In 1948, LePage (3) noted that, in tumor homogenates undergoing active anaerobic glycolysis, considerable pyruvate disappeared from the pyruvate-lactate pool. This problem was investigated further by Stoesz et al. (10), who made the first attempts at determining the products of the reaction. Under optimal conditions, the “pyruvate disappearance” approximated one-third of the lactate production, required diphosphopyridine nucleotide (DPN) and adenosine triphosphate (ATP), and was of the same magnitude both aerobically and anaerobically. Using C14-labeled pyruvate, Stoesz et al. (10) also found that only 5 per cent of the “pyruvate disappearance” could be accounted for by decarboxylation reactions and that no pyruvate was converted to oxaloacetate.

The purpose of these studies has been to determine the best conditions under which the reactions concerned with “pyruvate disappearance” occur in vitro and to establish the product or products resulting therefrom. The final optimal chemical medium used was found to differ only slightly from that described for maintaining phosphate bond energy in tumor homogenates (3).

METHODS AND RESULTS

For these in vitro studies, multiple transplants of the Flexner-Jobling carcinoma in female rats of the Holtzman-Rolfs-meyer strain were used throughout. The tumors were used 9–11 days after transplantation, since it was found that maximum activity was obtained when the tumors were essentially free of necrotic material and actively growing. The tumor weights ranged from 750 to 1,250 mg.

Immediately before each experiment, the tumor-bearing rat was decapitated, and the tumors were rapidly excised and placed in cold, isotonic KCl. After they had been freed of connective tissue and weighed, the tumors were homogenized in 9 volumes of cold, isotonic KCl in Potter-Elvehjem homogenizers.

The reactions were carried out in Warburg respirometer vessels, to which all the necessary constituents had been added previously to 0.8 ml. of the homogenate. The final volume in each reaction vessel was 3.0 ml. The flasks were incubated at 38°C for 60 minutes, and the reactions were stopped by the addition of 0.25 ml. of 26 per cent perchloric acid from the side-arm. All experiments were carried out under anaerobic conditions achieved by the evacuation technic (11).

It is desirable to work as rapidly as possible after the animal is killed, since the system under study is relatively labile, as illustrated by data in Table 1, which compares the “pyruvate disappearance” system to the glycolytic system.

Highly purified preparations of triphosphopyridine nucleotide (TPN) and concentrates of coenzyme A had no effect upon the fresh homogenates with regard to “pyruvate disappearance” and had no restorative effect upon the incubated homogenates.

<table>
<thead>
<tr>
<th>Incubation time (min.)</th>
<th>Lactate production (µM per flask)</th>
<th>Net P uptake (µM per flask)</th>
<th>Pyruvate disappearance (µM per flask)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.0</td>
<td>5.1</td>
<td>5.8</td>
</tr>
<tr>
<td>5</td>
<td>8.0</td>
<td>4.3</td>
<td>4.5</td>
</tr>
<tr>
<td>15</td>
<td>6.1</td>
<td>2.6</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The evaluation of the components in the medium is shown in Charts 1–8 and was based upon the measurements of CO2 evolution, phosphate uptake, and lactic acid production (cf. LePage and Umbreit [11]). Measurement of the extent of “pyruvate disappearance” was based on analyses for pyruvate uptake (2) and lactate production in the glycolytic system, blocked by fluoride at the phosphoglyceric acid step to the extent of 85–90 per cent (described below). Because of the fluoride block, pyruvate was added to the medium to act as hydrogen acceptor.

The medium of LePage (3) was used at first, and the components varied one at a time to determine optima. Charts 1–8 are plotted so that the amount of “pyruvate disappearance” from the pyruvate-lactate pool is related to the amount of each component added to each flask.
Pyruvate.—With fluoride present, added pyruvate serves as a hydrogen acceptor. Chart 1 shows that lowering the level of pyruvate tends to slow down the entire system, and higher than optimal levels decrease the amount of pyruvate that disappears. This may be due to a slight toxicity of the added pyruvate.

ATP and Inorganic Phosphate.—The need for phosphorylating conditions for “pyruvate disappearance” is seen in Charts 2 and 3. ATP is required to a much greater extent than is shown by the chart, since the presence of adenine nucleotide was demonstrated in the hexose diphosphate (HDP) used in this experiment. When HDP, which had been purified by ion-exchange chromatography on Dowex-1 (Cl form), was used, however, the addition of 1 μM of ATP per flask then stimulated the “pyruvate disappearance” by 250 per cent.

As the level of inorganic phosphate is increased, a point is reached where the amount of “pyruvate disappearance” increases at a rate greater than the increase in rate of lactate production.

Magnesium Ion.—Chart 4 shows the effect of added magnesium ion upon the system. If magnesium ion is required for “pyruvate disappearance”

charts 1–4.—The effects of varied concentrations of pyruvate, ATP, inorganic phosphate, and magnesium ion upon “pyruvate disappearance,” lactate production, and phosphate uptake.

charts 5–8.—The effects of varied concentrations of KF, DPN, HDP, and KHCO₃ upon “pyruvate disappearance,” lactate production, and phosphorus uptake.

Fluoride.—As is shown in Chart 5, fluoride has a multiple purpose: to partially inhibit glycolysis, to increase phosphorylative efficiency due to inhibition of phosphatases, and to stimulate “pyruvate disappearance.” At higher levels, however, the latter system is also inhibited. In addition, fluoride addition permits a quantitative estimation of the extent of the “pyruvate disappearance,” since little pyruvate is contributed to the pyruvate-lactate pool from triosephosphate metabolism. Since the degree to which fluoride inhibited at the phosphoglyceric acid step was important in determining the amount of “pyruvate disappearance,” experiments were carried out in which only priming levels of pyruvate were used, and the lactate production was measured at the various levels of fluoride. These data are presented in Table 2.

Diphosphopyridine nucleotide.—The effect of varied DPN concentration is shown in Chart 6. The requirement of DPN for “pyruvate disappearance” is seen to parallel closely that of the glycolytic system. When the glucose and hexose phosphate substrates were omitted, the system showed a definite requirement for reduced DPN.

The addition of approximately 10 μM of DPN·2H in a typical experiment yielded 4.7 μM of lactate and 2.6 μM of "pyruvate disappearance" products. Oxidized DPN had no effect under these circumstances. The addition of lactate had no effect upon this system, either in the presence of reduced DPN without substrate, or in the complete medium.

**Hexosediphosphate.**—The effect of varying the concentration of HDP is shown in Chart 7.

**Initial pH.**—The initial pH measurements were made as described by LePage (3). It may be seen in Chart 8 that the amount of pyruvate which disappears is dependent upon hydrogen ion concentration, there being an effective working range of 0.5–0.6 pH units.

### TABLE 2

<table>
<thead>
<tr>
<th>KF-final molarity</th>
<th>% inhib</th>
<th>at the phospho-</th>
<th>glyceric acid</th>
<th>step</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.00167</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>0.00333</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>0.00667</td>
<td>83</td>
<td>83</td>
<td>83</td>
<td>83</td>
</tr>
<tr>
<td>0.0100</td>
<td>88</td>
<td>88</td>
<td>88</td>
<td>88</td>
</tr>
</tbody>
</table>

* This figure was used to calculate the corrected pyruvate-lactate pool in subsequent experiments.

The final medium selected for further experiments is given in Table 3.

A number of experiments were done in an attempt to characterize and isolate the products of the system described above. It seemed likely that the "pyruvate disappearance" reactions involved a reduction, as demonstrated by the following type of experiment. With the use of the complete medium minus glucose, and the addition of 6 μM of HDP, the system was run until it failed. All the HDP was shown to be gone, and no triosephosphate remained. Yet only 7.5 μM of lactate appeared instead of a theoretical 12. Hence, the unknown products must have been formed by the addition of the extra 9 micro-equivalents of hydrogen to pyruvate or lactate, giving a molecule with at least two more hydrogens than pyruvate per 3 carbon atoms from the pyruvate-lactate pool.

Experiments to determine the extent of mixing through the fluoride block were conducted with pyruvate-1-C\(^14\) and pyruvate-2-C\(^14\). Reaction mixtures were incubated 0(T\(_0\)) and 60(T\(_{60}\)) minutes and were inactivated with perchloric acid. The pyruvic acid was isolated from each flask as the 2,4-dinitrophenylhydrazone by the method of LePage (5). The isolated derivatives were plated on paper and the specific activity (sp. act.) determined, with corrections for the self-absorption of the paper plate and analyses for the pyruvate derivative on the plate. In the T\(_0\) mixture, the pyruvate-1-C\(^14\) had a sp. act. of 1,650 counts/min/μM. This was checked by isolation of the phenylhydrazine derivative, which had a specific activity of 1,650 counts/min/μM upon combustion and counting as BaCO\(_3\). In the T\(_{60}\) mixture, the dinitrophenylhydrazones had sp. acts. of 1,380 counts/min/μM and 1,650 counts/min/μM for pyruvate-1-C\(^14\) and pyruvate-2-C\(^14\), respectively, which gave the ratios:

\[
\frac{T_{60}}{T_0} (\text{pyruvate-1-C}^{14}) = 0.835 ;
\]

\[
\frac{T_{60}}{T_0} (\text{pyruvate-2-C}^{14}) = 0.890 .
\]

From the T\(_{60}\) mixtures, lactic acid was also isolated as zinc lactate, which was purified, combusted, and counted as BaCO\(_3\). The ratios of the sp. acts. were as follows (using the sp. act. of the pyruvate for the T\(_0\)):

\[
\frac{T_{60}}{T_0} (\text{pyruvate-1-C}^{14}) = 0.885 ;
\]

\[
\frac{T_{60}}{T_0} (\text{pyruvate-2-C}^{14}) = 0.915 .
\]

Average of all four determinations = 0.881.

Thus, the leak through the fluoride block at 0.01 M KF was approximately 12 per cent, in agreement with that determined by a different method described earlier.

Moreover, mixing through the block does not
occur, as shown by the finding that a negligible amount of radioactivity occurred in the Ba-insoluble phosphate esters, which contain the phosphoglyceric acid of the reaction mixtures. For example, with pyruvate-1-C\(^{14}\) of sp. act. 1,650 counts/min/\(\mu\)M, 9.0 \(\mu\)M of lactate were found per flask and 3.0 \(\mu\)M of "pyruvate disappearance" products in a 60-minute incubation. Hence, greater than 12 \(\mu\)M of phosphoglyceric acid should be formed in order to account for the hydrogens. However, only 80 counts/min were found in the Ba-insoluble fraction out of 4,950 counts/min entering the "pyruvate disappearance" reactions.

Among the possibilities considered as a product of the "pyruvate disappearance" reactions was alanine. In an experiment with pyruvate-1-C\(^{14}\) (1,500 counts/min/\(\mu\)M), a "pyruvate disappearance" of 3.7 \(\mu\)M/flask was observed. Decarboxylation with ninhydrin was carried out by standard methods (7, 12). A 1.5-ml. aliquot of the flask contents was neutralized to pH 4.3–5.0 and diluted to 2.0 ml.; 200 mg. of KH\(_2\)PO\(_4\) and 100 mg. of ninhydrin were added, and the mixture boiled gently in a closed system for 30 minutes. The CO\(_2\) was collected in alkali, precipitated, and counted as BaCO\(_3\). In a T\(_0\) control, 68 counts/min/flask appeared in the CO\(_2\), whereas flasks incubated 60 minutes gave 192 counts/min/flask. Additional compounds reacting with ninhydrin to yield CO\(_2\), which was absorbed after the incubation, by injecting KOH through a rubber nipple on the side-arm vent of the respirometer vessel. In the flasks with actively metabolizing tissue, 2.9 \(\mu\)M of pyruvate disappeared per flask, and 194 counts/min appeared in the CO\(_2\). Thus, only 3.7 per cent of the pyruvate which disappeared was decarboxylated (Table 4).

Since pyruvate might have been converted into \(C_4\) acids by CO\(_2\)-fixation in these experiments, homogenates of liver and tumor were compared as to their rates of fixation. Duplicate flasks were used in each case, with \(C^{14}\)O\(_2\) added as K\(_2\)C\(^{14}\)O\(_3\). The reaction mixtures were deproteinized with perchloric acid after a 60-minute incubation and aerated vigorously for 1 hour; solid CO\(_2\) was added, and the aeration was repeated. The solutions were then combusted and counted for radioactivity. Since TPN is needed for one of the CO\(_2\) fixation reactions known to be present in liver (8), 200 \(\mu\)g.

### TABLE 5

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Counta/min/flask</th>
<th>Total counts/min entering the pyruv.</th>
<th>Per cent of total counts/min accounted for</th>
<th>Countb/min</th>
<th>Total counts/min fixed as (C^2) and (C_1)</th>
<th>CO(_2)-FIXATION IN 30-MINUTE EXPERIMENTS WITH 15 MG. OF RAT LIVER HOMOGENATE OR 30 MG. OF FLEXNER-JOBLING CARCINOMA HOMOGENATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine production</td>
<td>120</td>
<td>4,440</td>
<td>2.9</td>
<td>92</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>Formate production</td>
<td>988</td>
<td>4,070</td>
<td>5.9</td>
<td>770</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Decarboxylation</td>
<td>110</td>
<td>3,500</td>
<td>5.9</td>
<td>7,150</td>
<td>1.19</td>
<td></td>
</tr>
</tbody>
</table>

of TPN were added per flask to corresponding flasks of the experiment. The addition of TPN did not affect the "pyruvate disappearance." The results appear in Table 5. It is evident that the fixation reaction is not significant in the tumor homogenates, under conditions which permit good CO\(_2\)-fixation in liver homogenates.

The four previous experiments suggested a 3-carbon chain in the products of pyruvate metabolism. It was noted earlier that these products must be reduced compounds with the addition of at least two hydrogens per 3-carbon unit. Hence, the possibilities to be considered must be neutral compounds or their phosphorylated derivatives. The
following procedure was adopted: after a 60-minute incubation in the presence of pyruvate-2-\(^{14}C\), the flask contents were neutralized to pH 8.2 with concentrated KOH, and 0.1 ml of 1 M Ba\(\text{Cl}_2\) was added. The precipitate was collected by centrifugation in the cold. Four volumes of cold 95 per cent EtOH were then added to the supernate, and the precipitate was collected and washed with cold EtOH. The supernate was evaporated in vacuo to its original volume to remove the alcohol, then passed successively through columns of Dowex-50 (H form) and Dowex-1 (Acetate form). These three fractions, the Ba-insoluble, the Ba-soluble-alcohol-insoluble, and neutral fractions, respectively, were combusted and counted as BaCO\(_3\).

### TABLE 6

<table>
<thead>
<tr>
<th>Experiment</th>
<th>(\mu) of (^{14}C) found/\text{flask}</th>
<th>Ba-soluble-alc.-insol.</th>
<th>Ba-insoluble</th>
<th>alc.-insol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4.11</td>
<td>0.341</td>
<td>2.88</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>4.05</td>
<td>0.109</td>
<td>2.18</td>
<td></td>
</tr>
</tbody>
</table>

The results from two such experiments are given in Table 6. Since the amount of radioactivity found in the neutral fraction did not exceed that found in \(T_0\) mixtures, fractionated the same way, it was concluded that no free neutral metabolites of pyruvate were formed in this system, and this fraction was omitted from further consideration.

The Ba-soluble-alcohol-insoluble fraction contained radioactivity representing more than 50 per cent of the pyruvate which had disappeared, and a procedure was devised to account for a large proportion of the counts in this fraction. When these salts were dissolved in 1 N HCl and heated for 3 hours at 100°C, then reprecipitated with barium and alcohol, 18.5 per cent and 42.5 per cent of the radioactivity was retained in experiments I and II, respectively. The barium salt of 1,2-propanediol-1-phosphate (PDP)\(^a\) was then added as carrier, and the salts were dissolved in 1 N HCl and heated again for 3 hours at 100°C. The solution was cooled, made 0.5 N with respect to KOH, and again heated for \(\frac{1}{2}\) hour at 100°C. This procedure hydrolyzes all sugar phosphates and all other known phosphate esters except that of propanediol. The solution was cooled, neutralized to pH 8.2, and basic lead acetate added in excess. The lead salts were decomposed with H\(_2\)S, the PbS centrifuged off, and the solution aerated free of H\(_2\)S. The solution was neutralized to pH 8.2, and excess Ba\((\text{Ac})_2\)

was added, plus 4 volumes of cold 95 per cent EtOH. The precipitate was plated directly on thin aluminum plates and the specific activity of the PDP determined by counting and eluting each plate and analyzing for total phosphorus. These results are presented in Table 7. It is to be noted that the above procedure is not a method which accounts quantitatively for the total PDP formed, and thus the values given represent minimal values.

A preliminary investigation of these reactions was undertaken with several other tissues. In rat liver, brain, and kidney, the radioactivity disappearing from the pyruvate-lactate pool was accounted for mainly in the Ba-soluble-alcohol-insoluble fraction of the reaction mixtures.

### DISCUSSION

The metabolism of pyruvate has been shown to follow a great variety of pathways. Potter and Heidelberger (9) noted that at least eight different means of utilizing pyruvate are present in animal tissues. Among these are conversion to lactate, alanine, serine; condensation to C\(_4\) compounds; and decarboxylation to C\(_3\) and C\(_1\) fragments. In the tumor homogenate system used here, under anaerobic conditions, conversion to lactate was the main pathway. Except for this, all other known reactions concerning the metabolism of pyruvate were shown to be negligible, relative to a new reaction which resulted from a reduction of pyruvate and had a magnitude comparable to the conversion to lactate. Evidence obtained from carrier experiments strongly suggests that propanediol phosphate (CH\(_3\)CHOHCH\(_2\)OPO\(_3\)H\(_2\)) represents a large part of the reduced products. Further evidence of this identity is being accumulated. In addition, tracer experiments with the radiocarbon-labeled pyruvate indicate that a second component occurs in the Ba-soluble-alcohol-insoluble

\(^a\) Prepared by the method of Lindberg (6).
fraction, which is hydrolyzed by treatment with acid. Preliminary indications are that this is acetol phosphate (CH₃COCH₂OPO₃H₂), which would be a logical intermediate in the reduction of pyruvate to propanediol phosphate. It appears that the reduction of the carboxyl group is preceded by a phosphorylation.

Experiments are now in progress to isolate the proposed intermediate, acetol phosphate, and to evaluate the significance of these reactions to the metabolism of both normal and neoplastic tissues.

SUMMARY

The anaerobic glycolysis system with homogenates of Flexner-Jobling carcinoma was shown to metabolize pyruvate to reduced products other than lactate to an extent almost comparable in magnitude to the conversion to lactate. The reactions were studied with pyruvate-1-C¹⁴ and pyruvate-2-C¹⁴. The metabolism of pyruvate to alanine, serine, to C₄, C₂, and C₁ was shown to be negligible in this system. A new reaction was found in which the pyruvate was converted to propanediol phosphate, with acetol phosphate the probable intermediate. The significance of these findings to the metabolism of normal and neoplastic tissues is being investigated.

REFERENCES

Metabolism of Pyruvate in Tumor Homogenates


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