Phosphate Mediation of the Crabtree and Pasteur Effects

Does a change in energy metabolism enhance the potential for malignancy?

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The enhanced capacity of tumors to metabolize glucose (glycolysis) was observed in vivo by the Coris in 1925 (*1*). Four years later Henry Crabtree reported the phenomenon of glucose-induced respiratory inhibition in slices of solid sarcomas and carcinomas. He also made the observation that tumors were not the only tissues capable of enhanced aerobic glycolysis (*2*). A list of normal tissues, including blood cells, having more than the usual glycolytic capacity was presented by Ibsen in his history and discussion of the Crabtree effect (*3*). The ability of tumors to fulfill energy requirements by glycolysis enables them to grow where the oxygen supply is less than optimal, as in the peritoneal cavity. Here the cells may disperse on replicating instead of producing solid tumors having a vasculature and supporting stroma. Such growth provides for a harvest of relatively pure cancer cells, facilitating the study of metabolic patterns in malignancy. A review of these investigations has been presented by Wenner (*4*). In this article I describe the results of some experiments with tumor cells and mitochondria concerned specifically with the mechanism of the Crabtree and Pasteur effects, and suggest the implications of these results.

Observations on the Crabtree Effect

In 1936 Belitzer pointed out that competition between glycolysis and respiration for common intermediates might be the basis for the Crabtree effect (*5*). The potential competition for adenosine diphosphate (ADP) and phosphate (P_i) is shown in Fig. 1. Brin and McKee suggested that the competition was for P_i, because the amount of respiratory inhibition lessened on increasing the P_i concentration of the incubation medium (*6*). Bielka subsequently confirmed this concept by showing that the addition of sufficient P_i could even cancel the Crabtree effect in mouse leukemia cells (*7*).

Chance and Hess (*8*) raised an alternative possibility, suggesting that the limiting factor was ADP rather than P_i. This conclusion was based on experiments with an oxygen electrode by which continuous monitoring of oxygen consumption is possible. When glucose was added to a suspension of Ehrlich ascites cells, an immediate increase in respiration occurred analogous to mitochondrial state-3 oxidation (oxidative phosphorylation) which lasted about 60 seconds. This was followed by the typical inhibited phase of respiration with a concurrent decrease in the rate of glucose uptake by the cells. Thus the rapid phase of oxidation was terminated because the by-product of glucose phosphorylation, ADP, became limited (*8*). This interpretation posed a dilemma, because direct experimental observation indicated that P_i limitation was the controlling factor (*6, 7*), while analogy with isolated mitochondria suggested that ADP was the limiting factor for respiratory inhibition.

Packer and Golder observed that the addition of glucose to a suspension of Ehrlich ascites cells caused an increase in light scattering which reached a maximum at about the time respiratory inhibition occurred. These data indicated that the mitochondria were contracting (*9*), an interpretation that was given substance by the independent observations of Merker *et al.* using electron microscopy (*10*). The contraction of mitochondria apparently requires ADP (*11*), and this relationship of mitochondrial contraction to respiratory inhibition has been largely ignored by investigators probing for an explanation of the Crabtree effect.

Kun *et al.* (*12*) demonstrated the necessity for sufficient oxidizable substrate for induction of the Crabtree effect, and it was later found that succinate, oxalacetate, or pyruvate could restore respiration in substrate-depleted cells to a level that could again be inhibited by adding glucose. Some Krebs cycle intermediates (for example, citrate or isocitrate, α-ketoglutarate, malate) were virtually ineffective in restoring respiration (*13, 14*).

The Crabtree effect, then, is a manifestation of respiratory inhibition after the addition of glucose or another hexose that is capable of being phosphorylated by hexokinase. Phenomena that accompany the Crabtree effect include the contraction of mitochondria and a decrease in the rate of glucose utilization occurring at the time of respiratory inhibition. Glycolysis does not remain inhibited but increases to a steady state until all the glucose is phosphorylated (*15*).

Data Obtained with Intact Cells

By means of a Clark oxygen electrode, the consumption of approximately 2 microgram-atoms (equivalent to 1 micromole) of oxygen in cell suspensions can be monitored. For the experiments described here, Ehrlich-Lettré ascites cells derived from the mouse mammary carcinoma were used. The cells were first washed and then suspended in 54 m*M* P_i-Locke's solution (*13*). For the experiment shown in Fig. 2a, 5 m*M* oxalacetate was added to ensure the availability of oxidizable substrate for the duration of the experiment. The initial rate of respiration was 216 nanoatoms of oxygen per minute. The addition of 0.1 m*M* glucose induced an increased rate of oxidation (270 nanoatoms per minute) which lasted approximately 50 seconds. Respiration was then abruptly curtailed, with the rate now 36 percent less than the initial rate (before the addition of glucose). Four minutes later respiration had almost returned to the initial rate, indicating that the added glucose had been consumed (*6*).

The rapid phase of oxidation appears

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to involve oxidative phosphorylation, and was explained by Chance and Hess as follows. The hexokinase reaction, which utilizes the cells' complement of adenosine triphosphate (ATP) to phosphorylate the added glucose, yields ADP, the phosphate acceptor for oxidative phosphorylation, or state-3 oxidation (*16*). There can be little doubt that this is correct because as soon as glucose is added a large decrease in cellular ATP occurs simultaneously with an increase in ADP (*17, 18*).

To explain the cessation of the rapid oxidative phase, Chance and Hess suggested that the inhibition of glucose uptake by the cells would diminish the supply of ADP. The ADP that had been produced would already have been phosphorylated to ATP, so that the rate of respiration would decrease. Thus, state-3 oxidation would be curtailed for want of ADP, analogous to the results obtained in experiments with isolated mitochondria (*8, 16*). However, Ibsen *et al.* subsequently observed that respiratory inhibition began when ADP was present in excess of the capacity of both the respiratory chain or glycolysis to phosphorylate it (*17*). Hess and Chance later reported that the ratio of ATP to ADP (ATP/ADP) in glycolyzing (metabolizing glucose) cells was usually lower than the ratio in nonglycolyzing controls (*19*). Nevertheless, the concept of ADP being the limiting factor in respiratory inhibition has persisted.

Inhibition of the Crabtree Effect

Coe observed that iodoacetate (IAA), an inhibitor of glyceraldehyde 3-phosphate dehydrogenase, prevented the Crabtree effect elicited by low concentrations of glucose (*20*). In the presence of 2.8 m*M* IAA, 0.1 m*M* glucose caused a much higher rate of state-3 oxidation (500 nanoatoms per minute) (Fig. 2b) but the duration (50 seconds) of this rate was the same as the duration of state-3 oxidation in the absence of IAA. The rate then decreased abruptly, but remained slightly greater than the rate before the addition of glucose. Coe interpreted his results as indicating that IAA, by completely inhibiting glyceraldehyde 3-phosphate dehydrogenase, removed the competition for ADP by glycolysis (see Fig. 1); thus the respiratory chain could have access to all the ADP produced by hexose phosphorylation (*20*). However, an equally plausible interpretation would be that the competition for P_i would be eliminated by complete inhibition of the enzyme. The following experimental procedures should aid in resolving this dilemma.

Crabtree Effect in Isolated Mitochondria

The Crabtree effect was demonstrated in suspensions of whole cells by adding a relatively high concentration of a nonglycolyzable sugar, such as deoxyglucose (*17*). This indicated that the hexose phosphorylation reaction might be sufficient for induction of respiratory inhibition. It might therefore be possible to mimic the Crabtree effect in isolated mitochondria from tumor cells, because more than half of a cell's hexokinase activity is intimately associated with these organelles (*21*). The necessary enzymatic machinery could theoretically be isolated and studied with better control and understanding of the parameters concerned with respiratory inhibition.

The conditions under which Ehrlich ascites cells can be softened or broken are often detrimental to the production of well-functioning mitochondria. In our initial studies we used a hypotonic sucrose homogenization medium to obtain adequate mitochondrial preparations. By means of Warburg respirometry, we showed that the net effect of adding glucose to the tumor mitochondrial system was the removal of P_i with subsequent lowering of the ratio of ATP to ADP and associated depression of succinate oxidation (*22*). Hexose phosphorylation produced ADP with which the respiratory chain made ATP using the P_i of the medium. As the ATP was recycled by continued hexose phosphorylation, the P_i of the medium was depleted so that ADP accumulated. A similar observation had been made earlier by Lardy and Wellman who used liver mitochondria with added glucose and yeast hexokinase (*23*).

Changes in the respiratory rate that are characteristic of the Crabtree effect in mitochondria are shown in Fig. 3. The low rate of oxygen consumption by mitochondria with the substrate malate is typical of respiration in the presence of P_i without added ADP. The addition of ADP (0.17 m*M*) induced state-3 oxidation which ended rather abruptly in state-4 (endogenous or steady-state) oxidation, signaling completion of the phosphorylation of ADP to ATP. The addition of mannose (0.12 m*M*) after state-4 oxidation was established caused a prompt return to state-3 oxidation which was followed immediately by gradual slowing of oxygen consumption to a rate that was 24 percent less than that during state-4 oxidation. Analysis of the adenylates (*24*) at the end of the experiment showed the ratio of ATP to ADP to be 1.8 in the vessel to which mannose was added and 23 in the control vessel. The consumption of phosphate leading to this reduced ratio of ATP to ADP brings about a P_i deficiency which ap-

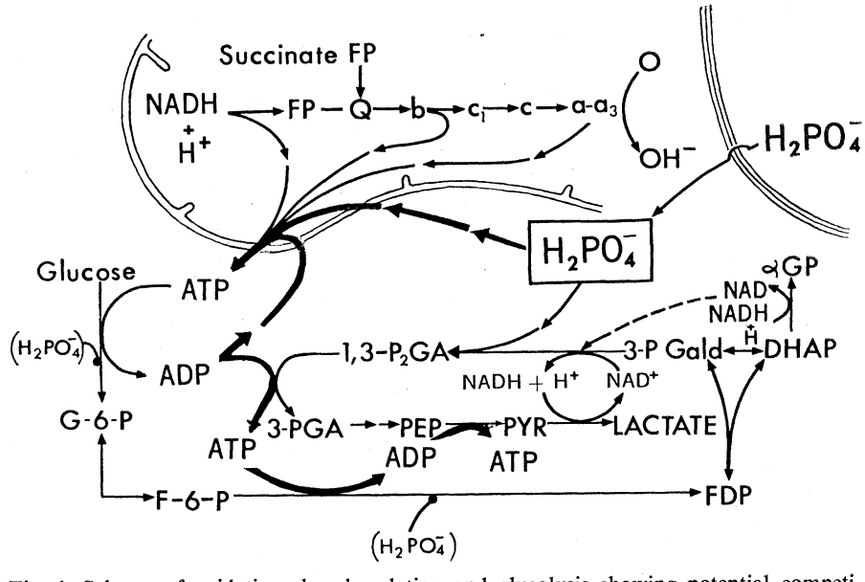

Fig. 1. Scheme of oxidative phosphorylation and glycolysis showing potential competition for phosphate and ADP. Abbreviations used are: G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; αGP, α-glycerophosphate; 3-P Gald, glyceraldehyde 3-phosphate; 1,3-P_2GA, 1,3-diphosphoglyceric acid; 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; FP, flavoprotein; Q, coenzyme Q; b, c_1, c, and a–a_3 are cytochromes.

pears similar to Chance's description of state-6 oxidation (*25*). Table 1 shows that succinate oxidation was also depressed significantly in association with a reduced ratio of ATP to ADP and, to a lesser degree, so was the oxidation of isocitrate and α-ketoglutarate. However, pyruvate oxidation was much less sensitive to the reduced ratio of ATP to ADP (*22*).

The rate of mitochondrial respiration was found to depend on both the amount of available phosphate and ATP/ADP (Fig. 4). Respiration was almost maximally depressed (23 percent) when the ratio of P_i to ADP was 0.6, which corresponds to ATP/ADP of 1.5 in this system. The lower rate of oxidation when ATP/ADP is 1.5, compared with the rate when the ratio is 9 (Fig. 4A), indicates that both a deficiency of P_i and a reduction in ATP/ADP are required for maximal inhibition of respiration. The influence of this ratio is emphasized by the fact that a much lower concentration of P_i and ADP can be added initially with the same results (Fig. 3). It must be remembered that a decrease in ATP/ADP indicates a deficit of P_i; otherwise, in the experiment illustrated in Fig. 4A the mitochondria would have reverted to state-3 (rapid) oxidation because adequate oxidative substrate was present. That succinate oxidation can be governed by ATP/ADP was indicated by the partial disengaging of the mitochondrial Crabtree effect when ATP was added (*22, 26*). In experiments similar to those in Fig. 4, but with malate as the substrate, the results were virtually identical to those with succinate (*27*).

When pyruvate was used as the substrate, the rate of oxidation increased as ATP/ADP declined (Fig. 4B). This explains the observation that higher concentrations of pyruvate added to intact cells reduced the amount of glucose-induced respiratory inhibition (*13, 14*). That is, the oxidation of pyruvate is not curtailed by glycolysis, apparently because pyruvate dehydrogenase activity is enhanced rather than depressed by a P_i deficiency and reduced ATP/ADP. This same observation was subsequently made by Linn *et al.* who used mitochondria from normal tissues (*28*). Oxalacetate oxidation in this same mitochondrial system produced a curve similar to that in Fig. 4B, suggesting that oxalacetate may readily yield pyruvate. McKee *et al.* showed, in fact, that the addition of oxalacetate to washed ascites cells resulted in an increase in

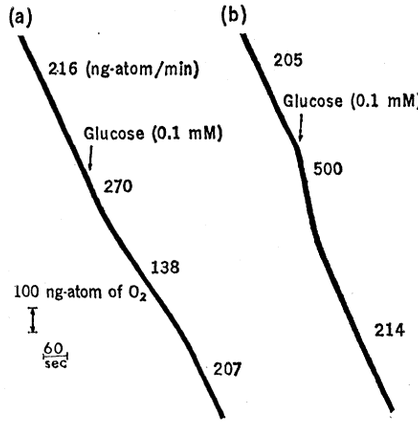

Fig. 2. The Crabtree effect in Ehrlich ascites cells (6.6 mg of cell protein) suspended in 54 m*M* phosphate-Locke's solution (*p*H 7.3); 5 m*M* oxalacetate was added to ensure availability of oxidizable substrate [(a) without and (b) with the addition of 2.8 m*M* iodoacetate]. Final volume was 6 ml.

pyruvate. Conversely, the addition of pyruvate to washed cells produced an increase in oxalacetate (*13*). From this information we may postulate that pyruvate and oxalacetate increase respiration in ascites cells by inducing Krebs cycle activity, the four oxidative steps of which are the actual sites of greater sensitivity to P_i deficiency and reduced ATP/ADP (Table 1). Although oxalacetate oxidation in the mitochondrial system was similar to that of pyruvate, oxalacetate in high concentrations did not diminish hexose-induced respiratory inhibition in whole cells (*13*). This difference can be explained by the observation that oxalacetate caused an immediate diminution in the concentration of reduced nicotinamide adenine dinucleotide (NADH) when added to the cytoplasmic fraction of ascites cells (*29*), and that when added to intact cells, oxalacetate caused an increase in the rate of glycolysis, presumably by oxidation of NADH (*30*). Thus oxalacetate is reduced in the cell to malate whose oxidation is inhibited by a P_i deficiency and low ATP/ADP (Fig. 3 and Table 1).

The Crabtree effect in mitochondria can be characterized as follows. Transition from state-4 oxidation to state-3 oxidation occurs on adding hexose, the same as postulated for whole cells by Maitra and Chance (*18*). State-3 oxidation gives way to inhibited (state 6?) oxidation as the mitochondria become deficient in P_i, and continued hexose phosphorylation reduces ATP/ADP. From these data it would appear that IAA cancels the Crabtree effect that 0.1 m*M* glucose may induce (Fig. 2) by reducing the competition for phosphate rather than ADP.

Maitra and Chance, however, proposed that state-3 oxidation in whole cells was terminated by lack of ADP rather than P_i; thus oxidation returns to state 4 instead of progressing to the P_i-deficient conditon (state 6?). They did not explain why the rate of state-4 oxidation after glucose addition should be so much less than the initial rate of state 4 (*18*). Experience with mitochondria from different tissues has shown that after multiple additions of ADP (in the presence of excess P_i) the respective rates of state-4 oxidation are very similar (*31*). It is therefore difficult to account for the significant decrease in the rate of oxidation below the initial rate of state 4 on the basis of an ADP deficiency.

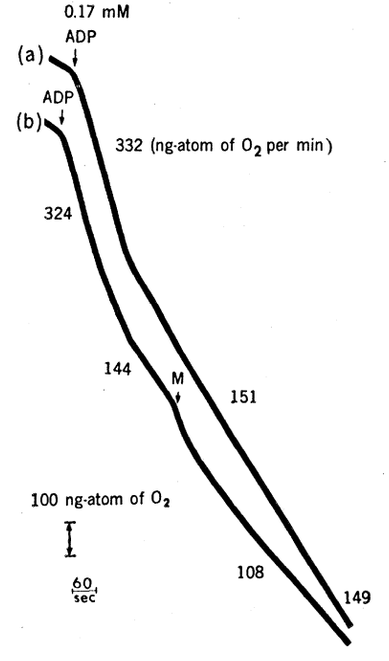

Fig. 3. The Crabtree effect in mitochondria. Traces were obtained simultaneously by means of Clark oxygen electrodes. Both vessels [(a) control and (b) experimental] contained 4 mg of mitochondrial protein and 5 m*M* malate. The numbers indicate slopes in nanoatoms of oxygen consumed per minute; 0.17 m*M* ADP was added to both vessels; 0.12 m*M* mannose (*M*) was added to (b) as indicated. The reaction medium contained 1.3 m*M* P_i, 34 m*M* tris (*p*H 7.2 at 37°C), 7 m*M* $MgCl_2$, 23 m*M* KCl, 60 m*M* sucrose, 0.6 m*M* ethylenediaminetetraacetic acid. Bovine serum albumin was added to the mitochondial preparation [1 percent; see (*22*)]; final concentration was 0.07 percent. Final volume was 6 ml.

Ratios of ATP to ADP and Intact Cell Respiration

Recognizing that the oxidation of succinate and malate by mitochondria isolated from tumor cells can be depressed (when deficient in P_i) by as much as 20 to 30 percent if ATP/ADP is reduced to 1.6 (Table 1), can we invoke this same mechanism for the Crabtree effect in the intact cell? Experiments have shown that when deoxyglucose or glucose plus IAA are added to suspensions of whole cells, ATP/ADP may be less than 1 (*32*). The results shown in Table 2 indicate that respiratory inhibition averaged about 58 percent with deoxyglucose and was associated with a marked depression of the cellular ATP/ADP. Thus experiments with isolated tumor mitochondria, which are probably not so well coupled as those in situ, provide some information about the mechanism of respiratory inhibition when hexose is merely phosphorylated.

The addition of 5 m*M* glucose to whole ascites cells caused only a slight reduction of ATP/ADP compared with the control cells (*17–19*) (Table 2). In agreement with this, about half as much respiratory inhibition occurred as with the addition of 5 m*M* deoxyglucose. Although an apparent correlation exists between the degree of respiratory inhibition and the amount of reduction in the ratio of ATP to ADP in whole cells, the inhibition of respiration on adding glucose is perhaps greater than should be expected for the modest decrease in ATP/ADP (Table 2). To reconcile this finding with the mechanism indicated by the Crabtree effect in mitochondria, we may suggest that the ratio of ATP to ADP, as determined for the whole cell, may not be a reflection of the ratio in the mitochondria. The presence of hexokinase on the mitochondria (*21*) helps reduce the ATP concentration locally, and ADP would accumulate if the respiratory chain is deficient in P_i.

That ADP is present in the mitochondria during glucose-induced respiratory inhibition, indicating a local P_i deficiency, is suggested by the finding that such inhibition begins when the ADP concentration is high (*17, 18*) and continues even during momentary increases (oscillations) of ADP to relatively high levels (*15*). Also, the cellular concentration of P_i decreases rapidly on adding hexose, whether or not glycolysis is occurring (*18, 33, 34*). Further evidence for the presence of ADP in the mitochondria is given in the following section.

Correlation of Mitochondrial Structure with Function

Experiments with intact Ehrlich ascites cells showed that increased light-scattering occurred when glucose was added, indicating that the mitochondria were contracting (*9*). Merker *et al.* demonstrated by electron microscopy that the mitochondria of ascites cells remained contracted as long as glucose was present. When the cells were washed to remove the excess glucose the mitochondria returned to the pre-glucose (usual) structure, and the respiratory rate increased 25 percent (*10*). These results were confirmed recently by Hackenbrock *et al.* who correlated increased light-scattering with mitochondrial contraction as demonstrated by conventional electron microscopy (*35*).

The morphologic appearances of mitochondria have been well correlated with some of the respiratory states and the presence of P_i, ATP, and ADP (*36, 37*). The contracted or highly condensed morphology is induced and maintained only by ADP (*11*), and has been observed in association with state-3 oxidation (*37*). State-4 oxidation (deficient in ADP) is observed with the usual mitochondrial configuration (*37*). Thus the suggestion that glucose-induced respiratory inhibition represents state-4 oxidation in mitochondria is difficult to reconcile with the fact that the mitochondria are contracted rather than in the usual state. Since the inhibited rate of respiration is less than the rate during state-4 oxidation and is very much less than the state-3 rate, we may conclude on the basis of the foregoing discussion that these slowly respiring, contracted mitochondria are in a phosphate-deficient condition.

Packer and Golder found that, along with mitochondrial contraction, more of cytochrome b was in the reduced state after the addition of glucose to ascites cells. Whether glucose, mannose, fructose, deoxyglucose, or glucose plus IAA was added, these same changes occurred (*9*). Chance's description of the P_i-deficient state of mitochondria (state 6) also included the observation that more cytochrome b was reduced while the other components of the respiratory chain were relatively more oxidized. This rate of state-6 oxidation, about one-fourth that of state 4, could be brought back to the state-4 rate by adding P_i (*25*).

It would appear that at no time after the addition of glucose to ascites cells can we say the mitochondria are lacking ADP until the added glucose has been

Table 1. Effect of the ATP/ADP ratio on the oxidation of various substrates in mitochondria. Average results of six experiments (see Fig. 3 for conditions) are presented for each substrate (ranges in parentheses). Mitochondrial protein ranged from 3.4 to 4.2 mg (1.7 to 2.1 mg for experiments with succinate). Final volume was 6 ml.

Substrate	Control		Experimental		Respiratory inhibition (%)	RCR*	ADP/O
	Total ATP (μmole)	ATP/ADP (state 4)	Total ATP (μmole)	ATP/ADP (state 6?)			
Succinate	0.88 (0.67–1.05)	15 (11–19)	0.55 (0.47–0.61)	1.6 (1.4–1.8)	21 (18–23)	2.5	1.2
Malate	0.91 (0.69–1.02)	15 (12–23)	0.55 (0.47–0.64)	1.6 (1.2–1.8)	27 (24–31)	2.2	1.7
α-Ketoglutarate	0.92 (0.49–1.11)	16 (10–22)	0.67 (0.57–0.74)	2.0 (1.5–2.4)	13 (7–24)	2.6	1.6
Isocitrate	0.80 (0.55–0.92)	11 (9–13)	0.54 (0.46–0.58)	1.6 (1.4–2.0)	16 (12–24)	2.2	1.6
Pyruvate	0.88 (0.59–1.07)	15 (8–22)	0.57 (0.47–0.64)	1.6 (1.2–2.3)	3 (0–10)	3.1	2.1

* Respiratory control ratio (RCR) is the ratio of state 3 to state 4.

utilized and the rate of respiration increases to the endogenous (state 4) rate. A local deficiency of P_i in the mitochondria is therefore indicated by a decreased respiratory rate in the manifested presence of ADP.

Phosphate Transport through the Cell Wall

If it is true that hexose-induced respiratory inhibition is caused by a deficiency of P_i in the mitochondria, there remains the question of how this deficiency is maintained in the presence of either active or inactive glyceraldehyde 3-phosphate dehydrogenase, a phosphate consumer. Ibsen *et al.* demonstrated that a low P_i concentration (1 m*M*) in the incubation medium increased the duration and degree of glucose-induced respiratory inhibition of ascites cells relative to a 5 m*M* concentration of P_i. On increasing the concentration of P_i to 10 m*M* the duration and degree of inhibition was greatly decreased; but addition of P_i above 10 m*M* made little subsequent difference in the duration or degree of the Crabtree effect (*38*). In addition to demonstrating phosphate control of both glycolysis and respiration, these experiments suggest that the cell wall limits transport of P_i from the incubation medium into the cell. In contrast, Bielka found that a high concentration of P_i in the medium abolished the Crabtree effect in leukemia cells (*7*). Thus, it is evident that tumor cells vary in their permeability to P_i. The Crabtree effect, then, can be appreciated as a phenomenon resulting from restricted phosphate entry into the cell.

The Rate of Phosphate Transport

Ibsen *et al.* observed that a low concentration of deoxyglucose did not induce respiratory inhibition while the same low concentration of glucose did (*39*). The results shown in Table 3 indicate that the concentration of deoxyglucose in the cell must be sufficient if phosphorylation is to proceed at a rate at which ADP will be produced faster than the rate at which P_i can enter the cell. In this way the ratio of ATP to ADP can be reduced with consequent respiratory inhibition.

A low concentration of glucose (0.1 m*M*) also did not induce respiratory inhibition when the activity of glyceraldehyde 3-phosphate dehydrogenase was suppressed by IAA (Fig. 2b). However, a concentration of 0.1 m*M* glucose was sufficient to cause a Crabtree effect (Fig. 2a); therefore it appears that the rate of phosphate transport cannot satisfy simultaneously the appetites of both glycolysis and respiration. Evidently, 0.1 m*M* glucose can induce the activity of glyceraldehyde 3-phosphate dehydrogenase to a sufficient extent to enable it to compete favorably with the mitochondria for phosphate.

The Form of Phosphate

When glucose is added to a suspension of ascites tumor cells there is a significant but variable drop in the P_i concentration of the cells (*17, 18*). However, the amount of P_i remaining in the cells is sometimes greater (*17*) than would be expected if phosphate exerts a controlling influence. Levinson has shown that HPO_4^{2-} ions are passively distributed between the medium and the cell interior and are therefore unlikely to influence metabolic control. He also found that the ascites tumor cell maintains a concentration of $H_2PO_4^-$ that is greater than would be predicted by passive diffusion (*40*). However, at a physiologic *p*H (7.4) the ratio of HPO_4^{2-} to $H_2PO_4^-$ is about 5. This indicates that the amount of available $H_2PO_4^-$ would be relatively limited in spite of the apparently enhanced capacity of the ascites cell to take in or produce $H_2PO_4^-$. Because the usual phosphate analyses do not distinguish between these ions, those instances in which the total phosphate does not drop so dramatically may indicate that the form primarily utilized by glyceraldehyde 3-phosphate dehydrogenase and the respiratory chain is $H_2PO_4^-$. Recent evidence has indicated that this is the case for oxidative phosphorylation (*41*).

When all the data are considered, hexose-induced inhibition of respiration in tumor cells can best be explained as the result of phosphate deficiency or control in the mitochondria, this allowing ADP to accumulate from the ATP consumed by local hexokinase activity. In terms of the mechanics of the process, limitation of phosphate only partially reduces the rate of electron transport, further inhibition being effected by subsequent reduction of the ratio of ATP to ADP in the mitochondria (Fig. 4A). Phosphate limitation in the mitochondria is brought about by the activity of glyceraldehyde 3-phosphate dehydrogenase or by an inadequate rate of phosphate entry into the cell.

Instances in which ascites cells or

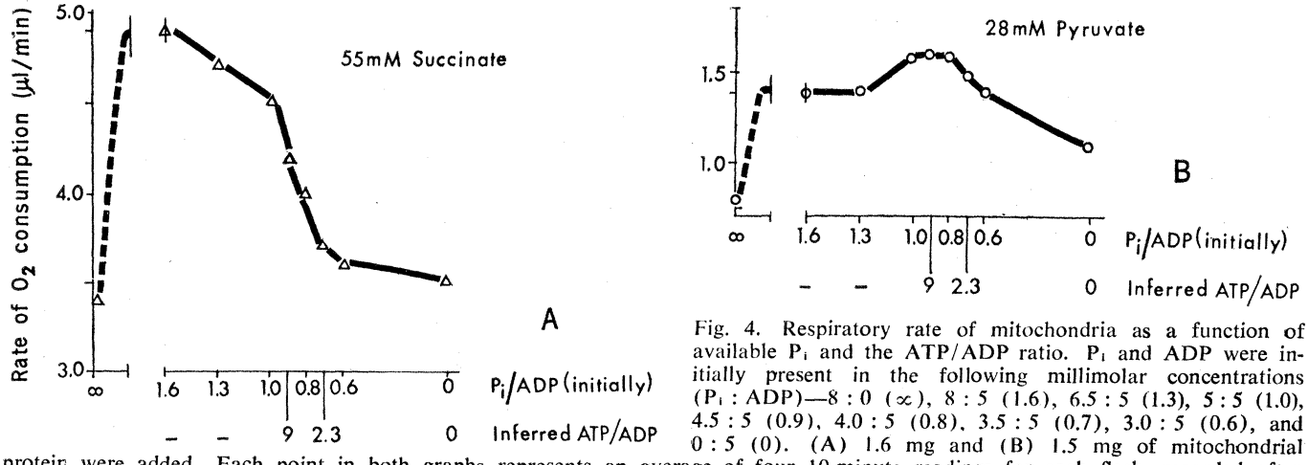

Fig. 4. Respiratory rate of mitochondria as a function of available P_i and the ATP/ADP ratio. P_i and ADP were initially present in the following millimolar concentrations (P_i : ADP)—8 : 0 (∞), 8 : 5 (1.6), 6.5 : 5 (1.3), 5 : 5 (1.0), 4.5 : 5 (0.9), 4.0 : 5 (0.8), 3.5 : 5 (0.7), 3.0 : 5 (0.6), and 0 : 5 (0). (A) 1.6 mg and (B) 1.5 mg of mitochondrial protein were added. Each point in both graphs represents an average of four 10-minute readings for each flask, recorded after state-3 oxidation had subsided. Thus the abscissas can also be expressed as the inferred ratio of ATP to ADP. [From Koobs and McKee (*22*); courtesy of Academic Press]

other malignant tumors do not demonstrate the Crabtree effect [see (*42*)] should be reevaluated with respect to the presence of sufficient oxidizable substrate, or permeability of the cells to P_i. Also, permeability to phosphate has been observed to vary from time to time in the same cell line (*38*).

Phosphate Control of Glycolysis

Chance and Hess observed that glucose utilization is decreased at about the same time respiration is inhibited (*8*). This is also the point in time at which the rate of ADP formation is greater than the combined rates of glycolytic and respiratory phosphorylation of ADP (*17*). Evidence presented here indicates that respiratory inhibition is the result of a P_i deficiency which allows for a reduction in ATP/ADP in the mitochondria, and that the cell wall limits the rate of phosphate transport (*38*) (Table 3). The simplest explanation for the reduction in the rate of glucose utilization would appear to be the same as that for respiratory inhibition.

Several investigators have shown that aerobic as well as anaerobic glycolysis in ascites cells is primarily regulated by the concentration of P_i (*33, 38, 43, 44*). An increase in the concentration of P_i in the incubation medium increased the rate of aerobic glycolysis as measured by glucose disappearance and lactate production (*33*).

As an alternative to P_i control, Maitra and Chance suggested that ADP might be the controlling factor in aerobic glycolysis, an idea based largely on the negative observation that triose phosphate did not accumulate as much as would be expected if P_i were the limiting factor (*18*). However, limitation of phosphate would also allow for the inhibition of hexokinase and phosphofructokinase by their respective products. Thus, the rate of glycolysis would be decreased at these two earlier steps, preventing excessive accumulation of the triose phosphates (*4, 33, 45*). Conversely, phosphate stimulates hexokinase and phosphofructokinase activity even in the presence of inhibiting amounts of their products or ATP (*33*). This lends further credence to the idea that phosphate controls or limits glycolysis.

Glyceraldehyde 3-phosphate dehydrogenase may also be inhibited by its product, 1,3-diphosphoglycerate (1,3-P_2GA), if the ADP concentration is not sufficient for phosphoglyceric acid kinase activity to remove it (see Fig. 1). Because the concentration of 1,3-P_2GA was too low for detection (*18*), the presence of inhibitory amounts could not be determined. Since NAD has not been found to be limiting (*15, 19*), control exerted by P_i would be favored as the reason for decreased activity of glyceraldehyde 3-phosphate dehydrogenase under the circumstances of the Crabtree effect.

Ibsen and Schiller have observed asynchronous fluctuations in the concentrations of ADP and ATP after adding glucose. The ADP maxima, which were concurrent with ATP minima, occurred without increase in the respiratory rate, emphasizing the P_i-deficient state of the mitochondria (*15*). Thus, the accumulation of ADP indicates operation of the first half of glycolysis consuming ATP; the subsequent accumulation of ATP indicates that the last half of glycolysis is operating. This agrees with Lynen's suggestion that ADP control of glycolysis is unlikely as long as ATP is available for hexose phosphorylation (*46*). It has been proposed that the sequestering of ADP, perhaps by mitochondria, might control glycolysis (*18*). However, the asynchronous fluctuation of the ATP and ADP concentrations during inhibited respiration is a good indication that neither ADP nor ATP is sequestered by the mitochondria during glycolysis (*4*).

Maitra and Chance pointed out that the data are more discrepant with respect to ADP control than P_i control because of the greater change in concentration of cellular P_i in contrast to minimal changes in the concentration of ADP (*18*). This lesser discrepancy with respect to phosphate control may be partly explained by the apparent preference of the respiratory chain for $H_2PO_4^-$. That the addition of such a low concentration of glucose (0.1 m*M*) can cause respiratory inhibition (Fig. 2) suggests that glyceraldehyde 3-phosphate dehydrogenase also may preferentially utilize $H_2PO_4^-$. Thus a relatively small change in the total phosphate concentration on adding glucose could be significant if $H_2PO_4^-$ is the preferred form of phosphate (*4, 46*).

Table 2. The relationship of the Crabtree effect to the ratio of ATP to ADP in whole cells. Cell protein (18 to 21 mg) was added to flasks containing 30 m*M* tris-Locke's solution (*p*H 7.3) at 37°C. The total volume was 6 ml plus 0.02 ml (with the addition of glucose or 2-deoxyglucose). Averages are shown in parentheses. The duration of each experiment was less than 2 minutes; the reaction was stopped with perchloric acid approximately 1 minute after the addition of hexose.

No. of experiment	Total ATP (μmole)	ATP/ADP	Crabtree effect (%)
	*Deoxyglucose (5 m*M)		
1	0.12	0.7	65
2	0.08	0.5	55
3	0.10	0.5	61
4	0.31	1.2	55
5	0.13	0.7	57
		(0.7)	(58.6)
	Control (no hexose added)		
1	0.53	7.6	
2	0.48	8.0	
3	0.42	6.0	
4	0.77	12.8	
5	0.64	9.1	
		(8.7)	
	*Glucose (5 m*M)		
1	0.49	7.0	28
2	0.45	7.5	21
3	0.39	3.9	29
4	0.72	10.3	20
5	0.59	6.6	31
		(7.0)	(25.8)

Pasteur Effect and Phosphate Control

Anaerobic glycolysis in ascites tumor cells may be four times faster than aerobic glycolysis (*33*). This difference (the Pasteur effect) can be decreased by increasing the concentration of P_i in the incubation medium (*44*). That is, glycolysis under aerobic conditions can be stimulated by increased phosphate, emphasizing the concept of competition for P_i between mitochondria and glyceraldehyde 3-phosphate dehydrogenase. Phosphate has also been considered as the controlling factor for the Pasteur effect in yeast (*46*). Thus, the Crabtree and Pasteur effects might be brought about by the same mechanism. The cell wall evidently does not allow sufficient influx of phosphate to satisfy simultaneously the potential requirements of glycolysis and oxidative phosphorylation. In the case of the Pasteur effect, mitochondria win the competition for P_i when oxygen is admitted, whereas the Crabtree effect demonstrates the presence of more than the usual amount of glycolytic enzyme protein.

Implications

The capacity of cancer cells to generate anaerobically the ATP necessary for growth leads us to inquire whether this metabolic program is intrinsic to

the genome or acquired. Huebner and Todaro have implicated C-type viral particles as a possible source of an "oncogene" which is thought to provide the information necessary for carcinogenesis (*47*). Whatever the mechanism of malignant transformation, the cancer cell shows a number of normal features. For example, a circumstance under which anaerobic energy production would be required is the development of the fertilized ovum or zygote, which, without a blood supply, immediately begins to grow and divide. This early product of conception continues division during its 1-week passage down the uterine tube and into the uterine cavity, and associates ultimately with the maternal blood supply sometime after implantation in the endometrium (invasion by trophoblast). Thus, some criteria of malignancy (growth, metastasis, and invasion) are fulfilled by the products of conception. The similarity of the trophoblast to cancer in appearance and activity was commented on many years ago by the embryologist J. Beard (*48*).

The explantation of normal body cells into culture media brings into operation glycolysis as the major mode of energy production, with loss of differentiated function and the onset of growth (*49*). Thus, cells in culture will demonstrate the Crabtree effect, indicating that a change in metabolic emphasis has occurred. The increase in amount of glycolytic enzyme proteins has been associated with an increase in ploidy (*50*), a commonly observed change in malignant cells. These similarities between cultured, cancer, and embryonic cells suggest that a significant part of the program of cancer is intrinsic to every cell of the body by virtue of the genetic endowment providing for procreation. Thus, anaerobic glycolysis is important for coping with the anatomy of conception. Ironically, the return to this primitive metabolic format may extinguish a life.

There remains the question of why growth accompanies this shift to glycolysis for energy production. With activation of the genome calling forth greater synthesis of the enzymes of glycolysis, perhaps there is an incumbent activation of a genome concerned with mitosis. A possible example of such an effect is hyperplasia of the prostate, the incidence of which increases with age. Thrombosis and marked sclerosis of prostatic blood vessels, commonly observed at necropsy in older persons, tend to reduce the blood supply. The consequent reduction in oxygen tension could conceivably turn on (derepress) the genomes concerned with anaerobic energy production and growth, producing an in vivo tissue culture, as it were. The incidence of cancer of the prostate also increases with age, and that there might be a relation between this and prostatic hyperplasia has been suggested (*51*). In this regard, hyperplastic liver nodules, while not malignant in character, have been implicated as a possible intermediate state in the development of chemically induced hepatomas (*52*). Perhaps carcinogens, including viruses, uproot the control mechanisms normally associated with the program of conception.

Because cancer cell metabolism and growth characterstics are so similar to the process of conception, it appears that the immunologic manipulation which allows the maternal host first to tolerate—then reject—the physiologic "tumor" is indeed a good model for investigating cancer therapy (*53*).

Table 3. Effect of varying concentrations of deoxyglucose on respiration in whole cells. See Fig. 2 legend for experimental conditions.

Cell protein (mg)	2-Deoxyglucose (m*M*)	Respiratory inhibition (%)
7.6	0.1	0
7.6	0.2	15
5.6	5.0	56

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