Some Factors Influencing Respiration and Glycolysis in Ehrlich Ascites Tumor Cells*

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SUMMARY

The paper presents a study of various environmental factors which influence respiration, glycolysis, and the Crabtree Effect in the Ehrlich ascites carcinoma cells. The factors examined for influence on respiration include: (1) pH in the presence and absence of glucose and (2) varying concentrations of inorganic phosphate in: (*a*) media used to wash the cells, (*b*) incubation media, with no supplementary substrate, with and without Tris buffer, (*c*) incubation medium containing 200 mg. per cent of DL-lactate, (*d*) incubation media containing 55 mg. per cent of glucose and 37 mM Tris, (*e*) incubation medium containing only 0.5 mg. per cent glucose, (*f*) incubation medium containing 110 mg. per cent of 2-deoxyglucose, and (*g*) incubation media containing 110 mg. per cent of glucose, 37 mM Tris and 7×10^{-5} M 2,4-dinitrophenol.

The most significant observations concern the changes in glycolysis, respiration, and the Crabtree Effect when inorganic phosphate concentration was lowered from 15 to 5 mM. Above 15 mM all were independent of phosphate, and below 5 mM all were dependent on phosphate concentration.

A suspension of Ehrlich ascites cells consumes oxygen at an appreciable rate without addition of exogenous substrate. Addition of certain hexoses or hexose analogs to such a respiring suspension will cause a transitory depression of respiration, a phenomenon often termed the Crabtree Effect. Contemporary workers have devoted much effort to studies of this effect, because elucidation of the mechanism involved could help explain how neoplastic cells regulate the various metabolic pathways contained within them. These studies have led to the proposal of several different mechanisms, including those involving a direct competition for an intermediate such as adenosinediphosphate (ADP) (6, 10, 16) inorganic phosphate (Pi), (1, 5, 12, 22), or diphosphopyridine nucleotide (DPN) (2), or a pH effect (4, 7, 20).

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To resolve some of the conflicting interpretations and data, more detailed information about environmental factors which may influence the respiratory and glycolytic pathways is needed. In this paper we attempt to examine some of these factors in detail. The results suggest that the mechanism may actually depend on the cells' environment and that more than one mechanism may operate to produce the Crabtree Effect.

MATERIALS AND METHODS

Tumor preparations.—Ehrlich ascites carcinoma cells were grown in C57BL mice bred in this laboratory. One-tenth ml. of tumor suspension ($1.0–1.3 \times 10^7$ cells) was inoculated intraperitoneally. The tumor was allowed to grow for 7 or 8 days, unless otherwise specified, and was withdrawn from the peritoneal cavities of the mice with a bulb pipette. In all the experiments except those involving different inorganic phosphate (Pi) concentrations the cells were washed twice in 55 mM phosphate Locke solution, pH 7.40, by the differential centrifugation technic of McKee *et al.* (14). When other Pi levels were used, the cells were washed in an isotonic solution containing all the components of Locke solution except Pi, and were buffered at pH 7.4 with 2 mM Tris. The washed

cells were added to isotonic Pi solutions to give the reported concentrations. Cell volumes were measured as previously described (14). In the experiments by the Warburg technic the cell volume was about 4.4 per cent, in the glycolytic systems it was about 2.2 per cent, and in the oxygen electrode studies about 1 per cent.

Oxygen consumption measurement.—Conventional Warburg manometry was used, with 7-ml. Warburg vessels containing 0.1 ml. 15 per cent KOH and a 15 × 15 mm. accordioned strip of filter paper in the center well, 0.8 ml. of cell suspension in the main chamber, and 0.1 ml. of substrate solution in the side arm. The incubation temperature was maintained at 37.9°–38.0° C. The substrate was tipped in after 15 minutes of endogenous respiration.

Oxygen electrode technic.—In these studies a Beckman model OM2 oxygen electrode was used in conjunction with a Brush DC amplifier and a Brush oscillograph. A suspension containing about 1 per cent (by volume) tumor cells was incubated in a 100-ml. beaker, which was held in a larger water bath by a wooden yoke. The temperature of this bath was maintained at 37.9°–38.0° C. by water circulating through it from a larger constant temperature bath. The cell suspension was stirred at a constant rate with a magnetic stirrer.

The incubation system was allowed to come into a steady state with the atmosphere, so that diffusion of oxygen into solution balanced removal from solution. Then differences in respiratory rate were detected by changes of oxygen tension from the original steady state to a new steady state, a shift toward increasing oxygen tension indicating a decreased respiratory rate. Although this method does not give a reliable estimate of the magnitude of change in rate, it is sensitive to very small changes and reveals whether there is a stimulation or depression of respiration. The response time appears to be almost instantaneous, since changes were seen within 1 second after addition of cells or solutions to the incubation system.

Chemical determinations and purifications.—Glucose was analyzed by a slight modification of the Somogyi method (19) with Nelson's color reagent (15) in barium hydroxide-zinc sulfate filtrates of tumor suspensions.

Lactate was determined in either 5 per cent trichloroacetic acid or barium hydroxide-zinc sulfate filtrates by the method of Barker and Summerson (3).

2-Deoxy-D-glucose was purified by cellulose chromatography.[1] A column 1 foot by ¾ inch was tightly packed with cellulose and wetted with water-saturated N-butanol. The 2-deoxy-D-glucose was placed on the column and eluted with the same solvent. Two-ml. aliquots were collected, and 0.25 ml. was removed and tested by the anthrone method (8), which can be used to distinguish between glucose and 2-deoxy-D-glucose, since the glucose absorption maximum is at 620 mμ and the 2-deoxy-D-glucose maximum at 520 mμ. Purity of the 2-deoxy-D-glucose was checked with paper chromatography (21). A 2 per cent contamination of the 2-deoxy-D-glucose with glucose was easily detectable. Since no glucose was demonstrable in the purified product, we concluded it was at least 98 per cent pure.

Tris(hydroxymethyl)aminomethane buffer (Tris) was recrystallized from hot methanol.

Chemicals.—All chemicals used were C.P. quality, unless otherwise stated. 2,4-Dinitrophenol (DNP) was a Matheson product. The Tris was Sigma 7–9 buffer. The 2-deoxy-D-glucose initially employed was obtained from Dr. Arne Wick and required no additional purification; later, 2-deoxy-D-glucose obtained from the Nutritional Biochemical Company was used and was purified as described above. Water redistilled in pyrex glass was used for making the reagent solutions.

RESULTS

The Effect of pH on Respiration in the Presence of Glucose

In Chart 1, the endogenous oxygen consumption in 55 mM phosphate buffer during 1 and 2 hours is plotted against the pH of the medium. The maximum consumption, at around pH 7.5, was taken as 100 per cent. At pH values above 7.6, the relative amount of oxygen utilization tended to be less at 2 hours than at 1 hour, suggesting gradual damage to cells exposed to alkaline conditions. We frequently observed clumping of cells incubated for long periods, and this inclination to clump became more pronounced as the pH and duration of incubation were increased.

Chart 2 presents an analogous study of cells incubated for an hour in the presence of glucose (initially 111 mg. per cent) and 55 mM phosphate. Since the pH changed during this period, owing to the accumulation of lactic acid, the mean pH over the interval was used. The top curve is given as per cent of the maximum oxygen consumption and may be compared with the lower curve in Chart 1. The shapes are noticeably different. The difference is reflected in the lower curve in Chart 2, which represents the relative Crabtree Effect over the hour interval. Since the respiration decreased more without glucose than with glucose as pH was lowered, the relative Crabtree Effect was less at lower

[1] Method suggested by Mr. Richard Guillory.

pH values. Scholefield (18) has also compared the changes in respiration with pH, and his results would indicate an even wider divergence between the endogenous and glucose respiration curves, perhaps owing to the greater pH change under his conditions.

Respiratory Changes with Varying Inorganic Phosphate Concentration

From the preceding section, it is obvious that both endogenous and glucose-inhibited respiration are rather sensitive to variations in pH. When Pi concentration is lowered, another buffering system must be used to avoid confusion resulting from large shifts in pH. We have used Tris buffer for this purpose, since the 37–55-mM level of Tris used

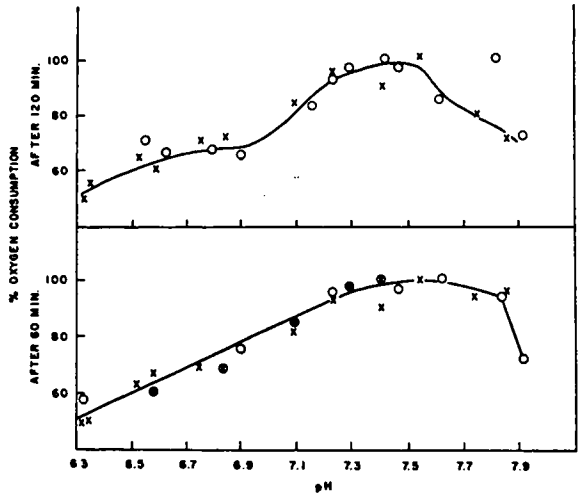

Chart 1.—The effect of pH on endogenous respiration. The values from three experiments, using 4.4 volumes per cent cells, are expressed as the per cent of maximum respiration. The lower part of the figure is for 60 minutes of respiration, whereas the upper part is for 120 minutes.

in the experiments presented did not greatly alter either respiration or glycolysis.

Phosphate and Tris are nearly equivalent in buffer capacity between pH 7.4 and 7.2, but the capacity of Tris decreases much more rapidly below 7.2. To have adequate buffer capacity in a Tris system, one must maintain the pH above 6.8. The initial pH in all experiments described below was 7.40 ± .06.

a) Respiration and glycolysis of cells washed in 0 and in 55 mM phosphate Locke solution.—In the investigations of the influence of Pi concentration described in the following sections, cells were washed in an isotonic phosphate-free solution (phosphate Locke with the phosphate replaced by an iso-osmolar amount of NaCl) and then were added to a solution which gave the reported final Pi and Tris concentrations. Since the cells are extremely permeable to Pi (10), it is possible that data obtained with cells washed in Pi solutions might not be strictly comparable to those obtained with cells washed in solutions lacking Pi. To test this possibility we have used two extreme cases,

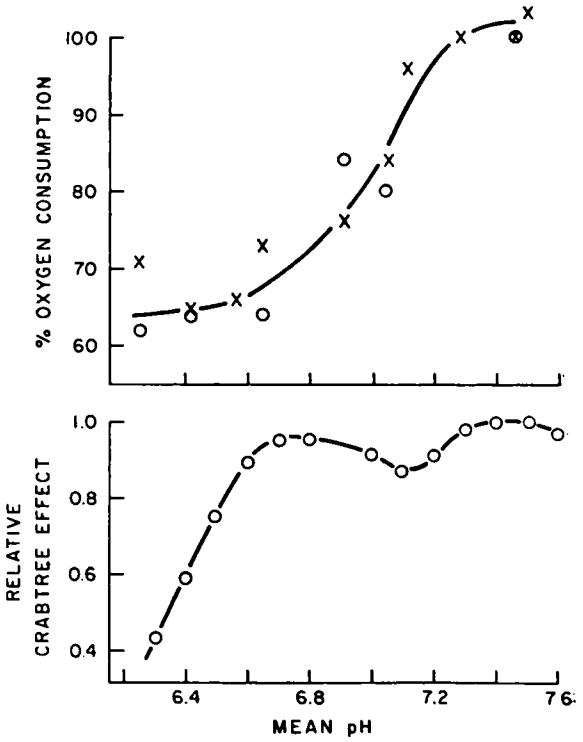

Chart 2.—The effect of pH on respiration in the presence of glucose and on the Crabtree Effect. The values from two 60-minute experiments with 4.4 vol. per cent cells in 111 mg. per cent glucose are expressed as the per cent of the maximum respiration in the upper half of the chart.

The lower curve was calculated from the curve in the upper half and from the 60-minute endogenous curve in Chart 1 by employing the observation that the maximal Crabtree Effect was 30 per cent around pH 7.4. Values taken from the curve of respiration in glucose were multiplied by 0.7 to bring them into proper relationship with the values from the endogenous curve, and then these decreased values were subtracted from the endogenous ones. The differences (30 at pH 7.4) were divided by 30 to give the Relative Crabtree Effect, or the fraction of the maximum.

one using cells washed in a Pi-free medium and the other using cells washed in a relatively high Pi concentration (55 mM). The results are presented in Table 1, where the endogenous respiration in two different final Pi concentrations is shown. It may be seen that, at the lower concentration (12 mM), the cells washed in the high Pi medium showed a higher rate of oxygen consumption. At the higher Pi concentration (42 mM) the difference

was less pronounced. However, even at a high concentration (55 mM), there was a significant difference in glycolysis: .04 ml. of cells at 38° C. used 800 μg. glucose and produced 728 μg. lactic acid per hour if washed in a phosphate-free medium, but they used 1,100 μg. glucose and produced 850 μg. lactic acid per hour if washed in 55 mM Pi. One may conclude that the wash solution used had a distinct influence on the cell's metabolism.

TABLE 1

THE EFFECT OF PHOSPHATE IN WASHING PROCEDURE ON RESPIRATION IN THE EHRLICH ASCITES CARCINOMA CELLS

Time (min.)	Final phosphate conc. (mM/liter*)	O_2 used per 0.04 ml. cells (cu. mm.)	
		Washed in 0 mM Pi	Washed in 55 mM Pi
0–60	12	54.1	62.1
0–60	42	66.8	66.0
0–120	12	91.2	106.0
0–120	42	109.6	115.3

* No Tris buffer was used.

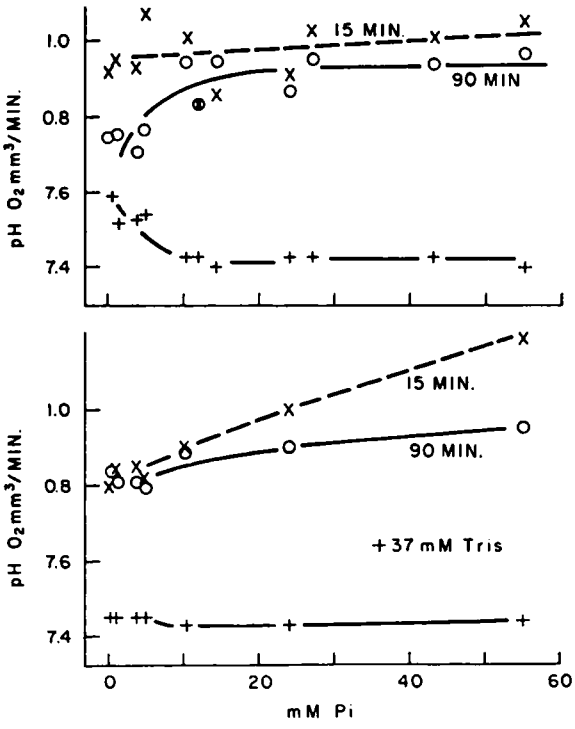

CHART 3.—The effect of phosphate and Tris buffer on endogenous respiration and pH. The system used for obtaining the lower set of curves contained 37 mM Tris and varying amounts of Pi. Only Pi was used for the system employed for obtaining the upper set of curves. The oxygen values are for 0.04 ml of cells

b) Phosphate concentration and endogenous respiration.—In Chart 3, the average rate of oxygen consumption of washed unsupplemented cells as well as the final (90 min.) pH of the medium are plotted vs. the final Pi concentration (mM). The upper set of curves represent Pi and cells with no additional buffer. The 90-min. average rate distinctly decreased below 10 mM Pi, and the decrease seems to correspond to the increase in pH. The fact that pH did rise in the weakly buffered range might be partly attributed to accumulation of ammonia from protein catabolism (17). The lower set of curves in Chart 3 compare results obtained in 37 mM Tris. The pH was maintained much better below 10 mM Pi, and the 90-min. average was correspondingly more constant; but the picture is somewhat confused by the 15-min. curve, which was quite elevated at higher Pi levels. The difference between the 15-min. and 90-min. averages suggests an initial stimulation and then a

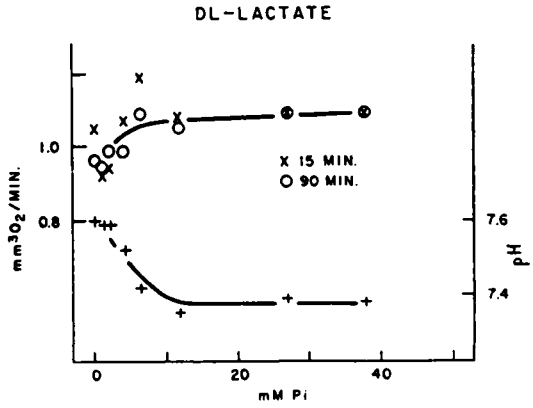

CHART 4.—The effect of phosphate and lactate on respiration and pH. The system contained 220 mg. per cent DL-lactate and varying amounts of Pi, without Tris. The oxygen values are for 0.04 ml. of cells (upper curve). The pH values are represented by the lower curve.

progressive inhibition by Tris in the presence of high Pi levels. In experiments employing concentrations of Tris up to 110 mM and Pi levels greater than 35 mM, we were able to demonstrate a more distinct inhibition of respiration (unpublished data).

c) Phosphate concentration and respiration in 220 mg. per cent DL lactic acid.—The curves of mean oxygen consumption and final pH of cells incubated in lactate presented in Chart 4 resemble those for endogenous respiration in the top part of Chart 3, with the exception that the 90-min. average was higher and experimentally indistinguishable from the 15-min. average when lactate was present, which indicates that supplementation with lactate prevented the decline in respiration.

d) Phosphate concentration and respiration in 55 mg. per cent glucose and 37 mM Tris.—Since respiration is depressed by glucose only as long as glucose is present, and 55 mg. per cent glucose was exhausted in much less than an hour under the conditions used, the data must be presented in a different manner. In Chart 5 the total and mean inhibitions, as well as the final pH, are plotted vs. the final Pi concentration. The total inhibition was computed by subtracting the total oxygen consumed during the whole period of glucose inhibition from the oxygen consumption which would be expected endogenously during the same period. Since the actual endogenous respiration declined somewhat during the period, the expected endogenous respiration was computed from the initial (0–15 min.) endogenous average rate. The justification for using the initial rate in this way arises from a comparison of the upper part of Charts 3 and of 4. Cells using glucose would produce a large supply of oxidizable lactate and would be able to maintain their initial oxidative rates. The mean inhibition is the total inhibition divided by the duration of inhibition in minutes and is equivalent to the difference between the initial endogenous rate and the rate in the presence of glucose expressed as the per minute rate.

It is apparent from Chart 5 that the total inhibition decreased sharply, about 64 per cent, as the Pi concentration was increased from 0 to about 5 mM, and then remained constant. The mean inhibition also decreased in this range, but much less remarkably—about 33 per cent. The difference between the mean and the total arises from the simple fact that the duration of inhibition was much greater at low Pi levels. Below 1 mM Pi, inhibition lasted about 45 minutes; as Pi was increased from 0 to 10 mM the duration decreased from 45 to 20 or 25 min. and then remained constant up to at least 40 mM Pi. The changes observed between 0 and 10 mM Pi could not be attributed to pH changes in the medium, since the final pH values were relatively constant over this range.

One may conclude that an increase of Pi concentration from about 10 mM to higher levels did not cause a decrease in the Crabtree Effect. The difference between this conclusion and the one previously reported by this laboratory (5) may be a result of the two different washing procedures used. Previously, the cells were washed in media containing the same Pi level as the incubation media, whereas in the present work a Pi-free solution was used for washing, and, as shown above, the Pi content of the washing medium can influence both respiration and glycolysis. Except at the highest glucose level this Pi effect should be greater than the pH effect. This pH effect was suggested by Scholefield (18) to be responsible for the stimulatory effect of Pi (5).

e) Phosphate concentrations and respiratory inhibition with 0.5 mg. per cent glucose.—We were able to demonstrate that 0.5 mg. per cent glucose in a 1 per cent suspension of cells incubated in 55 mM Pi would cause an inhibition of respiration. It seemed of interest to see if lowering the Pi of the medium would alter the effect of this low glucose level on respiration. The results are presented in Chart 6. No Tris buffer was used in this case be-

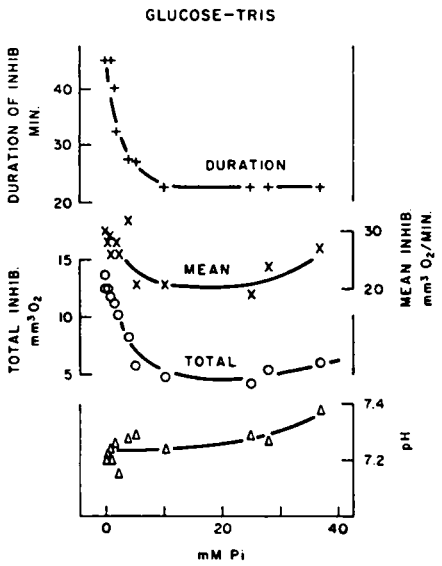

CHART 5.—The effect of phosphate on the duration, total and mean respiratory inhibitions, and pH changes produced by glucose addition. The system contained 55 mg. per cent glucose, 37 mM Tris, and varying amounts of Pi. The respiratory inhibitions are calculated on the basis of the initial 15-minute period of endogenous respiration (not indicated). The total inhibition is the cu. mm. O_2 difference between the endogenous and the glucose-containing systems for the total period of inhibition. The mean inhibition is the total divided by the duration in minutes. The oxygen values are for 0.04 ml. of cells.

cause of the small amount of glucose involved. In the 4–7-mM Pi range the Crabtree effect seemed to be absent, but, above and below these concentrations, inhibition was definitely present. At Pi levels from 7.1 mM Pi to at least 55 mM, the inhibition was transitory, but at the lowest Pi levels it seemed more permanent. We can therefore conclude again that there is some Pi-dependent change in metabolism which occurred when the Pi concentration was below 10 mM.

f) Phosphate concentration and respiration in 55 mg. per cent 2-deoxy-D-glucose, with and without

Tris.—Chart 7 gives the mean inhibition over a 2-hour period and final pH values in the presence of 2-deoxy-D-glucose. The total inhibition of oxygen consumption was impossible to estimate, since the cells were still in inhibition at the end of 2 hours (termination of experiment).

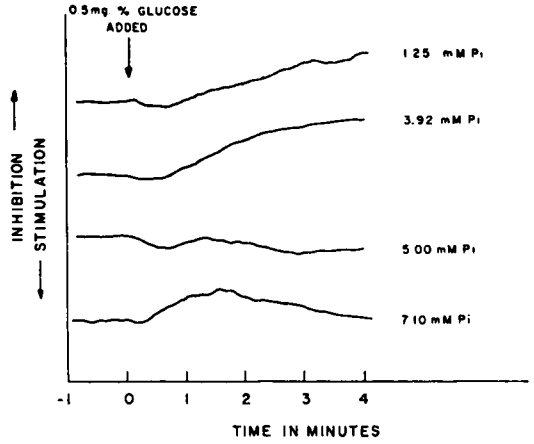

CHART 6.—The phosphate-induced change of response to 0.5 mg. per cent of glucose. This is measured with the oxygen electrode and a cell suspension of about 1 per cent. An upward deflection denotes inhibition, whereas a downward deflection indicates stimulation. No Tris buffer was used.

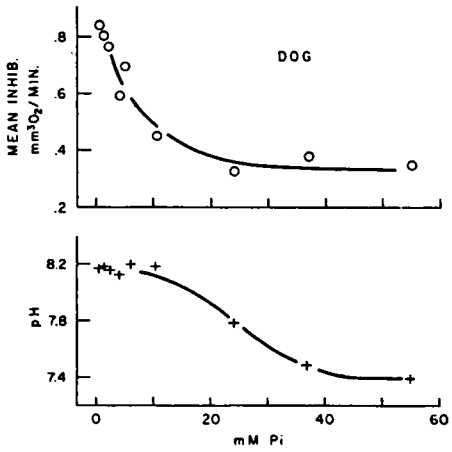

CHART 7.—The effect of phosphate on respiration and pH in the presence of 2-deoxyglucose. The system contained 55 mg. per cent of 2-deoxyglucose and no Tris buffer. The oxygen values are for 0.04 ml. of cells.

Several peculiarities of the 2-deoxy-D-glucose inhibition deserve mention. First, as can be seen by comparison of Charts 3, 4, and 7, the pH increase at low Pi levels was greater in the presence of 2-deoxy-D-glucose than in the presence of lactate or the absence of supplementary substrate. It seems that 2-deoxy-D-glucose accelerates the production of alkaline components from endogenous substrates. This may be correlated with the 2-deoxy-D-glucose-stimulated disappearance of acid-soluble pentose components (10) and could be associated with deamination of adenine nucleotides. Second, the decrease seen in the mean 2-deoxy-D-glucose-induced inhibition with increasing Pi qualitatively resembles the decrease seen in the glucose-induced inhibition (Chart 5). In data not presented it was observed that, while Tris helped to maintain the pH, only a small difference could be observed in the mean inhibition between identical systems with and without Tris. Therefore, it may be concluded that somehow Pi became limiting in phosphorylation. Perhaps Pi was trapped as deoxyglucose phosphate, thus making it unavailable for oxidative phosphorylation and ATP regeneration.

TABLE 2

THE INFLUENCE OF PHOSPHATE ON RESPIRATION OF EHRLICH ASCITES CARCINOMA IN THE PRESENCE OF DNP, GLUCOSE, AND TRIS BUFFER*

mM/LITER Pi	FINAL pH	PER CENT CHANGE FROM INITIAL ENDOGENOUS RESPIRATION	
		0–30 min.	30–60 min.
0.3	6.81	+15	+ 4
1.3	6.78	+16	+ 1
4.0	6.90	+13	+18
5.2	7.04	+ 4	0
6.9	7.02	+10	+24
10.3	7.07	+10	+34
24.7	7.13	+31	+32
37.0	7.18	+12	+27
55.0	7.18	+16	+22

* Each system contained 7×10^{-5} M/l DNP, 110 mg. per cent glucose and 37 mM/liter Tris. Each vessel contained 0.04 ml. of cells.

g) Phosphate concentration and respiration in 110 mg. per cent glucose, 37 mM Tris, and 7×10^{-5} M 2,4-dinitrophenol.—Table 2 shows the average respiration rate from 0 to 30 and 30 to 60 minutes and the final pH in the presence of glucose, Tris, and DNP vs. the Pi concentration. During the initial period of incubation the oxygen consumption was somewhat higher than that observed with lactic acid, demonstrating that 7×10^{-5} M DNP removed the glucose-induced inhibition at all Pi levels and even stimulated respiration. The later decrease in rate seen below 10 mM Pi could easily be a result of the lowered pH values. Kvamme (12) reported that this DNP level did not relieve the Crabtree Effect when the Pi level was under 20 mM. This difference probably is due to the large pH change (final pH, 5.4–5.8) in Kvamme's system. That the respiratory inhibition studied by

Kvamme does not appear until 10–15 minutes after glucose addition also indicates that this inhibition is due to pH.

FACTORS INFLUENCING GLYCOLYSIS

a) The effect of environmental pH on glycolysis in cells in 55 mM phosphate Locke solution.—Because of the great influence pH can have on respiration and the theoretical importance attached to pH effects (cf. 4), we deemed it important to examine the influence of pH on glycolysis as well. In Chart 8, the glucose consumed, the lactic acid produced, and the total change in pH brought about in 60 minutes are plotted against the mean pH of the medium. The pH change is a reflection of both the lactic acid production and the buffering capacity of 55 mM phosphate. Both glucose utilization and lactate production decreased with decreasing pH below pH 7.2. A comparison of Chart 8 with Charts 1 and 2 reveals that the slowing in glycolysis and respiration with a lowering of the pH are qualitatively similar.

b) The effect of varying inorganic phosphate concentration on glycolysis.—In Chart 9, the glucose utilization and lactic acid production by a 2.2 vol. per cent suspension of cells is plotted against the Pi concentration in the medium. The pH was maintained in the incubation mix with 55 mM Tris buffer, initially at pH 7.40, after the cells were washed in an isotonic Pi-free solution. Since the cells used in the study of pH effects described in the preceding section were washed in a 55 mM Pi solution, the glycolytic rates at high phosphate concentrations given in Chart 9 are somewhat lower than the rates around pH 7.2 given in Chart 8. Glucose utilization remained constant between 55 and 10 mM Pi, but between 10 and 0 mM it decreased with decreasing Pi concentration. The decrease in glucose utilization in this range (Chart 9) corresponds with the increase seen in the total inhibition of oxygen consumption (Chart 5). Lactic acid production began declining when the Pi concentration fell below about 15 mM.

Kvamme (12) reports that the rates of both glucose uptake and lactate production vary with Pi concentration over a wide range, whereas we find a rather sharp change around 5 or 10 mM Pi. The discrepancy may be attributed to the relatively large changes in pH which occur in Kvamme's

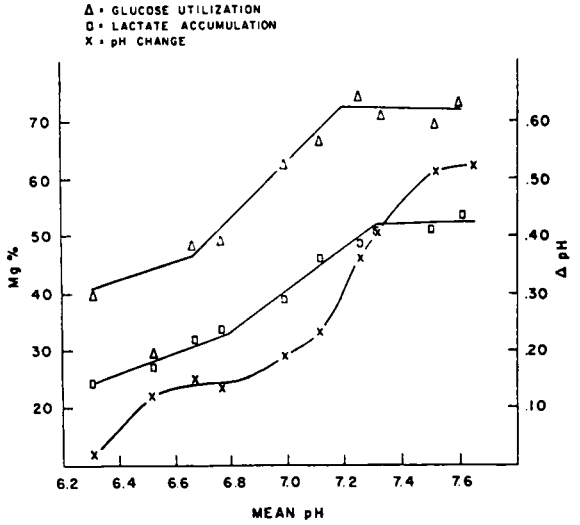

CHART 8.—The effect of pH upon glycolysis. The initial glucose concentration was 125 mg. per cent. The cells were washed and suspended (about 2.2 vol. per cent) in 55 mM phosphate solution without Tris. The duration of the experiment was 1 hour.

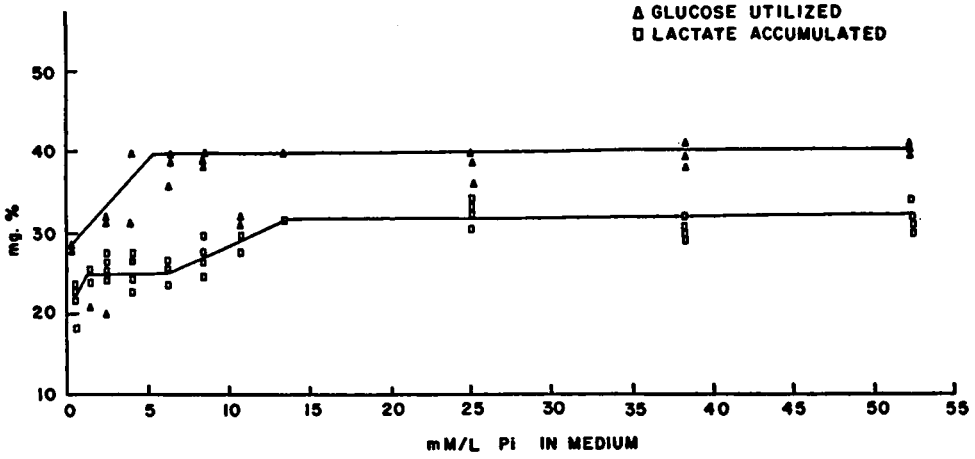

CHART 9.—The effect of varying phosphate concentrations on glycolysis. The initial glucose concentration was 125 mg. per cent. It should be noted that the cells in this experiment were washed in phosphate-free isotonic solution and then suspended (about 2.2 vol. per cent) in 55 mM Tris with varying amounts of Pi. The values are for 1 hour of glycolysis.

system, since it involved large amounts of glucose and a Tris buffering system which was ineffective below pH 7.0. At higher Pi levels, one might expect better buffering, a smaller drift in pH away from the optimal range, and a correspondingly higher rate of glycolysis such as he observed.

DISCUSSION

We have been able to demonstrate that the Pi concentration of the surrounding medium exerts a definite influence on the metabolism of Ehrlich ascites tumor cells. The Pi can exhibit its influence in three different ways: (*a*) by acting directly on the metabolic pathways during incubation, (*b*) by somehow changing the metabolic capacities of the cells while they are being washed, and (*c*) by buffering the cells so that effects due to pH changes are altered.

When the cells were incubated in a medium containing less than 5 mM Pi the rates of glucose utilization, lactate production, and respiration in the presence of glucose were clearly dependent on the Pi concentration (Charts 5, 6, and 9). Above 15 mM Pi, these three indices of metabolism were just as clearly independent of the Pi level. As Pi was lowered from 15 to 10 mM, lactate production began to decline, and between 10 and 5 mM glucose utilization began to slow. This progression of changes which occurred as Pi concentration was lowered might be explained in terms of the limitation of glycolysis by low Pi levels suggested by Wu and Racker (6) and the inhibition of hexose monophosphate shunt by high Pi levels shown by Kravitz and Guarino (20). Thus, as Pi was lowered below 15 mM, Pi-dependent steps of glycolysis slowed, but simultaneously the inhibition of the shunt was released so that the total rate of glucose utilization did not decline until below 10 mM. This slowing of glycolysis could be responsible for the inability of 0.5 mg. per cent glucose to cause an inhibition between 7 and 4 mM phosphate when measured by the oxygen electrode (Chart 6).

The effect of Pi on respiration in the presence of glucose was twofold. Because glucose utilization was slower below 7–10 mM, the inhibition of respiration by glucose lasted longer, and the total inhibition was greater. There also seems to be a direct effect on the respiration, judging from the increase in mean inhibition (Chart 5) and the renewed inhibition seen in the oxygen electrode tracings (Chart 6), when Pi levels were lowered. Such a direct effect on respiration might be mediated by a competition between glycolysis and respiration for Pi, although it should be noted that similar phenomena occurred when 2-deoxy-D-glucose was used in place of glucose (Chart 7).

Wu and Racker (22) have data indicating that the availability of Pi may control glycolysis and, in part, the Crabtree Effect at a concentration of 4 mM, whereas we (10) and Chance (6) have presented evidence suggesting that adenine mononucleotides are controlling agents when higher Pi levels are used. We can see now that these findings are not necessarily contradictory and may very well depend upon whether the Pi concentration was above or below about 10 mM.

Analysis of ascites fluid shows that only about 3.8 mM Pi is present (9). This might indicate that, *in vivo*, Pi is the rate-limiting factor; however, analysis of cells immediately after removal from the peritoneal cavity shows that the intracellular level is much higher (9), making the total availability of Pi difficult to evaluate *in vivo*.

As the data clearly show, Pi did not limit aerobic glycolysis or respiration when the external concentration was adequate. The fact that DNP stimulated respiration (Table 2) and glycolysis (10) suggests that the availability of ADP limits these pathways at higher Pi levels, and this in turn implies that the rate of ATP utilization limits the rate of ATP synthesis by respiration and glycolysis. One would expect, then, that initiation of glycolysis would show respiration and that the rate of ATP synthesis by glycolysis would be approximately equivalent to the decrease in rate of ATP synthesis by respiration, as has been demonstrated (11).

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