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Phosphorylation of 2-D-Deoxyglucose and Associated Inorganic Phosphate Uptake in Ascites Tumor Cells*

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ABSTRACT: The kinetics of 2-D-deoxyglucose and inorganic orthophosphate consumption, deoxyglucose 6-phosphate accumulation, adenine mononucleotide transformation, and respiration by Ehrlich ascites carcinoma cells incubated in 2.7–5.4 mM phosphate at 23° have been measured over a 3-min period after addition of 2-D-deoxyglucose. The estimate of P:O ratios based on deoxyglucose phosphorylation varies with time, being near 3 in the first 30 sec and declining thereafter. Consistent discrepancies between deoxyglucose phosphorylation and inorganic phosphate disappearance suggests either an unidentified reservoir of "high-energy phosphate" available for adenosine triphosphate regeneration or a release of inorganic phosphate from endogenous phosphate esters during the initial periods of rapid sugar phosphorylation. The latter interpretation is favored as being consistent with Lynen's phosphate cycle concept and other known features of ascites tumor cell metabolism. In these terms, the initial P:O ratio calculated from d(deoxyglucose 6-phosphate)/d*t*, after correction for phosphate derived from the declining adenosine triphosphate level, approaches 3 because the phosphorylation of deoxyglucose almost completely interrupts the preceding phosphorylation of endogenous substrates. As the phosphorylation of endogenous substrates increases again to compete effectively with deoxyglucose phosphorylation, the apparent P:O ratio declines. The very low P:O ratios calculated from inorganic phosphate and oxygen uptake over a 15-min interval are shown to be invalid because of the sharp decline in the rate of deoxyglucose phosphorylation after 3 min.

Recently, Morton and Lardy (1967a–c) described a technique for estimating intracellular oxidative phosphorylation by means of 2-deoxyglucose phosphorylation. The 2-deoxyglucose acted as a trap for the terminal phosphate of ATP and the deoxyglucose 6-phosphate thus formed neither inhibited hexokinase nor underwent further metabolism. A measurement of the inorganic orthophosphate taken up and the oxygen consumed could then be used to estimate the P:O ratio. The technique proved successful with bovine spermatozoa (1967a) and the P:O ratio approached the theoretical value of 3.0 in epididymal spermatozoa which had been treated to render the cell membrane permeable to ATP and protein (1967b). Application of the technique to ascites tumor cells yielded uniformly low P:O ratios (1.0 or less) which could be increased somewhat by addition of fluoride to the incubation medium (1967a). These last results raise some questions about the assumption that the intracellular P:O ratio is near 3 in ascites cells, which assumption has been used in calculation of theoretical rates of ATP synthesis in Ehrlich ascites cells (Coe, 1966a,b; Lee and Coe, 1967). A series of experiments was therefore undertaken to determine whether the low ratios estimated by the method of Morton and Lardy are valid for the ascites tumor cell system.

Experimental Procedures

Tumor Preparation. A hyperdiploid strain of Ehrlich ascites carcinoma cells was grown for 7–11 rays in Swiss white or strong A mice (*cf.* Table I) and was prepared and incubated in a tricine–phosphate buffer as described previously (Ibsen *et al.*, 1958, 1960).

Buffer Composition. Phosphate–Locke solution was modified by replacing the 54 mM phosphate buffer with 50 mM tricine (trishydroxymethylmethylglycine; General Biochemicals, Inc., Chagrin Falls, Ohio) and either 2.7 or 5.4 mM orthophosphate. Sodium chloride concentration was adjusted to make the solution isotonic, and final pH was adjusted to 7.4. The final concentrations of constituents were: tricine, 50 mM; sodium phos-

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TABLE I: Conditions Used in Experiments.[a]

Expt	Temp (°C)	Cell Concn (% v/v)	P_i Concn (mM)	2-D-Deoxyglucose Concn (mM)	2-D-Deoxyglucose μmoles/ml of Cells	Tumor Age	Mouse Strain
A	23	5.1	2.7	0.77	15.1	11	A
B	23	6.6	5.4	0.77	11.7	8	A
C	24	6.9	2.7	0.77	11.2	7	A
D	23	5.9	2.7	0.77	13.1	9	SW
E	23	7.9	2.7	3.85	48.8	8	SW

[a] The number of days between inoculation and harvesting of the tumor is given under *tumor age*. Two different strains of mouse were used: Strong A (A) and Swiss white (SW).

phate, 2.7 or 5.4 mM; NaCl, 95.6 or 91.5 mM (depending upon phosphate); $MgCl_2$, 0.49 mM; $CaCl_2$, 0.58 mM; KCl, 5.5 mM; $MnCl_2$, 0.016 mM.

Incubations. Temperatures and concentrations of cells, phosphate, and deoxyglucose used are summarized in Table I. The general procedure was the same in all cases: for each time interval, *t*, 1.0 ml of concentrated tumor suspension was added to 5.0 ml of buffer and allowed to stand for 2 min; at exactly 2 min 0.50 ml of 10 mM deoxyglucose, or 50 mM deoxyglucose in expt E, was blown in and mixed by swirling the flask; at *t* sec after deoxyglucose addition, 3.0 ml of cold 14% perchloric acid was blown in with mixing to quench the reaction. Zero-time samples were prepared by adding perchloric acid at 2 min and the deoxyglucose 15–30 sec later. The precipitated cells were centrifuged out, and 8.0 ml of the supernatant was neutralized with 20% KOH to pH 7.5–8.2. (Note: if filtrates were adjusted to pH 7.0 or less the enzymatic assay for deoxyglucose gave low, variable values, possibly because of interference from residual perchlorate; if filtrates were adjusted to 8.5 or higher appreciable loss of ATP and ADP was sometimes observed.) The $KClO_4$ was allowed to precipitate out overnight at 4°, and the neutralized supernatant was analyzed for deoxyglucose, deoxyglucose 6-phosphate, P_i, ATP, ADP, AMP, glucose 6-phosphate, fructose 6-phosphate, and lactate.

Rates of Respiration. The Gilson Medical Electronics Oxygraph, Model K, a polarographic device containing a bare, vibrating reed platinum cathode, was used for respiration studies as described previously (Coe, 1964). Buffer (1 ml) was added to the chamber followed by 0.20 ml of tumor suspension and, after 2 min, 0.10 ml of deoxyglucose in buffer. Recording was continued for 5 min or more and the respiratory rate was calculated directly from the decline in oxygen tension in the suspension. Conditions and relative concentrations were thus kept identical with those used in the incubation experiments. In control runs, 0.10 ml of buffer was added in place of deoxyglucose to obtain the effect of dilution and mixing. In expt E, where prolonged readings were required, the suspension was reaerated at 5-min intervals by means of a brief stream of bubbles blown through the suspension. To ensure identical conditions in the parallel incubation experiment in E, the suspensions in the flasks were also agitated at 5-min intervals.

Estimation of Metabolites. 2-Deoxyglucose was estimated by means of a commercially prepared glucose oxidase–peroxidase–chromogen combination ("Glucostat," Worthington Biochemical Corp., Freehold, N. J.), with the modification described previously (Coe, 1966a,b). It was found that free 2-deoxyglucose yielded about 70% of the absorbancy of equimolar amounts of glucose in this assay and gave linear, reproducible standard curves. Deoxyglucose 6-phosphate gave no reaction, and the background reaction of cell extracts without added deoxyglucose was low. Separate estimates of deoxyglucose 6-phosphate formed indicated that the deoxyglucose disappearing was entirely accounted for as deoxyglucose 6-phosphate (see below).

Deoxyglucose 6-phosphate was estimated colorimetrically after separation from deoxyglucose on an anion exchange resin, essentially as outlined by Letnansky (1964). A 1.0-ml aliquot of neutralized extract was diluted to 10 ml with water to decrease salt concentration, and the 10 ml was passed through a column of Dowex Cl 1-X8 2–3 cm high, by 1-cm diameter. The column was washed with three to five 1-ml additions of water and then with five 1-ml additions of 1 N HCl. Trials with standard deoxyglucose and deoxyglucose 6-phosphate solutions indicated that deoxyglucose was not bound to the column and that deoxyglucose 6-phosphate, which was initially bound, could be completely eluted with the 1 N HCl. The deoxyglucose 6-phosphate appeared mainly in the second HCl eluate and was barely detectable in the fifth eluate.

The colorimetric assay for deoxyglucose and deoxyglucose 6-phosphate was developed in this laboratory and will be reported in more detail elsewhere. In brief, a 1.0-ml sample, 0.10 ml of 1.5% cysteine-HCl, and 0.10 ml of 0.10 M fructose are mixed in an 18-mm test tube; the tube is immersed in ice water and 6.0 ml of H_2SO_4–H_2O (45:19) is added with shaking to prevent heating; the tube is allowed to stand at room temperature for 3 hr, after which the absorption at 590 mμ is read. A violet component with an absorption maximum at 590 mμ is characteristic of deoxyglucose; in a 20-mm cuvet in a Coleman Jr. spectrophotometer, the absorbancies of 0.10 μmole of deoxyglucose and deoxyglucose 6-phos-

phate are 0.4 and 0.3, respectively, under the described conditions. Other normally occurring sugars (glucose, galactose, ribose, etc.) do not interfere, although deoxyribose yields another chromogen with an absorption maximum near 500 mμ.

In estimations of deoxyglucose 6-phosphate eluted from the column, absorbancies of the five HCl eluates from a zero-time sample containing deoxyglucose but no deoxyglucose 6-phosphate were summed and subtracted from the summed absorbancies of the timed samples to correct for background absorption in the extracts.

Inorganic orthophosphate was estimated by reducing the phosphomolybdic acid formed after addition of molybdic acid with Elon (Gomori, 1941). Phosphorylated glycolytic intermediates and adenine mononucleotides were estimated by coupling specific enzymatic reactions with dehydrogenase reactions to reduce or oxidize pyridine nucleotides (Coe, 1966a,b). In particular, glucose 6-phosphate, fructose 6-phosphate, and ATP were estimated by sequential addition of glucose 6-phosphate dehydrogenase, phosphoglucose isomerase, and hexokinase in the presence of $NADP^+$, glucose, and Mg^{2+}; the increase in absorbancy at 340 mμ was followed with a Beckman DU-2 spectrophotometer. ADP and AMP were estimated by sequential addition of lactate dehydrogenase, pyruvate kinase, and adenylate kinase in the presence of Mg^{2+}, K^+, NADH, ATP, and excess phosphoenolpyruvate. Lactate was determined from NAD^+ reduction at pH 9 after addition of lactate dehydrogenase and glutamic–pyruvic transaminase in the presence of excess glutamate (Noll, 1966).

Materials. All nucleotides and most other biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) in the purest available form. Hexokinase was Sigma type C-302 or C-130; glucose 6-phosphate dehydrogenase was Sigma type V or X, or the Boehringer-Mannheim Corp. (New York, N. Y.) product; other enzymes were obtained in crystalline form from either Sigma Chemical Co. or Boehringer-Mannheim Corp.

2-D-Deoxyglucose, from Mann Research Laboratories, Inc. (New York, N. Y.), was stated to be chromatographically homogeneous. Incubation with hexokinase and ATP and chemical and enzymatic analysis of the products indicated that the deoxyglucose 6-phosphate formed contained less than a 1% contamination with glucose 6-phosphate or fructose 6-phosphate. With sufficient periods of incubation with cells, lower concentrations of deoxyglucose were totally consumed, demonstrating that there was so significant nonmetabolizable component.

Inorganic chemicals were all reagent grade. Water was distilled and deionized.

Results

Phosphate Uptake after 2-Deoxyglucose Addition. Addition of deoxyglucose to a tumor cell suspension in aerated buffer leads to a measurable decline in P_i over the first 3 min (Figure 1); addition of an equivalent volume of buffer without deoxyglucose to the suspension causes relatively little change in the P_i. Of the initial P_i level, 59 μmoles/ml of cells, about 51 μmoles is contributed by the buffer and about 8 μmoles is carried with the cells. Specifically, in the 6.5-ml incubation mixture, 6.17 ml is buffer containing 2.71 μmoles of P_i/ml and 0.33 ml is the volume occupied by the cells; 6.17 × 2.71/0.33 gives 50.7 μmoles of P_i/ml of cells added with the buffer, leaving 59.0–50.7 or 8.3 μmoles/ml of cells contributed by the cells themselves. If corrections are introduced for the trapped solvent in packed-cell volumes (Coe and Saha, 1966), the actual intracellular P_i concentration may be calculated as 10 mM, a value comparable with those reported by Wu and Racker (1959) and Levinson (1966). Intracellular P_i concentrations ranging from 10 to 15 mM were also observed in the other experiments described below.

The total decline in P_i over the 3-min period amounts to only about 10% of the initial level, which accentuates the necessity for high precision in phosphate analyses.

Changes in lactate level are also indicated in Figure 1. A slight accumulation of lactate is evident when buffer alone is added, but no accumulation is apparent after deoxyglucose addition. The transient but rapid accumulation of lactate after deoxyglucose addition reported previously (Ibsen *et al.*, 1958) is usually observed in media containing high (50 mM) phosphate concentrations but has not been evident in low concentrations of phosphate.

Phosphorylation of 2-D-Deoxyglucose. Figure 2 illustrates deoxyglucose phosphorylation as determined by two independent methods: the circles represent values calculated from the disappearance of deoxyglucose, estimated by the Glucostat method; the crosses represent deoxyglucose 6-phosphate accumulated, estimated by the colorimetric procedure after ion-exchange separation. A reasonably good agreement between the two methods may be noted. Since the total amount of deoxy-

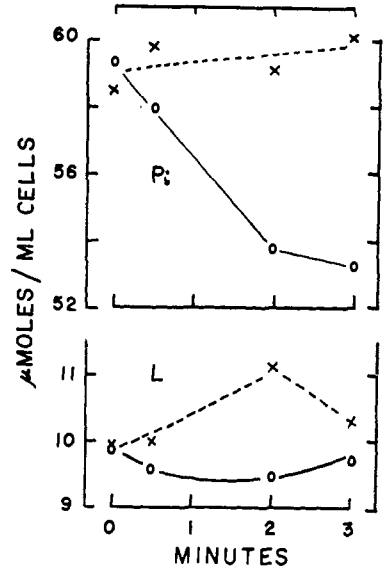

FIGURE 1: Changes in phosphate and lactate after addition of 2-deoxyglucose to Ehrlich ascites tumor cells. Data from expt A (see Table I). Upper frame gives change in P_i; lower frame gives change in lactate. Symbols (both frames): × ----, level after addition of buffer alone; O ——, level after addition of 2-deoxyglucose in buffer. Additions were at zero time.

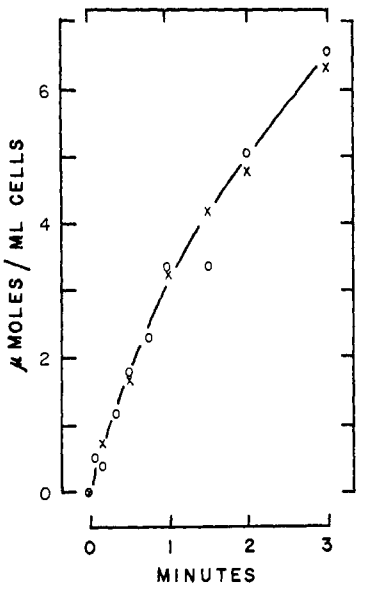

FIGURE 2: Deoxyglucose consumption and deoxyglucose 6-phosphate formation in ascites tumor cells. Data from expt C (Table I). Symbols: O, deoxyglucose disappearance; ×, deoxyglucose 6-phosphate accumulation.

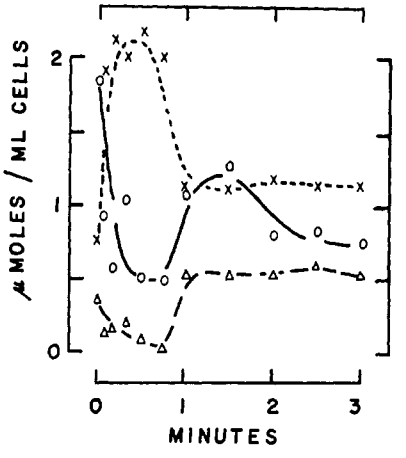

FIGURE 3: Changes in adenine mononucleotides after addition of deoxyglucose. Data from expt D (Table I). Symbols: O——, ATP; × ——, ADP; Δ——, AMP.

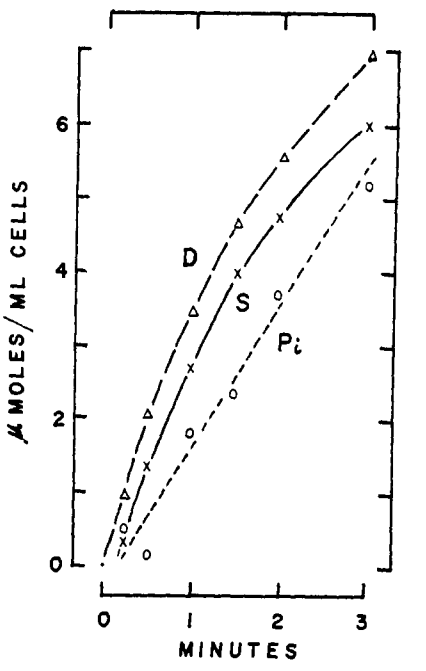

FIGURE 4: Comparison of deoxyglucose phosphorylation and P_i uptake. Averaged results from expt B–D. Curve D (Δ — —) shows deoxyglucose disappearance and deoxyglucose 6-phosphate accumulation, as in Figure 2. Curve S (× ——) represents phosphate esterified into deoxyglucose 6-phosphate and is calculated from curve D by subtracting the phosphate contributed from the declining ATP level (calculation described in text). Curve P_i (O - - - -) gives the inorganic orthosphosphate uptake.

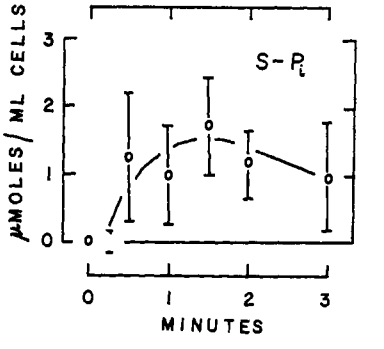

FIGURE 5: Difference between phosphate esterified into deoxyglucose 6-phosphate and P_i disappearance. Averaged results from expt B–D. At each time interval, the P_i uptake was subtracted from the esterified phosphate (S in Figure 4) and the differences from the three experiments were averaged; vertical bars give the mean deviation.

glucose initially present in this experiment (D, Table I) was 13.1 μmoles/ml of cells, about half the deoxyglucose was consumed in the 3-min interval; hence, less precision is required in the deoxyglucose estimates than in the P_i estimates.

Changes in Adenine Mononucleotide Levels. Typically, the ATP level declined from about 2 μmoles/ml of cells to about 1 μmole during the first minute where it stabilized while the ADP and AMP levels increased correspondingly during the first minute and then declined slowly. One of the more extreme variations in this pattern is illustrated in Figure 3. In this case, there is a distinct overshoot with ATP falling to 0.5 μmole and ADP rising to over 2 μmoles during the first 30 sec followed by a return of both nucleotides to about 1 μmole. AMP appears to follow the ATP curve rather than the ADP curve in a manner which suggests that adenylate kinase is ineffective in regulating the balance among the nucleotides until after 1 min. The more usual pattern resembled those reported by Letnansky (1964) and Overgaard-Hansen (1965) in which ATP fell sharply after deoxyglucose addition and then approached a lower stable value without the overshoot, and AMP tended to follow the ADP curve.

Relationship between Deoxyglucose Phosphorylation and Inorganic Orthophosphate Uptake. Since both phosphate and lactate changes were relatively small com-

pared with the initial levels (Figure 1), the measurements of these components were sensitive to small errors in the analyses. To obviate this difficulty, results from three separate experiments (B–D) were averaged to obtain the curves in Figure 4. Curve D represents actual deoxyglucose phosphorylation as estimated from both deoxyglucose disappearance and deoxyglucose 6-phosphate formation (Figure 2). Curve S represents deoxyglucose phosphorylation corrected for phosphate derived from preexisting ATP and ADP; specifically, for each point in each experiment, the change in ATP was added to and the change in AMP was subtracted from the change in deoxyglucose 6-phosphate: Δdeoxyglucose 6-phosphate + ΔATP − ΔAMP. Thus, if at 30 sec 2.0 μmoles of deoxyglucose 6-phosphate had been formed but ATP had declined by 0.5 μmole and AMP had increased by 0.2 μmole, 0.5 μmole of the phosphate in deoxyglucose 6-phosphate could be obtained at the expense of the ATP and another 0.2 μmole could have come from ATP derived from ADP *via* the adenylate kinase reaction; hence the S value would be only 1.3 μmoles of deoxyglucose 6-phosphate. This 1.3 μmoles should represent the phosphate esterified by way of oxidative phosphorylation during the 30-sec period and should therefore be equivalent to the P_i uptake, barring the existence of alternate "high-energy phosphate" sources such as creatine phosphate. It will be noted that the S curve extrapolates to zero at about 10 sec; this is attributable to the fact that the rise in deoxyglucose 6-phosphate is nearly equivalent to the decline in ATP over the first 10 sec. It will also be noted that P_i uptake is approximately linear over the first 3 min and is well below the S curve. Figure 5 illustrates that the discrepancy between the S and P_i curves is more than experimental error. Despite a substantial variation, the difference between S and P_i ($S - P_i$) is consistently positive after 15 sec.

The mean changes in lactate, ΔL, and glucose 6-phosphate (Figure 6) indicate that little, if any, glycolysis occurs. The initial dip in lactate varied in magnitude from one cell preparation to another but was consistently observed and could easily be attributable to the sharp increase in respiratory rate immediately after deoxyglucose addition (see below). Glucose 6-phosphate accumulation amounted to only 0.1 μmole/ml of cells over the 3-min period, and fructose 6-phosphate accumulation was even less. Transient but significant accumulations of both lactate (Ibsen *et al.*, 1958) and the hexose monophosphates (E. L. Coe and I.-Y. Lee, unpublished observations) during the first minute after deoxyglucose addition have been observed, but such accumulations have always occurred in cells suspended in media containing high phosphate concentrations. The effect of phosphate concentration on the possible mobilization of endogenous glycolytic reserves is presently being investigated.

Figure 6 also illustrates the total adenine mononucleotide content declines by about 8% over the 3-min period. The alteration in mononucleotide levels after deoxyglucose addition has been extensively studied by Overgaard-Hansen (1965), who observed a similar decline in total nucleotide content. Overgaard-Hansen found a total adenine mononucleotide level about twice as high as that reported here, but this difference is probably attributable to a difference in tumor strain. Values obtained previously in this laboratory with a hypotetraploid strain were consistently around 5–6 μmoles/ml of cells for total mononucleotide and around 3 μmoles for

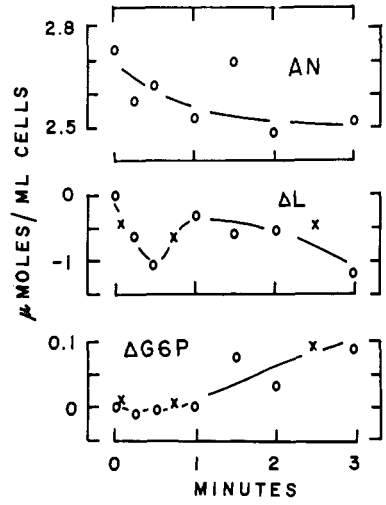

FIGURE 6: Changes in total adenine mononucleotide, lactate, and glucose 6-phosphate after deoxyglucose addition. Top frame shows change in ATP + ADP + AMP; middle frame gives change in lactate; bottom frame shows change in glucose 6-phosphate. Symbols: O, average from expt B–D; ×, average from expt C and D.

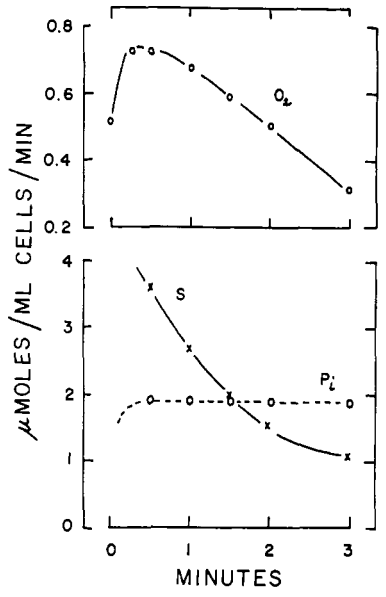

FIGURE 7: Rates of oxygen consumption, phosphate esterification into deoxyglucose 6-phosphate, and P_i disappearance. Averaged results from expt B–D. Upper frame (O ——) gives rate of oxygen consumption, as determined from oxygen electrode measurements. Lower frame shows the rate of phosphate esterification (× ——) estimated by taking slopes to curve S in Figure 4, and the rate of P_i uptake (O – – – –), equal to the slope of line P_i in Figure 4.

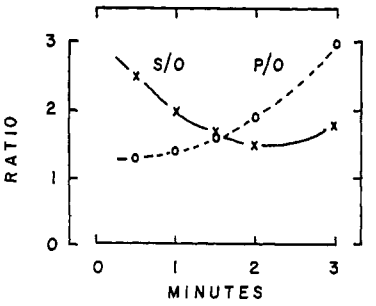

FIGURE 8: P:O ratios calculated from phosphate esterification into deoxyglucose and P_i disappearance. Curve S/O (× ——) equals $1/2(dS/dt)/(dO_2/dt)$; curve P/O (O – – – –) equals $1/2(dP_i/dt)/(dO_2/dt)$. The rates used are shown in Figure 7.

ATP (*cf.* Lee *et al.*, 1967, for example), which were in the range reported by Overgaard-Hansen. The present work was carried out with a hyperdiploid strain which has consistently shown an ATP level of 1.5–2 μmoles/ml of cells and a total adenine mononucleotide level around 3 μmoles. These latter values are more comparable with those found by Letnansky (1964). Overgaard-Hansen (1965) established that the decline in these nucleotides was attributable to a further degradation of AMP to IMP, inosine, and ultimately free hypoxanthine. Since the analyses in the experiments described above were carried out on perchloric acid extracts of cell suspensions, they include nucleotides from both the cells and the medium and therefore suggest a net disappearance of nucleotides. In the course of other experiments undertaken to evaluate a filtration procedure for separating cells from their medium (Coe and Saha, 1966) it has been found that leakage of nucleotides from the cells is negligible over short incubation periods, and hence the observed changes are intracellular.

Rates of Oxygen and Phosphate Uptake and Deoxyglucose Phosphorylation. After addition of deoxyglucose to aerated cells, the cellular respiration rate increases by about 40% within the first 15 sec and then declines steadily (Figure 7). Respiration is stimulated above the initial endogenous rate for 2 min and then falls below this rate, entering into the deoxyglucose "Crabtree effect" (Seelich and Letnansky, 1961; Ibsen *et al.*, 1962; Yushok, 1964). During the 3-min period the rate in cells receiving an addition of buffer in place of deoxyglucose declines gradually to about 0.4 μmole/ml of cells per min.

The rate of phosphate esterification into deoxyglucose 6-phosphate, obtained by taking tangent slopes to curve S in Figure 4, decreases by more than a factor of 3 during the first 3 min, whereas the rate of P_i uptake remains constant at 1.9 μmoles/ml of cells per min (Figure 7). Studies of P_i disappearance over longer periods (see Figure 9) suggest that the rate may be declining by 3 min, although this is not evident in Figure 4. Division of dS/dt and dP_i/dt by $2dO_2/dt$ gives two different estimates of the apparent P:O ratios which disagree (Figure 8): that calculated from S begins near 3 and falls to about 1.5, while that from P_i begins near 1 and rises toward 3 at 3 min. The question of which ratio is the more reliable index of the true intracellular P:O ratio will be considered in the Discussion. However, both ratios indicate that the intracellular P:O ratio may approach 3 at certain times.

Oxygen, Deoxyglucose, and Phosphate Uptake over Longer Time Intervals. Since the measurements of Morton and Lardy (1967a,c) were carried out over incubation periods of 15 min, a comparison of the results obtained over the longer period was undertaken (Figure 9). By 5 min both O_2 and deoxyglucose utilization had slowed to only a fraction of the initial rates and P_i had started to increase again. The sharp slowing in the deoxyglucose utilization after the first minute or two even in the presence of a large excess of deoxyglucose has been observed repeatedly in Ehrlich ascites tumor cells (Ibsen *et al.*, 1958; Letnansky, 1964) as well as in Krebs II ascites tumor cells (McComb and Yushok, 1964a,b) and therefore appears to be quite characteristic of the ascites tumor cell system. Although oxygen consumption has also slowed by 5 min, it continues at a rate of 0.11 μmole/ml of cells per min while deoxyglucose consumption continues at a rate of only 0.09; hence, it is apparent that even P:O ratios calculated directly from deoxyglucose utilization will decrease with time. Table II compares ratios calculated from total changes over both a 2- and 15-min period in expt E with ratios over a 2-min period obtained from the averaged results of expt B–D. Over the 2-min interval the ratios from $\Delta P_i/\Delta O_2$ in expt E and the expt B–D average agree well, but the ratio in expt E over the 15-min interval is much lower. The low 15-min ratio is comparable with those calculated by Morton and Lardy, however. A similar relationship is observed among ratios calculated from the deoxyglucose phosphorylation ($\Delta P_{est}/\Delta O_2$), although the ratios from expt E were calculated directly from the deoxyglucose utilization without the correction for declining ATP and therefore represent maximal values; application of the mean correction estimated in expt B–D would lower the ratios in expt E by about 0.5. One may conclude

TABLE II: P:O Ratios Calculated from P_i Disappearance and Phosphate Ester Accumulation over 2 and 15 min.[a]

Parameter	Expt	Time Period 0–2 min	Time Period 0–15 min
ΔP_i	E	1.66	0.65
	B–D	1.55	
ΔP_{est}	E	<2.4	<1.3
	B–D	1.93	

[a] The ratios given after ΔP_i were calculated from the total disappearance of P_i and oxygen over the time interval indicated. The ratios given after ΔP_{est} were calculated from the phosphate esterified into deoxyglucose 6-phosphate over the same intervals; results from expt E were not corrected for phosphate contributed by the declining ATP level and therefore represent maximum values.

that the low ratios calculated by Morton and Lardy are a consequence of the long time interval used.

Discussion

The Discrepancy between P:O Ratios Calculated from Phosphate Disappearance and Deoxyglucose Phosphorylation. The differing ratios in Figure 8 are a consequence of the greatly differing rates of phosphate esterification into deoxyglucose 6-phosphate (curve S, Figure 7) and inorganic phosphate uptake (curve P_i, Figure 7). It is obvious that during the first minute, more phosphate is appearing in deoxyglucose 6-phosphate than can be accounted for from the decline in ATP and the disappearance of P_i, and the problem therefore reduces to a determination of the phosphate source. There are two general possibilities: (a) the cells contain an alternate source of "high-energy phosphate" such as creatine phosphate which serves to regenerate the ATP when its concentration declines; or (b) P_i is released from phosphate esters inside the cell by assorted phosphatase activities, and this is then rapidly consumed by oxidative phosphorylation.

If case a holds true, then the P:O ratio calculated from P_i disappearance is the more valid index of the true P:O ratio, because the extra deoxyglucose 6-phosphate formed during the first minute depends upon the alternate source of high-energy phosphate and not on additional oxidative phosphorylation. On the other hand, if case b hold true, then the P:O ratio calculated from phosphate esterification into deoxyglucose 6-phosphate is the more valid index because the extra deoxyglucose 6-phosphate formed depends upon the release of P_i within the cell and its subsequent esterification by way of oxidative phosphorylation. At present, a firm decision between the two alternatives is impossible. A detailed analysis of the theoretical rates of ATP synthesis by respiration and glycolysis after addition of small loads of glucose has indicated that the total rate of ATP synthesis from both sources increases above the endogenous rate during the early period of glycolysis and then decreases below the endogenous rate for 30 sec or so after glycolysis has ceased (Lee and Coe, 1967). This sequence suggests that a phosphagen, if present, is equilibrating with glycolytically generated ATP rather than with oxidatively generated ATP and therefore argues against alternative a, although the argument is by no means conclusive. In support of alternative a, Egawa (1966) has found an acid-labile phosphate ester fraction equivalent to about 1 μmole/ml of cells which he tentatively identifies as creatine phosphate. The nature of this fraction is uncertain, however, and so this argument is not conclusive, either.

Allowing the possibility that an unidentified phosphate anhydride or ester could provide a means of regenerating a limited amount of ATP, one can still interpret the results entirely in terms of alternative b without invoking the unidentified phosphagen source, and the interpretation is consistent with Lynen's "phosphate cycle" concept (Lynen, 1941; Lynen *et al.*, 1959) as well as with other known features of ascites tumor cell metabolism. First, it is apparent from studies such as the one

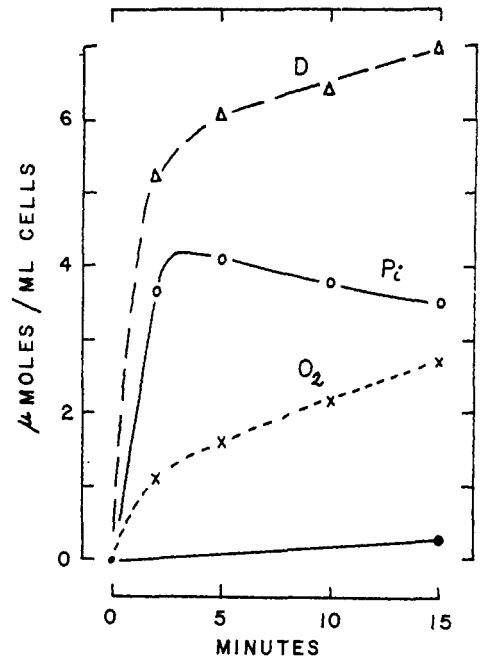

FIGURE 9: Deoxyglucose, P_i, and oxygen uptake over a 15-min period. Data from expt E (Table I). Symbols: D, △ — —, deoxyglucose disappearance; P_i, ○ ——, phosphate uptake after deoxyglucose addition; ● ——, phosphate uptake after addition of buffer only; O_2, × - - - -, oxygen consumption after addition of deoxyglucose.

shown in Figure 1 that the P_i level remains relatively constant in cells oxidizing their own endogenous substrates despite the facts that respiration, and therefore presumably oxidative phosphorylation, is continuing, and ATP is maintained at a level which usually exceeds the levels of ADP and AMP. These observations imply that the "phosphate cycle" concept applies, and that there is a continual release of P_i within the cells which is balanced by a reesterification by way of oxidative phosphorylation. Given such a situation, one would predict that any sudden interruption or diversion of the phosphorylation of endogenous substrates by the cycle would lead to a transient increase in P_i due to the continuation of P_i-releasing (or phosphatase) activities. Eventually, as the phosphate esters declined, the phosphatase activity would slow and a new balance would be established. In the case where introduction of deoxyglucose caused the diversion of the phosphorylation of endogenous substrates, the released P_i would not accumulate but would be rapidly taken up and used for synthesis of deoxyglucose 6-phosphate.

Effect of Deoxyglucose Phosphorylation on the Phosphorylation of Endogenous Substrates. The calculation of the P:O ratio from either the phosphate esterified in deoxyglucose 6-phosphate or the P_i uptake assumes that once deoxyglucose is added, the total phosphorylative capacity of the cell is devoted to deoxyglucose 6-phosphate synthesis. As discussed above, however, the cell is probably phosphorylating a variety of endogenous substrates involved in the cell's maintenance, and there is no reason to believe that all these reactions immediately and permanently decline to zero. Rather, a com-

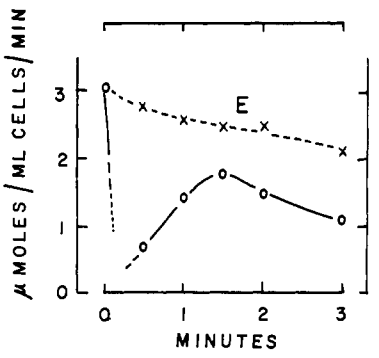

FIGURE 10: Calculated effect of deoxyglucose phosphorylation on the phosphorylation of endogenous substrates. Curve E (× ----) equals $6(dO_2/dt)$ for cells receiving an addition of buffer in place of deoxyglucose in buffer and should be equivalent to the uninterrupted rate of phosphorylation of endogenous substrates under the experimental condition used. The other curve (O ——) equals $[6(dO_2/dt) - dS/dt]$ for cells receiving an addition of deoxyglucose at zero time and should be equivalent to the rate of phosphorylation of endogenous substrates continuing after deoxyglucose addition. Calculations assume that the P:O ratio is 3.0 and that all the phosphate esterified (S) came by way of oxidative phosphorylation.

petition among the various ATP-requiring reactions probably develops. In order to evaluate, approximately, the effect that deoxyglucose phosphorylation might have on the preexisting reactions, the extreme situation in alternative b, described above, was assumed, and the possible effect calculated therefrom is shown in Figure 10. The theoretical P:O ratio of 3.0 was also assumed, and $6dO_2/dt$ for cells with buffer only added is compared with $(6dO_2/dt) - (dS/dt)$ for cells in the presence of deoxyglucose. Provided the assumptions are valid, the lower curve would represent the composite rate of the normal ATP utilizing of the cell in the presence of deoxyglucose; the upper curve (E) indicates what the rate would have been in the absence of deoxyglucose. Phosphorylation of the endogenous substrates appears to be interrupted by deoxyglucose for about 30 sec after which it recovers to 50–70% of the endogenous rate. Assumption of a lower P:O ratio would lower both curves and imply a greater suppression of endogenous phosphorylation by deoxyglucose. Contribution of phosphate from an unidentified phosphagen source would decrease the effect and therefore this calculation represents the maximum possible effect at any given P:O ratio.

Hypothetical Sequence of Phosphorylative Events after Addition of Deoxyglucose. From the rates shown in Figure 8 and the relationships illustrated in Figure 10, a hypothetical sequence of events can be reconstructed, and these are shown in Figure 11. The situation prior to deoxyglucose addition is shown in frame a; general endogenous components X are being phosphorylated at a rate of 3.0 μmoles/ml of cells per min to X–P; X–P components are converted into Y–P components which are then dephosphorylated at a rate of 3.0 μmoles/min to yield Y components and P_i; the whole system is balanced, and there is no net change in P_i. In the early period (10–30 sec) after deoxyglucose addition, the conversion of X into X–P is slowed to 0.5 μmole/min by the severe competition from deoxyglucose phosphorylation, but the dephosphorylation of Y–P continues at nearly the original rate (2.5 μmoles/min), leading to a decline in X–P and Y–P (frame b); the total rate of oxidative phosphorylation increases to 4.5 μmoles/min, being mainly dependent upon the supply of ADP provided by the rapid deoxyglucose phosphorylation, and a net decline in P_i begins. Between 60 and 120 sec (frame c), something approaching a new steady-state develops;

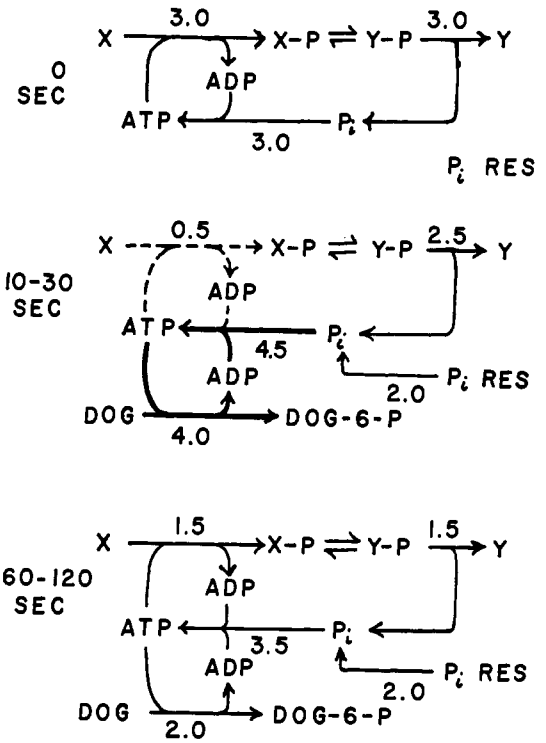

FIGURE 11: Hypothetical sequence of phosphorylative events after addition of 2-deoxyglucose. Based on same assumptions used in Figure 10. The situation before addition of deoxyglucose is shown in top frame: endogenous substrates X are being phosphorylated to X–P at a rate of 3.0 μmoles/ml of cells per min; X–P components are converted to Y–P components which are being dephosphorylated at the same rate to Y and P_i; P_i is being taken up by oxidative phosphorylation to form ATP, also at the same rate; no net change in P_i is occurring, and the phosphate reserves (P_i RES) are left untouched. In the early periods after deoxyglucose addition (10–30 sec; middle frame), the reaction of deoxyglucose catalyzed by hexokinase drains the ATP away from other phosphorylative reactions which decline to a rate of only 0.5 μmole/ml of cells per min; dephosphorylation of the Y–P components continues at a declining but higher rate at the expense of the pool of X–P and Y–P components; oxidative phosphorylation increases to a rate of 4.5 μmoles/min, which exceeds the P_i released from Y–P, and hence, the P_i reserves are drawn upon at a rate of 2.0 μmoles/min. In the period 60–120 sec after deoxyglucose addition (bottom frame) something approaching a new steady state is established with respect to phosphorylation of endogenous components; phosphorylation of X competes favorably with phosphorylation of deoxyglucose, rising to 1.5 μmoles/ml of cells per min, while the concentration of the Y–P components has fallen to a point where dephosphorylation of Y–P also equals 1.5 μmoles/min; oxidative phosphorylation still exceeds the release of P_i, and P_i from the reserves continues to disappear but now at a rate equal to that of deoxyglucose phosphorylation.

phosphorylation of the X components begins to compete favorably with the phosphorylation of deoxyglucose, which has declined in rate, and the X–P and Y–P components have decreased in concentration to a point where dephosphorylation approximately matches phosphorylation at 1.5 μmoles/min. At later times, it is apparent from Figure 10 that there is a general slowing of phosphorylation reactions and, probably, a general decline in all cellular activities, a consequence possibly of the general loss of nucleotides (Overgaard-Hansen, 1965). Figure 11 is meant to provide an approximation of the events based on actual rate measurements and a few simplifying assumptions, and is subject to modification as new information becomes available.

"True" Intracellular P:O Ratios. From Figure 8, it may be concluded that the P:O ratio is greater than 1.0 and could easily be as high as the theoretical 3.0. From the foregoing discussion, it is likewise apparent that no precise evaluation is possible until the extent of the competing endogenous reactions are known. Such complications render the approach of Morton and Lardy (1967a–c) inapplicable to cells such as the Ehrlich ascites tumor cells without a considerable body of supplementary information. Morton and Lardy (1967a) attribute low P:O ratios to a low phosphorylation efficiency brought about by barriers to diffusion of phosphorylated compounds through intracellular compartments and across cell membranes, and support their conclusion with the observation the physical or chemical damage to cell membranes leads to greater P_i uptake and higher P:O ratios. The interpretation derived from the evidence in the present paper would be somewhat different. Normally the phosphorylation of endogenous substrates (X to X–P, Figure 11) competes effectively with the phosphorylation of deoxyglucose, and P_i released from Y–P tends to recycle. Damage to the cell membranes disrupts this cycle through the endogenous substrates by causing a loss of enzymes or cofactors or by disrupting some critical spatial arrangement and allows the cell to devote its full phosphorylative capacity to deoxyglucose. Recycling of P_i ceases and the net uptake of P_i increases.

Certain lines of evidence argue against the idea that barriers to diffusion decrease the phosphorylation efficiency. During the 3-min period of most of the experiments described above, entry of P_i into the cell probably never becomes a limiting factor because the total P_i disappearance is less than the amount carried by the cells, although it may become a factor at later times. Moreover, a wealth of indirect evidence has indicated that the true intracellular P:O ratio remains near 3. From comparisons of steady-state rates of anaerobic glycolysis and endogenous respiration (Quastel and Bickis, 1959) or from the depression of respiration in the Crabtree effect and the rate of aerobic glycolysis (Ibsen *et al.*, 1959) P:O ratios between 2.5 and 3.0 may be obtained. Phosphate and oxygen consumption during the period of sharp respiratory stimulation immediately after glucose addition also indicate a ratio of 3 (Hess and Chance, 1959), as does the magnitude of respiratory stimulation after addition of small amounts of 2-deoxyglucose (Coe, 1968). Such information suggests that agents which damage cell membranes may be doing something other than changing the efficiency of phosphorylation by removing barriers to diffusion.

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