ± 0.32	0.48 ± 0.06	4.49 ± 0.63	7.88	1754

* These values were approximated from a graph for the P_i concentration vs. time since it was experimentally impossible to obtain data at the 8—10 s time point.

higher than those of Glc-6-*P*. Moreover, lower maximal concentrations of Fru-1,6-P_2 were attained in cells incubated in a medium containing zero P_i compared to cells incubated in a medium containing 1, 13 or 26 mM P_i. The reverse was observed for Glc-6-*P*.

The effect of addition of glucose on intracellular P_i. The initial concentration of intracellular phosphate was dependent on the concentration of P_i in the suspending medium. It was 8.6 ± 1.4 mM in the medium containing 26 mM P_i; 6.6 ± 1 mM in the medium containing 13 mM P_i; 4.5 ± 0.3 mM in the medium containing 1 mM P_i; and 3.6 ± 0.4 mM in the medium containing zero P_i. The addition of glucose caused a rapid decline in the intracellular P_i concentration at all concentrations of extracellular phosphate although the absolute values were dependent on the level of the extracellular P_i concentration. The decline in the intracellular P_i was a mirror image of the behavior of the two phosphorylated sugars and especially that of Fru-1,6-P_2. The P_i concentration declined rapidly as the two phosphorylated sugars accumulated, attaining its lowest steady-state concentration at approximately the same time as they reached their maxima. Thereafter the intracellular P_i concentration began to increase. The increase began approx. 3—4 min after the addition of glucose and was readily observed when the measurements were carried out to approx. 10 min (Tables II and III). In each case the increase in intracellular P_i concentration appeared to correlate with a decrease in the concentrations Glc-6-*P* and Fru-1,6-P_2. In the presence of 10 mM and greater P_i in the suspending medium the intracellular P_i concentration increased more rapidly and to higher levels (Table III). This suggests that the increase in the intracellular P_i concentration observed at longer time intervals is contributed from two sources: uptake of phosphate from the suspending medium and breakdown of phosphorylated intermediates.

It should be mentioned here that upon addition of glucose to cells suspended in media containing less than 1 mM phosphate the intracellular phosphate concentration fell to about 1 mM. This low intracellular concentration of P_i may severely impair or limit metabolic activity of the cell.

The effect of glucose on lactate production. Extracellular levels of lactate were higher in the media containing no added phosphate (0.46 ± 0.03 mM) and lower in the media containing added P_i (0.24 ± 0.01 mM at 1 mM; 0.27 ± 0.02 mM at 13 mM, and 0.18 ± 0.04 mM at 26 mM P_i). Addition of glucose was followed by an increase in extracellular lactate concentration (which is indicative of its increased production) after a lag of 10—20 s. In the absence of P_i in the medium (or at the very low levels) extracellular lactate concentration increased by about 20% and leveled off when the intracellular phosphate concentration dropped to its lowest level. It was resumed, although at a very slow rate, when the Glc-6-*P* and Fru-1,6-P_2 concentrations started to decline and the intracellular P_i concentration began to rise (Table II). At high extracellular P_i concentration, the concentration of lactate steadily increased and was doubled in about 8 min in 13 mM P_i (Table III) and almost tripled in the same time in 26 mM P_i (not shown).

Effect of glucose addition on the levels of adenine nucleotides. The total concentrations of intracellular adenine nucleotides were between 4.8 mM and 6.3 mM, similar to those found in other types of cells [21]. Addition of glucose

to cells suspended at the various P_i concentrations caused an abrupt, approx. 2-fold increase in ADP concentration and a small decline in ATP. At the end of the burst of respiration, the concentration of ADP began to decline and in media containing high P_i it returned to its original level. In media containing low phosphate levels, the ADP concentration remained elevated.

The behavior of [ATP]/[ADP] followed that of ADP because changes in ATP concentration were rather small i.e. the [ATP]/[ADP] decreased to about half of the value observed prior to glucose addition and then returned to the original level in the media containing high phosphate but remained lower in low-phosphate media. The [ATP]/[ADP][P_i] decreased during the initial burst of respiration and then increased until respiration became inhibited. The [ATP]/[ADP][P_i] were always higher in the low-phosphate media than in the high-P_i media both before and after glucose addition, in agreement with the observed lower rates of respiration. It should be noted that prior to the addition of glucose the concentrations of ATP, ADP and P_i remained constant during 5—15 min preincubation (straight line in Fig. 2).

Effects of galactose and fructose addition on the metabolism of ascites tumor cells. Addition of either 4 mM galactose or 4 mM fructose to cell suspensions in a medium containing 1 mM P_i showed essentially no effect on the measured parameters. The Glc-6-*P* and Fru-1,6-P_2 concentrations remained at very low (less than 0.05 mM and 0.1 mM, respectively) and constant levels throughout the entire experiment. There were no changes either in respiration or in the levels of adenine nucleotides and P_i. This indicates that the reactions which lead to channeling of these substrates into the main glycolytic pathway (transport across the membrane, phosphorylation of the respective sugar, epimerization of Gal-6-*P* and aldolase splitting of Fru-1-*P*) are considerably slower in sarcoma 180 cells than is the phosphorylation of glucose.

The concentrations of the cytochromes and the redox state changes of cytochrome c. The cytochrome content and the turnover number of cytochrome *c* were calculated for three separate cell suspensions in 1 mM P_i medium. The concentration of cytochrome *c* was found to be the same as in liver cells (0.02 nmol/mg wet wt.) whereas the content of cytochrome *a* was one half that of liver cells. The turnover number for cytochrome *a* of 11.4 s^{-1} is equal to that in liver cells whereas that for cytochrome *c* is somewhat lower (3.0 s^{-1} compared to 6 s^{-1}).

The redox changes of cytochrome *c* following glucose additions were investigated in separate, parallel experiments in cells suspended in media containing low (1 mM) and high (10 mM) P_i. Changes in the redox state of cytochrome *c* were measured at 550 nm minus 540 nm using a dual-wavelength spectrophotometer. This method gives good signal-to-noise ratio and allows measurement of the time dependence of the absorbance changes. The wavelength dependence of the absorbance changes (Fig. 3) identifies them as arising from the oxidation and reduction of cytochrome *c*.

The addition of glucose to cell suspensions in media with low P_i caused a small highly reproducible reduction of cytochrome *c* which was followed by reoxidation to a level slightly beyond that observed prior to glucose addition (representative experiment shown in Fig. 4). This reoxidation of cytochrome *c* corresponded in time to the inhibition of respiration recorded with an oxygen

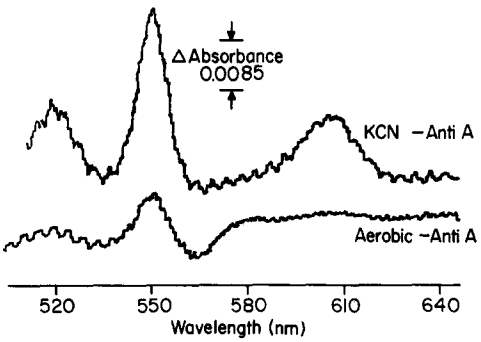

Fig. 3. Steady-state absorption spectra of the cytochromes of an ascites tumor cells suspension containing 1 mM P_i. The cells were suspended at 82.6 mg wet weight/ml in a Krebs-Henseleit solution. Suspension was continuously mixed with a vibrating stirrer and the spectrum of the aerobic cell suspension was recorded and stored in the computer memory. Spectra were then measured after addition of 7.5 μg antimycin A/ml and after addition of 1 mM KCN. The computer was then used to subtract the spectrum after antimycin A addition from each of the others and to plot the resulting difference spectra.

electrode. The steady-state level of reduction of cytochrome *c* prior to the addition of glucose was 17 ± 2% reduced.

The addition of glucose to cells suspended in a medium containing high P_i caused a slight progressive oxidation of cytochrome *c* during the initial 1—2 min period. There may be a very transient reduction of cytochrome *c* immediately after glucose addition but this is at the noise level of the measurement and is not reproducible from experiment to experiment. The steady-state

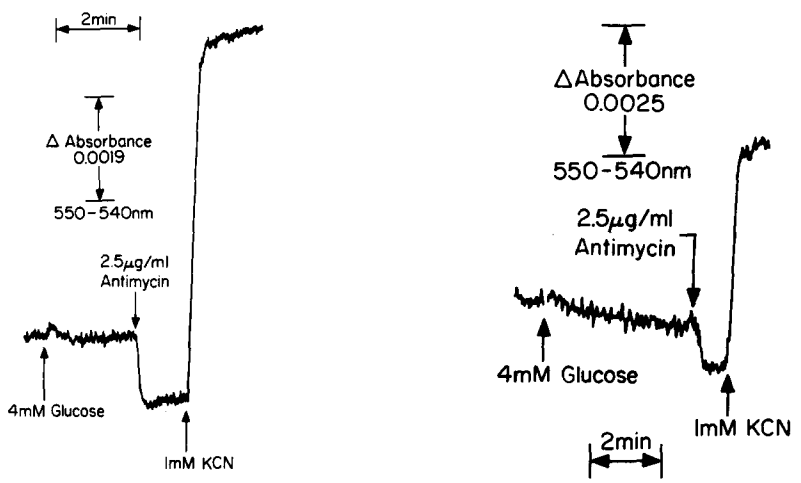

Fig. 4. Measurements of redox state of cytochrome *c* in suspensions of ascites tumor cells in media contaning 1 mM P_i. Cells were suspended at 19.6 mg wet weight/ml in a Krebs-Henseleit solution containing 1 mM P_i. The suspension was continuously mixed with a vibrating stirrer and additions were made as indicated in the figure. The light path was 1.0 cm and the measuring light had a bandwidth at half-intensity of 1.6 nm.

Fig. 5. Measurements of redox state of cytochrome *c* in suspensions of ascites tumor cells containing 10 mM P_i. Cells were suspended at 23.6 mg wet weight/ml in a Krebs-Henseleit solution containing 10 mM P_i. Suspension was continuously mixed with a vibrating stirrer and additions were made as indicated in figure.

level reduction of cytochrome *c* before glucose addition was 29 ± 2% and this fell to a level of 17 ± 2% by approx. 1 min after glucose addition (representative experiment shown in Fig. 5).

Measurement of extracellular pH. The extracellular pH of cell suspensions containing 1 mM and 13 mM P_i was monitored with a pH electrode. It was found that in 1 mM P_i the extracellular pH was between 7.25 and 7.30 and remained essentially constant for 5 min after the addition of glucose. In medium containing 13 mM P_i the starting pH was 7.30 and decreased about 0.1 pH unit during the 10 min incubation period following the addition of glucose.

Discussion

The results described above show that there is more than a single variable which responds to addition of glucose to starved ascites tumor cells. This may explain the diversity of proposals put forward in explanation of the Crabtree effect. Several authors have emphasized the role of intracellular inorganic phosphate [7,8,12,13], others stressed the changes in concentrations of the adenine nucleotides [10] or possible damage to the respiratory chain [9]. However, careful analysis of all these variable shows that none of these proposals taken individually can explain in a consistent manner the observed changes in cellular energy-producing systems.

Changes in ATP concentration cannot explain the metabolic consequences of glucose addition to sarcoma 180 cells because the levels of this adenine nucleotide remain essentially unaltered during the time course of the experiments. Although the ADP concentration increases initially consistent with respiratory stimulation it does not decrease enough to explain the subsequent inhibition phase. The same applies to the behavior of the [ATP]/[ADP] ratio. The decline in the intracellular P_i concentration is in the opposite direction to that which would be expected for the stimulation of respiration (and glycolysis) but may contribute to the inhibition of respiratory activity over longer time intervals in media containing low levels of phosphate. However, in media containing high concentrations of phosphate, the intracellular P_i levels never fall low enough to be the reason for the inhibition of respiration.

The observed metabolic changes are most closely correlated with [ATP]/[ADP][P_i] which falls when respiration is stimulated and rises when respiration is inhibited[22—24]. The changes seen in the [ATP]/[ADP][P_i] during the initial burst of respiration are large enough to account for all the increase in respiratory rate. Moreover, the values presented in Tables I—III may overestimate the real situation because the time required for the passage of a sample through the silicone oil in the measurement of P_i is longer than that necessary to quench the cells for the measurements of the adenine nucleotides. Thus the P_i values for calculation of the [ATP]/[ADP][P_i] are lower than their actual intracellular concentrations at the time when a sample was taken for the determination of the [ATP]/[ADP]. Since the changes in the P_i concentration are rather fast during the initial phase this may contribute a substantial error to the measured [ATP]/[ADP][P_i].

At longer time intervals, during the phase of inhibited respiration, these

differences in quenching do not lead to a significant error because the levels of the adenine nucleotides as well as those of inorganic phosphate are changing slowly (over a period of minutes). In this phase of cellular response to glucose addition the $[ATP]/[ADP][P_i]$ ratios increase above the level observed before glucose addition and in media containing low phosphate levels reach the values which severely inhibit respiration.

Another factor which may contribute to the inhibition of respiration is the oxidation of the intramitochondrial pyridine nucleotides. This is suggested by the oxidation of cytochrome *c* shown in Fig. 5.

The question which necessarily arises is why only certain types of cells exhibit the Crabtree effect. The following considerations may help to throw some light on the problem. Rat heart, perfused with glucose produces 60 μmol ATP/min per g wet wt. of tissue; 58 μmol are provided by oxidative phosphorylation and 2 μmol are provided by glycolysis [25]. During the burst of respiration and glycolysis that occurs in ascites tumor cells incubated in high-phosphate medium, 12 μmol of ATP are produced/min per g of cells, 50% of which are contributed by oxidative phosphorylation and 50% by glycolysis. Two things are immediately obvious. The first is that the total ATP production is much higher in the heart than it is in the ascites tumor cells and this is due to the high rates of oxidative phosphorylation in the former. The second is that the glycolytic production of ATP is much higher in the ascites cells both on an absolute and relative basis. Thus the occurrence of the Crabtree effect in the latter may be attributed to the very high glycolytic capacity of the ascites cells combined with their low capacity for oxidative phosphorylation as suggested earlier by Krebs [26]. This suggestion is substantiated by the finding that ascites cells have lower concentrations of key respiratory chain carriers such as cytochrome oxidase. This means that in order to achieve the same respiratory rate the mitochondria of the ascites tumor cells must operate at a lower $[ATP]/[ADP][P_i]$ or $[NAD^+]/[NADH]$ than do mitochondria of other types of cells.

It can also be seen that glycolysis is regulated by essentially the same parameters as is oxidative phosphorylation. An increase in ADP and a decrease in ATP stimulate phosphofructokinase whereas low phosphate levels prevent the deinhibition of this enzyme by ATP [27] and lead to a decrease in glycolytic activity at the time when the $[ATP]/[ADP][P_i]$ is increased. Moreover in the experimental system described here the role of intracellular P_i as an independent regulatory parameter can be clearly observed. When the concentration of intracellular phosphate falls to a low level (1 mM or less) cellular ATP utilization becomes severly inhibited leading to a large increase in the $[ATP]/[ADP][P_i]$ and inhibition of both glycolytic and mitochondrial ATP synthesis. Such a depletion of intracellular phosphate must rarely occur in vivo and is the result of rather non-physiological experimental conditions in which starved cells suspended in very low-phosphate medium are suddenly exposed to glucose. Under most cellular conditions glucose is always present, and does not precipitously change in concentration. This decreases the variation in phosphorylated intermediates and therefore of the intracellular P_i concentration. Under these conditions the transport of P_i across the plasma membrane can compensate for changes in the intracellular P_i concentration. The transport

of phosphate across the cellular membrane appears to be controlled so that the gradient is maintained between the extracellular and intracellular spaces. At low extracellular phosphate its concentration inside is higher than outside (although the actual intracellular P_i concentration does depend somewhat on its concentration in the medium) and the opposite gradient exists at high extracellular P_i. It is also interesting that high intracellular phosphate levels stimulate the rate of ATP utilization. This is indicated by the fact that at high intracellular P_i the steady-state ATP levels are lower and the rate of respiration and glycolysis are higher. This may result from the increase in active transport required to maintain proper intracellular ion balance (P_i, Na^+, K^+, Ca^{2+}, etc.).

It has been reported that AMP deaminase is also inhibited by P_i [28]. One would therefore expect a decrease in the total levels of adenine nucleotides when the intracellular P_i concentration is lowered. Such a situation is observed in liver cells incubated with fructose in which the fall in the cytosolic P_i concentration is accompanied by a large decrease in the adenine nucleotide concentrations [20]. However, Sarcoma 180 cells differ from liver cells because the levels of adenine nucleotides in the former cells remained essentially constant when the intracellular P_i decreased. This suggests that Sarcoma cells must have relatively low AMP deaminase activity.

In studies with Ehrlich ascites tumor cells it has been shown that fructose elicits the Crabtree effect [29,30]. In the Sarcoma 180 tumor cell line investigated here, neither galactose nor fructose cause any measurable effect on respiration or glycolysis. Metabolism of either galactose or fructose is too slow in this cell type to cause substantial accumulation of their phosphorylated derivatives and thus a depletion of the intracellular P_i concentration.

The data presented in this paper suggest that the cytoplasmic [ATP]/[ADP]-[P_i] is the primary regulatory parameter of cellular energy metabolism, controlling both respiration and glycolysis. This does not imply that glycolysis is regulated solely by this parameter but rather that the [ATP]/[ADP][P_i] is responsible for coupling respiration and glycolysis in cellular energy metabolism. Regulation of glycolysis by other parameters, such as glucose 6-phosphate and fructose 1,6-bisphosphate represents an important form of independent control which regulates the pattern of carbon flow for cellular metabolism. 'Local control' of glycolysis by parameters other than [ATP]/[ADP]-[P_i] is particularly evident in the initial burst of metabolic activity following glucose addition. In glucose-starved cells, hexokinase and phosphofructokinase are in an activated 'state' because of the low level of glycolytic intermediates. For the first few seconds following glucose additions these enzymes utilize ATP to make phosphorylated sugars much faster than they can be further metabolized. The duration of the burst of metabolic activity is determined by the time required for 'local control' to lower the activities of hexokinase and phosphofructokinase to the appropriate levels for steady-state operation in the presence of glucose (see Ref. 31). The [ATP]/[ADP][P_i] then rises and inhibits the overall glycolytic flux as well as respiration.

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