P³² Incorporation by Ehrlich Ascites Cells in Vitro

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It was shown by Crabtree in 1929 (8) that addition of glucose to tumor slices led to a decrease in their respiratory activity, and this effect has been confirmed (6, 10). The inhibitory effect of glucose on the respiratory activity of ascites cells is much greater and may approach 40 per cent (for reviews of the "Crabtree" or "reversed Pasteur" effect in ascites tumor cells, the recent papers by Bloch-Frankenthal and Weinhouse [3] and by Ibsen, Coe, and McKee [18] should be consulted). The mechanism of this effect has been studied by Racker (21) and by Chance and Hess (7), both of whom seem to favor some form of imbalance of the adenine nucleotides through the interplay of cytoplasmic glycolysis and mitochondrial respiration. However, the results of Aisenberg, Reinafarje, and Potter (1) and Aisenberg and Potter (2) indicate that, in a system consisting of a mixture of a glycolyzing preparation with liver mitochondria, the phenomenon of the Pasteur effect may be observed but there is no detectable change in the levels of each of the three adenosine phosphates. On the other hand, the observations and calculations of Quastel and Biexis (19) suggest that the depletion of adenosine triphosphate (ATP) resulting from the inhibition of respiration by glucose is exactly counterbalanced by the yield of ATP from aerobic glycolysis. A similar series of sparing actions has been described by Medes and Weinhouse (16), who observed that added substrates such as acetate and other fatty acids may "spare" the oxidation of endogenous carbon compounds and that added glucose was a preferred substrate for oxidation by ascites cells.

It was felt that a detailed study of the rate of turnover of P³² in the various acid-soluble components of Ehrlich ascites cells would shed some light on the effects of glucose in this system. Such in vitro studies of P³² turnover have been carried out by Harrington and Lavik (11, 12) and by Thomson, Snellie, and Davidson (24) using Ehrlich ascites cells. Similar studies have been made by Reddy, Breiger, and Orchen (22) using slices of human ovarian tumors and by Mirollo and Cornatzer (17, 18) using a CSH ascites tumor. However, in the foregoing studies, emphasis was placed on the turnover of P³² in the acid-insoluble fractions, and no attempt was made to separate the acid-soluble fraction into its various constituents. In the present experiments attention has also been paid to the rate of turnover of P³² in the adenosine phosphates present in the acid-soluble fraction of the cell.

MATERIALS AND METHODS

The strain of Ehrlich ascites carcinoma cells used in the present work was originally obtained from Lederle Laboratories (courtesy Dr. J. S. Colter) and was maintained by weekly intraperitoneal injection in CFI mice. In order to insure that the ascitic form remained in a state of both high and constant metabolic activity it was re-isolated every 8 weeks from the solid form, which was obtained by subcutaneous injection. The ascites cells were removed from the host after 6-8 days' growth and freed from ascitic fluid and blood elements by repeated washings with isotonic saline; finally the packed cells were diluted to 8 times their volume with buffer-free, calcium-free Krebs-Ringer solution ("salts solution"). In all the experiments in which P³² was used, 1 ml. of such a suspension was added per 8-ml. total volume and also a total of approximately 100 μC. P³².

After incubation, the samples were added to 5 ml. of ice-cold salts solution, spun down, washed in a further 5-ml. salts solution, and finally precipitated with 5 ml. 5 per cent (w/v) trichloroacetic acid (TCA) and maintained at a temperature of 0°C. for 30 min. The supernatant after centrifugation was decanted and a sample plated and counted. This fraction, called the acid-soluble phosphate fraction, was extracted 3 times with twice its volume of ether, reduced to dryness, redissolved in a small volume of water, and subjected to two-dimensional chromatography on Whatman No. 1 filter paper as described by Bolton, Britten, Cowie, Creaser, and Roberts (4). Radioactive areas on the chromatograms were located by radioautography with Kodak "No Screen" x-ray film and counted on the paper.

The acid-insoluble material was washed twice with 5-ml. portions of 5 per cent TCA, extracted with 5 ml. 80 per cent ethanol at 40°-50°C. for 15 min. and with 5 ml. 1:1 ether—80 per cent ethanol at 40°-50°C. for 15 min. Samples of these extractions were plated and counted to determine the radioactivity of the phospholipide fraction. The material still remaining was then dissolved in M KOH and incubated overnight (23). Protein and DNA were precipitated from this solution by bringing the pH to 3.5 with perchloric acid (9). This precipitate was removed by centrifugation, and a sample of the supernatant was plated and counted. The supernatant.

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fluid contained the 2',3'-ribonucleotides derived from RNA and also inorganic phosphate which was assumed to result from hydrolysis of phosphoprotein (PP). The inorganic and nucleotide phosphates were separated by electrophoresis of the mixture at pH 3.5 in 0.05 M ammonium formate (15), the different bands being located by radioautography and their relative radioactivities counted on the paper. In some cases the mixture was not resolved but was counted directly as RNA + PP. The precipitate resulting from digestion with KOH and precipitation by perchloric acid was washed with cold 5 per cent TCA, resuspended in water, plated, and counted. This is referred to as the DNA fraction.

RESULTS

When the Ehrlich ascites cells were incubated as above for 30 min., the incorporated radioactivity was found to be distributed as shown in Table 1. The acid-soluble compounds which became labeled were AMP (adenosine monophosphate), ADP (adenosine diphosphate), ATP, and the triphosphates of uridine and guanine (UTP and GTP). From 70 to 90 per cent of the radioactivity of the organic phosphates was present in the ADP and ATP. Two other phosphate esters which could not be identified (X fraction) also became labeled, particularly in the presence of glucose, either aerobically or anaerobically, but they were only found in significant amounts when the cells were extracted with 80 per cent ethanol prior to TCA. Ethanol extraction was not used routinely, since it did not give quantitative removal of the acid-soluble phosphates such as the nucleotides. Overexposure of the radioautographs revealed the presence of several other radioactive components of the acid-soluble fraction, but the activity of these fractions was very low.

Time course on P32 uptake and turnover.—Ehrlich ascites cells were incubated in the presence of 20 mM glucose in air and samples removed at various times. These samples were analyzed as described above, and the distribution of radioactivity in the various acid-soluble and acid-insoluble fractions was measured. The results obtained are shown in Chart 1, in which the total P32 uptake is classified under the headings of inorganic phosphate (obtained from the chromatograms), total soluble organic phosphate (total acid-soluble radioactivity minus radioactivity of inorganic phosphate), and total radioactivity of the acid-insoluble fraction. It may be seen that the activity

<table>
<thead>
<tr>
<th>Exp.</th>
<th>CONDITION</th>
<th>GLUCOSE CONCENTRATION</th>
<th>M/MOLS P32 IN ORGANIC PHOSPHATE/ML PACKED CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Air</td>
<td>Nil</td>
<td>5.220, 1.220, 770, 210, 100, 28, 193, 19</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>20 mM</td>
<td>8.300, 1.480, 1.140, 13, 230, 168, 700, 46, 270, 21</td>
</tr>
<tr>
<td>2</td>
<td>N2</td>
<td>Nil</td>
<td>5.210, 177, 177, 50, 43, 82, 8</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>20 mM</td>
<td>7.220, 1.500, 930, 344, 170, 280, 99, 350, 26</td>
</tr>
</tbody>
</table>

The cells were incubated at 37° C. for 30 min. in Krebs-Ringer solution containing 0.01 M phosphate, pH 7.4, and 100 #u. P32. After incubation the cells and their contents were fractionated as described.

* Abbreviations used: Pi, inorganic phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; GTP, guanine triphosphate; UTP, uridine triphosphate; X, unidentified phosphate esters; PL, phospholipide; PP+RNA, phosphoprotein+ribonucleic acid; DNA, deoxyribonucleic acid.

**TABLE 1**

**THE EFFECT OF GLUCOSE AND OXYGEN ON THE INCORPORATION OF P32 INTO THE VARIOUS FRACTIONS OF EHRlich ASCITES CELLS**

**CHART 1**—The rate of incorporation of P32 into various phosphorus-containing fractions of Ehrlich ascites cells in the presence of 20 mM glucose.

A: Inorganic phosphate.
B: Total acid-soluble organic phosphate.
C: Acid-insoluble phosphate compounds.

The standard incubation medium, containing a total of 34.5 #mole phosphate and 100 #u. P32 in 8 ml., was employed. The incubation was carried out at 37° C., and samples were removed at the times indicated.
of the inorganic phosphate continues to increase for the duration of the experiment quoted, although at a decreasing rate. In other, longer-term experiments the total activity of this fraction reached a maximum value after 60–90 min. and remained at that value for periods up to 4 hours. The radioactivity in the organic phosphate fraction reached a maximum value within 20–60 min., while the radioactivity in the total acid-insoluble fraction continued to increase for at least one hour.

The acid-soluble fraction was separated into its various constituents by the chromatographic method described under "Methods and Materials," and the activities of these fractions are shown in Table 1. The incorporation of P\textsuperscript{32} into ATP is seen to be the most rapid, but in most experiments the level of radioactivity reached a maximum value in 20–45 min. (Chart 2) and subsequently dropped below this value. In experiments of up to 5 hours' duration it became apparent that the level of radioactivity in the ATP could vary between quite wide limits but never rose above this initial maximum level and never fell to a level of less than one half of it. The level of radioactivity in the ADP was somewhat less than that in the ATP in Table 1 but was sometimes a little higher. It was always of the same order and always became constant after 20–40 min. The results obtained for the incorporation of P\textsuperscript{32} into GTP and UTP are typified by those shown in Table 1. Incorporation of radioactivity into AMP could not be detected for 30–30 min. but rose slowly after that interval.

The incorporation of P\textsuperscript{32} into the various acid-insoluble fractions is shown in Chart 3 and will be discussed in greater detail later.

The effect of glucose on P\textsuperscript{32} labeling under aerobic and anaerobic conditions.—Ascites cells were incubated for 30 min. in the presence and absence of glucose under aerobic and under anaerobic conditions. The various cellular constituents were then isolated, plated, and counted as described above, and the results shown in Table 1 were obtained.

It is at once apparent that, despite its effect on respiratory activity, there was no inhibitory effect of glucose on P\textsuperscript{32} turnover in the ascites cells. In fact, the presence of glucose always led to a stimulation of P\textsuperscript{32} uptake and incorporation which varied from 5 per cent to 50 per cent. These results are in harmony with the suggestion that the rate of ATP synthesis and turnover in ascites cells is not inhibited by the presence of glucose under aerobic conditions.

The results of Rabinovitz, Olsen, and Greenberg (20) demonstrated that amino acid incorporation in ascites cells is supported under anaerobic conditions if glucose is present. Further, the calculations of Quastel and Bickis (19) indicate that the rate of production of ATP should be the same under both aerobic and anaerobic conditions in the presence of glucose. It was, therefore, of interest to determine the effect of glucose on the incorporation of P\textsuperscript{32} under anaerobic conditions, and the results obtained in one such experiment are quoted in Experiment 2 of Table 1. A time course experiment was also conducted anaerobically in the presence and absence of glucose, and the course of labeling of the ATP is shown in Chart 3.

It may be seen from the results shown in Table 1 that incorporation of P\textsuperscript{32} into the various cellular fractions was minimal when both glucose and air were absent. If glucose was present the incorporation into the various phosphate compounds was not significantly affected by the presence or absence of oxygen. These effects are more clearly seen in Chart 3, where the incorporation of P\textsuperscript{32} into ATP is plotted against time. Again it can be seen that the incorporation into ATP under an-
aerobic conditions in the absence of glucose was only 5 per cent of that obtained when glucose, air, or both were present.

In the experiments quoted in Table 1 the effects of glucose were constant over at least the 1st hour of incubation, but on occasion a different type of response was obtained when the labeling of ATP was examined. In these cases, as shown in Chart 2, the amount of labeled phosphate incorporated into ATP reached a maximum after 20 min. and then declined. This result was observed when glucose was present under either aerobic or anaerobic conditions. However, the stimulation by glucose of the initial rate of incorporation into ATP was in the same range (20-50 per cent) as that observed in the cases in which no subsequent decrease in the labeling of the ATP was observed.

The effect of phosphate concentration.—In view of the possibility that the presence of glucose led to an increase in the actual phosphate content of the ascites cells, the effect of various levels of extracellular phosphate on the \( P^{32} \) turnover was examined. The results obtained are quoted throughout.

![Chart 3](image)

**Chart 3.**—The course of the labeling with \( P^{32} \) of the various acid-insoluble, phosphorus-containing fractions of Ehrlich ascites cells. Broken lines—no ascitic fluid added; full lines—ascitic fluid added, the amount being equal to half the final volume of the incubation medium; 20 mM glucose was present.

They indicate that increase in the extracellular concentration of inorganic phosphate led to an increase in the amount of radioactive phosphate associated with the intracellular inorganic phosphate and that the presence of glucose led to a still further increase. On the other hand, the addition of increasing amounts of inorganic phosphate had less effect on the equilibrium levels of ADP and ATP when these are expressed in terms of mmoles of \( P^{32} \) incorporated. When the final concentration of inorganic phosphate in the medium was below 8.2 mM there was evidence of a lesser incorporation of \( P^{32} \) into the adenine nucleotides. It was concluded that, if alteration of the inorganic phosphate level within the cell occurred, it could not be responsible for any change in the extent of labeling of the adenosine polyphosphates with \( P^{32} \), provided that the extracellular phosphate concentration was at least 8 mM. It is of interest to note that the addition of 20 mM glucose led to a decrease in the extent of labeling of the organic phosphate fractions of the cell when the phosphate concentration was 8.2 mM or less. At higher levels of phosphate...
the presence of 20 mM glucose caused a slight increase in the extent of labeling of the organic phosphates with $^{32}P$. The reason for the decrease at low levels of phosphate is probably the drop in pH as a result of the metabolism of glucose in an insufficiently buffered system (3, 5).

The effect of ascitic fluid.—It was shown by Harrington and Lavik (12) that addition of ascitic fluid to an in vitro preparation of Ehrlich ascites cells increased the rate of incorporation of radioactivity from formate-$C^{14}$ into the nucleic acids. Thomson et al. (24) have reported that a similar effect was obtained when an extract of liver cytoplasm was substituted for ascitic fluid. In the present work the incorporation of $^{32}P$ into the various fractions of Ehrlich ascites cells in the presence and absence of ascitic fluid has been studied. It was found that coagulation of the cells often occurred when they were incubated with ascitic fluid but that this coagulation could be prevented by addition to the medium of heparin, versene, or sodium oxalate at a concentration of 60 $\mu$g/ml. Addition of any of these anticoagulants did not affect the incorporation of $^{32}P$ into the cells (cf. ref. 14) and, in all subsequent experiments with ascitic fluid, heparin was added at this concentration. Since ascitic fluid was found to contain 5–7 mM inorganic phosphate, due allowance was made by adding less carrier phosphate when ascitic fluid was to be present (assuming the phosphate concentration of the fluid to be 6 mM).

When ascites cells were incubated with $^{32}P$ in the standard incubation medium in the presence and absence of ascitic fluid, there was found to be little effect of the ascitic fluid on the incorporation of radioactivity into the acid-soluble constituents of the cell. However, there was a definite effect on the incorporation of radioactivity into the various acid-insoluble fractions. The results from such an experiment are shown in Chart 3, which indicates the incorporation of $^{32}P$ at various times into the RNA, phosphoprotein (PP), phospholipide (PL), and DNA fractions of the ascites cells. It may be seen that the rate of incorporation of $^{32}P$ into these fractions decreased after 30 min. when the cells were incubated in

### TABLE 2

<table>
<thead>
<tr>
<th>Final phosphate concentration (mM)</th>
<th>Glucose concentration (mM)</th>
<th>mmoles P$_{32}$ phosphate taken up/ML cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic phosphate</td>
<td>Total organic phosphate</td>
<td>ADP</td>
</tr>
<tr>
<td>1.5</td>
<td>0</td>
<td>1,200</td>
</tr>
<tr>
<td>4.8</td>
<td>0</td>
<td>2,300</td>
</tr>
<tr>
<td>8.2</td>
<td>0</td>
<td>3,380</td>
</tr>
<tr>
<td>11.5</td>
<td>0</td>
<td>4,760</td>
</tr>
<tr>
<td>14.8</td>
<td>0</td>
<td>6,510</td>
</tr>
<tr>
<td>18.2</td>
<td>0</td>
<td>8,640</td>
</tr>
<tr>
<td>1.5</td>
<td>20</td>
<td>1,060</td>
</tr>
<tr>
<td>4.8</td>
<td>20</td>
<td>3,120</td>
</tr>
<tr>
<td>8.2</td>
<td>20</td>
<td>4,620</td>
</tr>
<tr>
<td>11.5</td>
<td>20</td>
<td>5,840</td>
</tr>
<tr>
<td>14.8</td>
<td>20</td>
<td>7,160</td>
</tr>
<tr>
<td>18.2</td>
<td>20</td>
<td>8,160</td>
</tr>
</tbody>
</table>

The cells were incubated aerobically at 37°C for 30 min. in Krebs-Ringer solution, the amounts of phosphate indicated, and approximately 100 $\mu$c. $^{32}P$. After incubation the cells and their contents were fractionated as described above.

The turnover of $^{32}P$ in labeled Ehrlich ascites cells.—Bolton et al. (4) have shown that it is possible to gain information concerning the pathways of phosphate metabolism by incubating cells for a short time in a medium containing radioactive phosphate and then following the redistribution of the isotope after replacement of the radioactive phosphate in the medium by nonradioactive...
phosphate. This technic has been applied to the study of P$^{32}$ incorporation in Ehrlich ascites cells by incubating the cells in the standard incubation medium containing P$^{32}$ and 20 mM glucose for 30 min. At the end of this period the cells were removed from the incubation medium by centrifugation, washed once with salts solution, and finally resuspended in fresh incubation medium containing 20 mM glucose but no P$^{32}$. The results obtained from such an experiment are shown in Chart 1. It will be noted that the radioactivity in all the acid-soluble and -insoluble fractions fell after the P$^{32}$ in the medium was replaced by P$^{31}$. However, when the experiment was repeated with the further addition throughout of ascitic fluid to the incubation medium the other results shown in Chart 4 were obtained. In this case, after replacement of the radioactive phosphate, the radioactivity of all the components of the soluble fraction fell, but the radioactivity of all the acid-insoluble fractions actually continued to rise after replacement of the P$^{32}$ in the external medium. This rise was probably at the expense of the acid-soluble fraction.

**DISCUSSION**

The results reported above have been developed in three main phases: (a) an investigation of the incorporation of P$^{32}$ into the acid-soluble and acid-insoluble fractions, (b) determination of the over-all effects of glucose on P$^{32}$ uptake and incorporation both aerobically and anaerobically, and (c) an investigation of the effects of ascitic fluid on P$^{32}$ metabolism.

It is apparent from the results shown in Chart 1 that the total radioactivity associated with the inorganic phosphate had usually approached a
limiting value after approximately 45 minutes of incubation. The simplest explanation of this result is that at this time the inorganic phosphate in the medium and that present inside the cell had come into isotopic equilibrium with each other, i.e., that their specific activities had become the same. Further, it would appear that the exchangeable phosphate of the ATP and the ADP had also come into isotopic equilibrium with the intracellular inorganic phosphate. The relative lack of radioactivity on the chromatograms in the ultraviolet-absorbing area occupied by adenylic acid indicates that only the pyrophosphate groups of the adenosine phosphates turn over at an appreciable rate. The results obtained in long-term experiments indicated that the final level of phosphate reached in the presence of glucose closely approximated that present in the incubation medium. In the absence of glucose the phosphate concentration inside the cell reached a lower level. The results obtained in experiments in which the phosphate concentration was varied tended to confirm this conclusion. The observations of Ibsen, Coe, and McKee (13) also indicate that the phosphate concentration inside the cells becomes equal to that of the medium. However, other results obtained by Ibsen et al. indicated that this equilibrium was attained within 5-15 minutes, and there was little effect of glucose. No immediate explanation for this discrepancy is apparent.

The results quoted in Experiment 1 of Table 1 are typical of those obtained in studies of the effects of 20 mM glucose on P32 turnover. The stimulation of the incorporation of P32 into the inorganic phosphate was 60 per cent, but the presence of 20 mM glucose led to a stimulation of only 20 per cent in the incorporation into the ATP. In other experiments the range of stimulation of the rate of labeling of the ATP was 10-50 per cent. The inhibition of respiration of these cells by glucose (the Crabtree effect) also seems to be without effect on the pattern of labeling of the ATP, ADP, and the organic phosphates. These results are in agreement with the calculations of Quastel and Bickis (19) concerning the effect of glucose on the production of ATP. Further, it is of interest that the turnover of P32 in the adenosine polyphosphates in the presence of 20 mM glucose under anaerobic and aerobic conditions was quantitatively and qualitatively constant. Quastel and Bickis (19) observed that, under similar circumstances, the theoretical yields of ATP were similar, as were the observed extents of incorporation of glycine-1-C14 into protein.

Previous studies (11, 12, 17, 18, 22, 24) on P32 incorporation into the acid-insoluble fractions of cells and particularly in ascites cells (11, 12, 17, 18, 24) have indicated that the kinetics of the incorporation into the acid-insoluble fractions were similar. The present results are in general agreement with this conclusion. It is clear from Chart 3 that the presence of ascitic fluid does not affect the rate of P32 incorporation into the acid-insoluble components but does affect the total amount incorporated by prolonging the period during which incorporation takes place at the initial high rate. There are two possible mechanisms by which P32 may be incorporated into the acid-insoluble components of the cell. First, incorporation would be expected to occur during de novo synthesis of these compounds, and, secondly, they may acquire radioactivity by exchange of their phosphate groups with radioactive phosphate groups present in certain components of the acid-soluble fraction. In the former case (synthesis) the flow of radioactivity would be unidirectional, whereas in the case of an exchange reaction it would be expected that the flow would be reversed if the isotopic equilibrium were altered. To assess the relative contributions of these two processes to the labeling of the acid-insoluble components in Ehrlich ascites cells, the experiments shown in Chart 4 were performed. In these experiments the radioactive phosphate in the medium was replaced by unlabeled phosphate after 30 minutes' incubation. As would be expected, all the components of the acid-soluble fraction, being in equilibrium with inorganic phosphate, rapidly exchanged radioactivity with that of the medium until a new lower equilibrium level of radioactivity was reached. When the experiment was performed with cells incubated under the standard conditions, the amount of radioactivity in the acid-insoluble fractions also fell to a new and constant level, indicating that these components were also in equilibrium with the phosphate of the medium. On the other hand, when the cells were incubated in the presence of ascitic fluid, although the radioactivity of the acid-soluble materials fell to a new equilibrium level, the radioactivity of the acid-insoluble fractions rose appreciably after the replacement. This striking difference indicates that incorporation into these components probably occurred mainly by exchange when the cells were incubated in salts alone, whereas much greater de novo synthesis occurred when ascitic fluid was present. A similar observation has been made by Harrington and Lavik (11), who obtained evidence concerning the effect of radiation on the incorporation of P32 into DNA in vivo and in vitro. They concluded that under in vitro conditions exchange was predominant but under in vivo con-
tions most of the incorporation occurred as a result of de novo synthesis.

SUMMARY

1. The addition of 20 mM glucose to respiring Ehrlich ascites carcinoma cells was accompanied by an increase in the uptake of P32-labeled phosphate and an increase in its incorporation into the various phosphorus-containing cellular constituents.

2. Under anaerobic conditions the incorporation of P32 was negligible in the absence of glucose, but in the presence of this metabolite both the uptake and the incorporation of P32 returned to the aerobic levels.

3. Increase in the concentration of inorganic phosphate in the incubation medium led to a corresponding increase in the concentration of P32 phosphate within the cell. The phosphate level did not influence the amount of P32 incorporated into the ATP or ADP, provided that it was 8 mM or greater.

4. Replacement of the P32 in the medium by P31 led to results which indicate that, in the presence of ascitic fluid, de novo synthesis of the acid-insoluble fractions accounted for most of the P32 incorporated. In the absence of ascitic fluid the incorporation of P32 took place largely as the result of freely reversible exchange processes.

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