The Oxidative Metabolic Pattern of Mouse Hepatoma C-954 as Studied with C\textsuperscript{14}-labeled Acetates, Propionate, Octanoate, and Glucose*

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Weinhouse and co-workers (39–42) have established that the Krebs tricarboxylic acid cycle operates in a variety of experimental tumors and that fatty acid oxidation, believed to be nonexistent in tumors (9), provides 2-carbon fragments for continuous operation of this cycle in neoplastic tissues. Our studies with the mouse hepatoma C-954, carried by mice of the C57-Leaden strain, also led us to conclude that the oxidation of octanoate by tumor tissue followed the same course as in normal tissues (4, 8). The labeling of acetoacetate, formed after incubation of tumor slices with octanoate-1-C\textsuperscript{14}, was consistent with the mechanism of \( \beta \)-oxidation-cleavage-condensation observed in normal tissues (4, 8).

The present experiments were undertaken to determine whether any well defined derangement existed in the metabolism of a neoplastic tissue. Comparisons were made of normal mouse liver, host liver, and hepatoma C-954, all of which were allowed to oxidize radioactive substrates in vitro. Acetate-1-C\textsuperscript{14}, acetate-2-C\textsuperscript{14}, octanoate-1-C\textsuperscript{14}, propionate-1-C\textsuperscript{14}, pyruvate-2-C\textsuperscript{14}, and uniformly labeled glucose were employed as substrates. Incorporation of C\textsuperscript{14} from the substrates into various intermediates was studied by a chromatographic-autoradiographic technic. The present experiments with hepatoma C-954 fully confirm, in general, the observations of Weinhouse and co-workers (39–42) and extend those observations to include, in the metabolic spectrum of tumor tissue, several reactions peripheral to the tricarboxylic acid cycle. Most reactions observed in the normal liver proceed in the hepatoma, although certain differences do exist.

MATERIALS AND METHODS

**Labeled substrates.**—Sodium acetate-1-C\textsuperscript{14} and -2-C\textsuperscript{14} and sodium propionate-1-C\textsuperscript{14} were synthesized in this laboratory by Dr. S. Abraham, by conventional methods. Sodium octanoate-1-C\textsuperscript{14} was kindly supplied by Dr. W. G. Dauben of the Department of Chemistry. Dr. Abraham also prepared uniformly labeled glucose (8) photosynthetically by the method of Putman and Hassid (81). Pyruvate-8-C\textsuperscript{14} was purchased from Isotope Specialties, Inc.

**Buffers.**—Krebs-Ringer phosphate and Krebs-Henseleit bicarbonate buffers were prepared as described by Umbreit et al. (84).

**Animals and their tissues.**—The hepatocarcinoma C-954 carried by mice of the C57-Leaden strain was originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. We are indebted to Mr. Carl Emanuel for maintaining this tumor by serial subcutaneous transplantation. Tumors and livers were removed about 2 weeks after implantation of the hepatoma. (The mice rarely survive more than 8 weeks after implantation.) The tumors selected in this study always appeared to be free of necrosis.

In vitro procedures.—Slices of normal liver, hepatoma, or host liver were prepared either free-hand or with the aid of a McIlwain-Buddle (86) automatic tissue slicing machine (Mickle Co.). Whenever the tumor tissue was not solid enough to slice, the loose tissue was merely suspended in the incubation medium. Flasks containing center wells for collection of respiratory CO\textsubscript{2} were employed. The tops of the flasks were closed with rubber caps (19) through which acid could be injected to stop reactions. Pertinent details of the incubation in the several experiments are given in the tables.

**Analytical procedures.**—Respiratory CO\textsubscript{2}, collected in the alkaline center wells of the flasks, was precipitated as BaCO\textsubscript{3} and assayed for C\textsuperscript{14} (18). Aqueous extracts of the reaction mixture were examined by the chromatographic-autoradiographic technic (8) as applied to animal tissues (77). After the extracts were desalted electrolytically (10), they were subjected to two-dimensional chromatography on Whatman No. 1 filter paper sheets (18 X 22 in.). The solvents employed were phenol saturated with water, in the short dimension, and n-butanol-acetic acid-water (50:11:22) in the long dimension. Autoradiograms were prepared by placing the air-dried paper chromatograms in contact with 14 X 17-in. "No-Screen" x-ray films (Eastman Kodak Co.). Radioactive areas of the chromatograms were outlined and were assayed for C\textsuperscript{14} with a 2.5-inch diameter, thin end-window Geiger tube (8). Amino acid areas were detected or verified with ninhydrin, glucose
with anilino-trichloroacetic acid (or tetrazolium chloride), and acid areas with bromcresol green. Spots obtained on the autoradiograms and/or chromatograms were identified by reference to standard chromatographic maps. Intermediates were identified by their relative positions, rather than by their Rf's in the solvents (5).

Where applicable, aliquots of aqueous extracts were steam-distilled in the presence of H2SO4. Acetocetate is lost in this step. The volatile fraction obtained corresponds to unreacted, volatile, isotopic substrates (acetate, octanoate, propionate), while the nonvolatile fraction corresponds to those compounds appearing on the paper chromatograms. (When glucose was employed as substrate, no distillation was carried out.) Aliquots of the acid-volatile and nonvolatile fractions were made basic and counted directly on aluminum planchets. Appropriate mass correction factors were used whenever necessary.

The incorporation of isotope into each compound resolved by the chromatographic-autoradiographic method was first expressed as a fraction or percentage of the total counts/min found on the chromatograms. The per cent of the incubated counts/min incorporated into any compound was then given by the expression: per cent of incubated counts/min found in nonvolatile fraction X (counts/min in compound on paper divided by total counts/min on paper).

It was observed that unreacted octanoate-1-C14 was not completely volatilised from the chromatograms. Therefore, in expressing the percentage of the total counts/min recovered in any compound on the chromatograms, the C14 of the area corresponding to isotopic octanoate was not included in the total counts/min.

The per cent of incubated counts/min found for any compound is independent of the total counts/min on the paper chromatograms. Values in the tables usually represent the average percentages obtained from several chromatograms. The reproducibility which may be attained is illustrated by the average percentages obtained from several chromatograms. Values in the tables usually represent the average percentages obtained from several chromatograms. The reproducibility which may be attained is illustrated by the average percentages obtained from several chromatograms. 

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Resired CO2 fraction</th>
<th>Nonvolatile</th>
<th>Volatile</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver</td>
<td>48.4</td>
<td>38.5</td>
<td>12.8</td>
<td>93.7</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>38.7</td>
<td>22.5</td>
<td>2.4</td>
<td>63.6</td>
</tr>
<tr>
<td>Host liver</td>
<td>40.4</td>
<td>36.7</td>
<td>7.3</td>
<td>93.4</td>
</tr>
</tbody>
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</table>
the glutamate and glutamine areas after spraying with ninhydrin, these two amino acids were present on the chromatograms in approximately the same amount. It seems that the rate of conversion of glutamate to glutamine is limited in the hepatoma.

The hepatoma seemed to have little capacity to form radioactive glucose, although detectable quantities of glucose (as revealed by spray reagents) were present on the paper chromatograms. This glucose may have been derived from the blood stream. It is thus conceivable that, even in vivo, the hepatoma synthesizes little or no glucose. This point requires further study. In this connection it should be noted that Zamecnik et al. (46) also found that gluconeogenesis (from pyruvate-2-C14) in a rat hepatoma was much less than in slices of normal liver.

In this preliminary experiment the percentage of isotope recovered in glucose in the case of normal liver, while higher than that for hepatoma, was anomalously low.

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A COMPARISON OF THE OXIDATION OF ACETATE-1-C14 AND OCTANOATE-1-C14 IN HEPATOMA AND HOST LIVER SLICES

According to reports from other laboratories (5, 30), acetate appears to be poorly activated to the reactive metabolic 2-carbon fragment (acetyl-S-CoA) by neoplastic tissues. However, the short-chain fatty acids, butyrate and octanoate, were shown to be readily converted to CO2 and acetoacetate by the mouse hepatoma C54 (8). In the preliminary experiments reported above it was shown that the isotope of octanoate-1-C14 was incorporated into intermediates by the hepatoma in a fashion similar to that of normal or host liver. It was, therefore, of interest to extend these observations to see how acetate compared with octanoate in its ability to label various intermediates. The experimental protocol and the results are presented in Table 3.

In both liver and hepatomas, octanoate-1-C14 was utilized in preference to acetate as judged by (a) conversion to C4O2, (b) incorporation into the

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TABLE 2
INCORPORATION OF ISOTOPE FROM OCTANOATE-1-C14 INTO NONVOLATILE COMPOUNDS

Corresponds to experiments reported in Table 1)

<table>
<thead>
<tr>
<th>COMPOUNDS</th>
<th>NORMAL LIVER</th>
<th>HEPTOMA C54</th>
<th>HOST LIVER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per cent of nonvolatile counts/min</td>
<td>Per cent of incubated counts/min</td>
<td>Per cent of nonvolatile counts/min</td>
</tr>
<tr>
<td>β-Hydroxybutyrate</td>
<td>33.5</td>
<td>19.0</td>
<td>71.7</td>
</tr>
<tr>
<td>Glutamate</td>
<td>14.1</td>
<td>4.6</td>
<td>40.3</td>
</tr>
<tr>
<td>Glutamine</td>
<td>6.7</td>
<td>2.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Di- and tri-COOH acids</td>
<td>6.0</td>
<td>2.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Aspartate</td>
<td>3.3</td>
<td>1.1</td>
<td>3.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.2</td>
<td>0.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.2</td>
<td>0.7</td>
<td>4.6</td>
</tr>
<tr>
<td>Pyrrolidone-COOH acid</td>
<td>1.2</td>
<td>0.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.1</td>
<td>0.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Urea</td>
<td>1.1</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Others (unidentified)</td>
<td>0.8</td>
<td>0.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Glutathione</td>
<td>0.8</td>
<td>0.3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

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TABLE 3
INCORPORATION OF ISOPTOE FROM ACETATE-1-C14 AND OCTANOATE-1-C14 INTO VARIOUS FRACTIONS BY HOST LIVER AND HEPATOMA C54 OF MICE

(Bicarbonate Buffer)

(0.50 gm. slices [wet wt.] incubated for 3 hours in 5.0 ml. Krebs-Henseleit bicarbonate buffer, pH 7.4, 37° C.; 95 per cent O2-5 per cent CO2 as gas phase. Concentration of acetate, 0.008 M; of octanoate, 0.002 M. Reactions stopped with HCl + heat. Tissues from several animals were pooled.)

<table>
<thead>
<tr>
<th>TISSUE AND SUBSTRATE</th>
<th>RECOVERED ACETOCETATE</th>
<th>PER CENT OF INCUBATED C14 RECOVERED AS:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Requirements</td>
<td>Nonvolatile fraction</td>
</tr>
<tr>
<td>Liver:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate-1-C14</td>
<td>8.8</td>
<td>1.1</td>
</tr>
<tr>
<td>Octanoate-1-C14</td>
<td>20.5</td>
<td>16.3</td>
</tr>
<tr>
<td>Acetate-1-C14+octanoate</td>
<td>5.1</td>
<td>0.67</td>
</tr>
<tr>
<td>Hepatoma:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate-1-C14</td>
<td>3.3</td>
<td>0.15</td>
</tr>
<tr>
<td>Octanoate-1-C14</td>
<td>16.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Acetate-1-C14+octanoate</td>
<td>1.4</td>
<td>0.10</td>
</tr>
</tbody>
</table>

* Determined from slices incubated under conditions identical with those from which the nonvolatile and volatile fractions were analyzed. Carrier acetocetate acid was added before analysis.
nonvolatile fraction, (c) incorporation into individual intermediary products, and (d) conversion to acetoacetate. The molar concentration of the labeled acetate incubated was 4 times that of the octanoate, so that an equivalent number of potential 2-carbon fragments were represented. When nonisotopic octanoate was incubated with acetate-1-C\textsubscript{14}, an inhibition in the conversion of the isotope to CO\textsubscript{2}, nonvolatile fraction, intermediary products, and acetoacetate was observed for both liver and hepatoma.

It is clear from the data of Table 3 that the utilization of acetate for CO\textsubscript{2}, acetoacetate, and nonvolatile fraction was less in the hepatoma than in liver. It may be calculated that labeled CO\textsubscript{2} was produced from the substrate at the rate of 39 m\textmu M X (gm. wet wt. X min.)\textsuperscript{-1} by host liver, but at only 15 m\textmu M X (gm. wet wt. X min.)\textsuperscript{-1} by the hepatoma.

The nonvolatile compounds in the experiments of Table 3 were examined, and the data are summarized in Table 4. In general, the relative distribution of isotope in the various compounds of the nonvolatile residue was the same with acetate-1-C\textsubscript{14} and octanoate-1-C\textsubscript{14}. These compounds have been arranged arbitrarily in three groups according to the percentages of isotope (with respect to the total C\textsubscript{14} in all compounds found on the paper chromatograms). The three arbitrary groups are: major incorporation products, >5 per cent; intermediate incorporation products, 1–5 per cent; and minor incorporation products, <1 per cent; these values represent percentages of isotope of the nonvolatile residues.

**Host liver.—**Glutamine, \(\beta\)-hydroxybutyrate, glucose, and glutamate were major incorporation products for acetate-1-C\textsubscript{14}, octanoate-1-C\textsubscript{14}, and for acetate-1-C\textsubscript{14} in the presence of nonisotopic octanoate (Table 4). While \(\beta\)-hydroxybutyrate (22.2 per cent) and glutamine (23.4 per cent) were about the same when derived from acetate-1-C\textsubscript{14}, a marked difference in distribution of isotope was observed when \(\beta\)-hydroxybutyrate (57.9 per cent) and glutamine (12.0 per cent) were derived from octanoate-1-C\textsubscript{14}. Decrease of isotope from octanoate-1-C\textsubscript{14} in glutamine and most compounds (except \(\beta\)-hydroxybutyrate) was a consequence of the greater ketogenic activity of octanoate as compared with acetate.

Intermediate incorporation products in host liver were lactate, alanine, and urea. The di- and tricarboxylic acids (taken as a group) showed some variability.

Minor incorporation products were glutathione, aspartate, and unidentified compounds at the origin. Since there was no evidence of appreciable amounts of radioactive glycine or cysteine, the glutathione probably contained only the labeled amino acid residue, glutamate.

When acetate-1-C\textsubscript{14} was incubated with nonisotopic octanoate, the distribution of C\textsubscript{14} among the compounds on the chromatogram did not resemble that when acetate-1-C\textsubscript{14} alone or octanoate-1-C\textsubscript{14} alone was incubated. The conclusion may be drawn that 2-carbon fragments derived from acetate-1-C\textsubscript{14} interacted with those derived from nonisotopic octanoate, and the C\textsubscript{14} thereby assumed a metabolic pattern somewhere between that of the two labeled substrates.

It can be noted that, when acetate-1-C\textsubscript{14} was incubated with nonisotopic octanoate, acetate participated in the ketogenic activity of octanoate.

### Table 4

**INCORPORATION OF ACETATE-1-C\textsubscript{14} AND OCTANOATE-1-C\textsubscript{14} INTO NONVOLATILE COMPOUNDS**

\[
\begin{array}{|c|c|c|c|}
\hline
\text{INTEGRATION PRODUCTS} & \text{HOST LIVER SLICES} & \text{HEPATOMA SLICES} & \text{ACETATE-1-C\textsubscript{14}} \\
\hline
\text{Major:} & \text{Glutamine (25.4)} & \text{Glutamine (58.7)} & \text{Glutamate (40.0)} \\
>5 \text{ per cent of nonvolatile counts/min} & \beta\text{-Hydroxybutyrate (97.3)} & \beta\text{-Hydroxybutyrate (60.4)} & \text{Di- and tri-COOH acids (18.5)} \\
& \text{Glucose (8.5)} & \text{Glucose (10.5)} & \text{Glucose (10.5)} \\
& \text{Pyruvate (10.5)} & \text{Pyruvate (10.5)} & \text{Glucose (10.5)} \\
& \text{Lactate (5.7)} & \text{Lactate (5.7)} & \text{Lactate (5.7)} \\
& \text{Aspartate (5.2)} & \text{Aspartate (5.2)} & \text{Aspartate (5.2)} \\
\hline
\text{Intermediate:} & \text{Lactate (4.7)} & \text{Lactate (4.7)} & \text{Pyruvate (4.8)} \\
1–5 \text{ per cent of nonvolatile counts/min} & \text{Glutamate (1.7)} & \text{Glutamate (1.7)} & \text{Glutamate (4.8)} \\
& \text{Alanine (1.4)} & \text{Alanine (1.4)} & \text{Alanine (1.4)} \\
& \text{Urea (1.4)} & \text{Urea (1.4)} & \text{Urea (1.4)} \\
& \text{Acetate (1.4)} & \text{Acetate (1.4)} & \text{Acetate (1.4)} \\
\hline
\text{Minor:} & \text{Glutathione (0.9)} & \text{Glutathione (0.9)} & \text{Alanine (0.9)} \\
<1 \text{ per cent of nonvolatile counts/min} & \text{Aspartate (0.7)} & \text{Aspartate (0.7)} & \text{Pyruvate (0.7)} \\
& \text{Origin (0.8)} & \text{Origin (0.8)} & \text{Glutamic acid (0.8)} \\
& \text{Aspartate (trace)} & \text{Aspartate (trace)} & \text{Glutamine (0.8)} \\
\hline
\end{array}
\]

* Percentages of isotope incorporation do not necessarily add up to 100 per cent, since they represent average values.

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This special property of octanoate may be traced to the terminal-type 2-carbon fragment (11) peculiar to fatty acids of the even series. The different patterns for acetate and octanoate are traceable, in some measure, to the predilection of this 2-carbon fragment for the carbonyl moiety of acetoacetate and of β-hydroxybutyrate. The reactivities of the 2-carbon fragments of acetate and fatty acids are discussed at length elsewhere (7, 11, 24).

**Hepatoma C₅₄.**—The incorporation of the C¹⁴ of acetate-1-C¹⁴ into CO₂, acetoacetate, and nonvolatile compounds by the hepatoma was less than one-third that observed for host liver (Table 3). β-Hydroxybutyrate was the predominant radioactive compound in host liver (as was glutamine in experiments with acetate-1-C¹⁴), but the major incorporation products from tumor with octanoate-1-C¹⁴ were, in generally decreasing order: glutamate, di- and tricarboxylic acids (taken as a group), β-hydroxybutyrate, lactate, aspartate, glutamine. A few inversions in this order occurred with acetate-1-C¹⁴, but the six compounds were nevertheless members of the major incorporation products. Intermediate incorporation products observed in tumor experiments with octanoate-1-C¹⁴ were alanine, glutathione, and the origin.

Several features of the metabolic pattern of the hepatoma deserve comment. The experiments with octanoate-1-C¹⁴ will be discussed here, although the comments also apply generally to experiments with acetate-1-C¹⁴. In the experiment under consideration ketogenesis was greater in the host liver than in the hepatoma as judged by acetoacetate (Table 3) or β-hydroxybutyrate formation (Table 4).

The data of Table 4 emphasize that (aside from β-hydroxybutyrate, glutamine, and glucose) all compounds in the hepatoma experiment contained greater percentages of the nonvolatile C¹⁴ than was the case with host liver. Quite striking is the difference in the value for the glutamate-C¹⁴/glutamine-C¹⁴ ratio. This ratio had a value of 0.58 in host liver, but a value of 6.75 in hepatoma as a result of both an increased isotope incorporation into glutamate and a decreased incorporation into glutamine. Note that glutamate accounted for one-third of the isotope of the nonvolatile fraction in the case of hepatoma.

Another metabolic shift found in the hepatoma was evidenced by the relatively larger amounts of isotope incorporated into the di- and tricarboxylic acids. While these acids constituted a borderline case in the major incorporation products for host liver, they represented, in the hepatoma, 19 per cent of the nonvolatile residue. The relative incorporation of isotope into lactate, aspartate, alanine, and glutathione was also higher, presumably at the expense of isotope in β-hydroxybutyrate (Table 4).

In the experiment with host liver appreciable amounts of isotope were incorporated into glucose. In the case of hepatoma, glucose again contained little isotope (see above). Nonincorporation of isotope in glucose does not seem to be the result of a failure to deliver isotope from cycle intermediates to pyruvate or phospho-enol-pyruvate, because both lactate and alanine contained isotope. To establish further that pyruvate-C¹⁴ contributed negligibly to glucose-C¹⁴ formation, pyruvate-2-C¹⁴ was incubated with hepatoma and host liver slices. The hepatoma formed radioactive lactic acid, but yielded little, if any, glucose. Host liver, on the other hand, did convert pyruvate-2-C¹⁴ to glucose as well as to lactate.

The utilization of octanoate-1-C¹⁴ by hepatoma, observed in experiments reported in this section, was lower than that reported in the previous section (Table 1). The preliminary experiments were carried out in a phosphate buffer instead of with a bicarbonate buffer. It seemed desirable to determine whether a difference in buffer could account for the observed difference in utilization.

**Incubation of host liver and hepatoma slices in phosphate buffer.**—As judged by the isotope in the volatile fraction (unreacted substrate), in respired CO₂, and in the nonvolatile fraction, utilization of octanoate-1-C¹⁴ by hepatoma in phosphate buffer (Table 5) again exceeded that observed in bicarbonate buffer (Table 3). The incorporation of isotope into the nonvolatile residue was higher in phosphate than in bicarbonate buffer for hepatoma, while the reverse was true for host liver. In the experiments shown in Table 5, an appreciable

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**TABLE 5**

INTEGRATION OF ISOTOPE FROM OCTANOATE-1-C¹⁴ INTO VARIOUS FRACTIONS BY HOST LIVER AND HEPATOMA C₅₄ OF MICE

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Required</th>
<th>Nonvolatile</th>
<th>Volatile</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host liver</td>
<td>14.0</td>
<td>32.3</td>
<td>9.6</td>
<td>64.9</td>
</tr>
<tr>
<td>Host liver</td>
<td>14.7</td>
<td>19.4</td>
<td>5.6</td>
<td>64.7</td>
</tr>
<tr>
<td>Hepatoma</td>
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</tr>
<tr>
<td>Hepatoma</td>
<td>20.6</td>
<td>14.1</td>
<td>19.3</td>
<td>60.0</td>
</tr>
</tbody>
</table>

(phosphate buffer)

(0.5 gm. of slices [wet wt.] of liver or tissue dispersion of hepatoma, incubated for 2 hours in Ca²⁺-free Krebs-Ringer phosphate buffer, pH 7.5, 37.5° C. Oxygen as gas phase, alkali in center well. Concentration of octanoate, 0.002 M; 1.97 × 10⁴ counts/min incubated per flask. Four animals employed. Reactions stopped with HCl + heat.)

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fraction (about 85 per cent) is not accounted for. This isotope may have resided mostly in acetoacetate, which was not analyzed.

Compounds of the nonvolatile residue that contained C14 are presented in Table 6. Characteristically, the conversion of isotope to \( \beta \)-hydroxybutyrate, for hepatoma, was higher with phosphate than with bicarbonate buffer. The metabolic pattern observed with hepatoma incubated in the bicarbonate buffer held when this tumor was incubated in the phosphate buffer—for example, high glutamate-C14/glutamine-C14 ratio, small amount of isotope in glucose, and elevated aspartate-C14 and di- and tricarboxylic acid isotope levels. Variations observed from experiment to experiment, whether due to buffer, nutritional state, substrate concentrations, or age of the hepatoma, seemed not to affect the general, over-all metabolic picture.

**TABLE 6**

**INCORPORATION PRODUCTS FROM OCTANOATE-1-C14**

<table>
<thead>
<tr>
<th>Incorporation products</th>
<th>Host liver</th>
<th>Hepatoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major: 5-5 per cent of nonvolatile counts/min</td>
<td>( \beta )-hydroxybutyrate (48.4)</td>
<td>Glutamate (50.4)</td>
</tr>
<tr>
<td></td>
<td>Glutamate (11.5)</td>
<td>( \beta )-hydroxybutyrate (48.8)</td>
</tr>
<tr>
<td></td>
<td>Glucose (7.6)</td>
<td>Di-and tri-COOH acids (6.6)</td>
</tr>
<tr>
<td></td>
<td>Glutamine (6.6)</td>
<td>Lactate (5.7)</td>
</tr>
<tr>
<td></td>
<td>Lactate (5.6)</td>
<td>Aspartate (5.5)</td>
</tr>
<tr>
<td>Intermediate: 1-5 per cent of nonvolatile counts/min</td>
<td>Urea (4.4)</td>
<td>Glutathione (5.9)</td>
</tr>
<tr>
<td></td>
<td>Pyrrolidone-COOH acid (3.8)</td>
<td>Pyrrolidone-COOH acid (3.8)</td>
</tr>
<tr>
<td></td>
<td>Alanine (5.1)</td>
<td>Alanine (5.7)</td>
</tr>
<tr>
<td></td>
<td>Di-and tri-COOH acids (4.2)</td>
<td>Origin (1.8)</td>
</tr>
<tr>
<td></td>
<td>Glutathione (5.0)</td>
<td>Glutamine (1.6)</td>
</tr>
<tr>
<td></td>
<td>Aspartate (2.9)</td>
<td>Urea? (1.0)</td>
</tr>
<tr>
<td>Minor: &lt;1 per cent of nonvolatile counts/min</td>
<td>Origin (0.8)</td>
<td></td>
</tr>
</tbody>
</table>

We also found a low rate of acetate oxidation by mouse hepatoma. It is not enigmatic that acetate is poorly utilized while octanoate is readily oxidized, for these substrates are activated by different enzymes (15). Poor utilization of acetate by this as well as by other hepatomas (5, 30) may not be a general property of tumors, for other workers have found the utilization to be about the same as or higher than (29) that of normal liver.

**TABLE 7**

**FATTY ACID INHIBITION OF ACETATE OXIDATION IN HEPATOMA**

<table>
<thead>
<tr>
<th>Fatty acid added with acetate-1-C14</th>
<th>Per cent inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Butyrate</td>
<td>77</td>
</tr>
<tr>
<td>Hexanoate</td>
<td>70</td>
</tr>
<tr>
<td>Octanoate</td>
<td>70</td>
</tr>
<tr>
<td>Decanoate</td>
<td>67</td>
</tr>
<tr>
<td>Laurate</td>
<td>70</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

\[
\text{Per cent C14O2 in absence of acid} = 100 \times \left(1 - \frac{\text{Per cent C14O2}}{\text{Per cent C14O2 in absence of acid}}\right)
\]

We also found a low rate of acetate oxidation by mouse hepatoma. It is not enigmatic that acetate is poorly utilized while octanoate is readily oxidized, for these substrates are activated by different enzymes (15). Poor utilization of acetate by this as well as by other hepatomas (5, 30) may not be a general property of tumors, for other workers have found the utilization to be about the same as or higher than (29) that of normal liver.

**Inhibition of acetate-1-C14 by short-chain fatty acids.**—A reduction in the utilization of acetate-1-C14 by hepatoma C954 in the presence of octanoate was observed above (Table 3). Octanoate was found by Lehninger (29), Walkenstein and Weinhouse (37), and by Vestling et al. (36) to inhibit markedly the tricarboxylic acid cycle and to decrease oxygen uptake in slices or homoge-
nates of normal tissues. The effectiveness of octanoate and other short-chain fatty acids in inhibiting the oxidation of acetate-1-C\textsuperscript{14} by the mouse hepatoma was tested. The experimental conditions and results appear in Table 7. In the presence of either butyrate, hexanoate, octanoate, decanoate, or laurate, the conversion to C\textsuperscript{14}O\textsubscript{2} was inhibited about 70 per cent. Similarly, the incorporation of isotope into the nonvolatile residue was depressed by the short-chain fatty acids. If the depression in C\textsuperscript{14}O\textsubscript{2} production were due merely to dilution of isotopic 2-carbon fragments by nonisotopic fragments generated from the short-chain fatty acids, one would expect the C\textsuperscript{14}O\textsubscript{2} production to vary with the chain length of the fatty acids (assuming each was converted to 2-carbon fragments at the same rate). But the percentage inhibition was remarkably constant (see Table 7).

The results point to an actual depression in the activation of acetate by the short-chain fatty acids. This may be owing to competition for activation as the acetylSCoA. If, however, the short-chain fatty acids do limit the oxidative disposal of 2-carbon fragments from acetate by inhibiting some step in the tricarboxylic acid cycle, then the rapid utilization of the short-chain fatty acids themselves remains unexplained.

Utilization of acetate-2-C\textsuperscript{14} by mouse hepatoma.—

The dynamics of the tricarboxylic acid cycle are such that the methyl carbon of a 2-carbon fragment is incorporated into intermediates of the cycle to a greater extent than is the carboxyl carbon (19, 38). This leads to a greater incorporation of the acetate methyl carbon into glycolytic intermediates, since these may be derived from acetate only after the latter has entered the cycle.

The pattern of labeling of intermediates from acetate-2-C\textsuperscript{14} by hepatoma was studied and compared with that obtained from a carboxyl-labeled, 2-carbon fragment (e.g., acetate-1-C\textsuperscript{14} or octanoate-1-C\textsuperscript{14}). It was anticipated that denser radioactive areas on the autoradiograms would be obtained with acetate-2-C\textsuperscript{14} at comparable levels of isotope in the incubation medium. The chromatograms were to be examined specifically for the presence of radioactive glycine (see below).

Table 8 summarizes the data on the isotope incorporation into various compounds. A typical autoradiogram of a chromatogram from which the data of Table 8 were compiled appears as Figure 4. In the present experiment, 3.9 per cent of the isotope appeared in the nonvolatile components, and 5.0 per cent appeared in C\textsuperscript{14}O\textsubscript{2}.

The major incorporation products with acetate-2-C\textsuperscript{14} were glutamate, lactate, alanine, aspartate, di- and tricarboxylic acids (as a group), and \(\beta\)-hydroxybutyrate. The principal difference noted in the present experiment, as compared with the experiment in which acetate-1-C\textsuperscript{14} was employed, is that alanine and lactate contain much more isotope. While 30 per cent of the counts/min on the chromatograms resided in lactate + alanine, again little or no isotope was found in glucose. Only 1.8 per cent of the nonvolatile counts/min resided in the area occupied by glucose, glycine, and serine. Because of the low percentage incorporation into this area, it was not feasible to determine which of these three compounds contained the activity.

The area corresponding to citrate was eluted and rechromatographed one-dimensionally in 1-
and the large conversion of isotope to glycine in his experiments was no doubt facilitated by the "trapping" of isotopic glycine in this pool.

The heavy labeling of alanine and lactate, derivable from pyruvate by transamination and reduction, respectively, indicates that considerable isotope passed through the keto acid.

As much as 1.4 per cent of the nonvolatile residue resided in a ninhydrin-positive area which traveled close to the solvent front with phenol-water but which was immobile in the other direction. This is presumed to indicate the presence of a peptide, although this identification is tentative.

**Utilization of Propionate-1-C14 by the Hepatoma**

Two pathways have been proposed for the utilization of propionate by animal tissues: (a) oxidation to lactate via acrylate (16, 25) and (b) CO₂ fixation to yield succinate (22). 2-Methyl malonate (isoxosuccinate) has been shown to accumulate along with the succinate (14, 18). The present experiment was undertaken to see what radioactive compounds would arise from propionate-1-C¹⁴ incubated in the presence of slices of the hepatoma. The reproduction of the autoradiogram appears in Figure 5, and the data are given in Table 9. The nonvolatile fraction contained 1.5 per cent of the incubated isotope, while a much larger amount appeared in the respired CO₂, namely, 7.9 per cent.

The most dominant radioactive compound on the chromatograms had a position identical with that of lactate. Over 40 per cent of the isotope of the nonvolatile residue was recovered in this compound. The next most active compound was alanine, with 18 per cent. The ratio of lactate-C¹⁴ to alanine-C¹⁴ was approximately equal to 3, a value close to that observed in experiments with acetate-1-C¹⁴ (Table 4). Acetate carbon can appear in lactate and alanine via pyruvate, and the fact that the ratio of lactate-C¹⁴ to alanine-C¹⁴ was the same, whether the two compounds were derived from propionate-1-C¹⁴ or acetate-1-C¹⁴, suggests that propionate carbon passed through pyruvate before being converted to lactate. If this assumption is correct, α-oxidation of propionate to lactate (16, 25) is ruled out.

Eight per cent of the nonvolatile residue was recovered in an area close to that normally occupied by β-hydroxybutyrate. This is probably β-hydroxyvalerate, for Daus et al. (12) have shown that propionate-C¹⁴ is converted to this hydroxy acid by mouse liver slices, presumably from condensation of propionylSCoA with acetylSCoA followed by decylation and reduction.

**Oxidation of Uniformly Labeled Glucose by Slices of Hepatoma and Host Liver**

The previous experiments were largely concerned with reactions closely associated with the tricarboxylic acid cycle. It was shown that the hepatoma cannot convert cycle intermediates, or even pyruvate, to glucose, at least not to any considerable extent. As it has long been observed that tumor tissues are active in glycolysis (38, 39), it became of interest to study the pattern of this glucose utilization by the chromatographic-autoradiographic procedure. Slices of hepatoma and host liver were incubated with a trace amount of uniformly labeled glucose, and the respiratory CO₂ and buffer extracts were analyzed. Typical autoradiograms for the hepatoma and host liver appear in Figures 6 and 7. The data are presented in Table 10.

Over 65 per cent of the isotope incubated with the hepatoma appeared as C¹⁴O₂. This extraordinary ability of hepatoma to oxidize glucose was further substantiated upon inspection of the autoradiogram prepared from the chromatogram of the buffer extract. More isotope appeared in lactate and alanine than appeared in the residual glucose. On the other hand, the host liver converted only 13 per cent of the isotope to C¹⁴O₂, indicating a much smaller utilization. In the case of host liver, very little isotope appeared in lactate or alanine, most of it residing in unreacted glucose. No exact measure was made of the per cent of residual glucose in either case. However, comparison of the two tissues with regard to isotope incorporation into lactate and alanine may be made by comparing the ratios of counts/min
in product: counts/min in residual glucose, as measured directly on the paper chromatograms (Table 10). The results indicate that hepatoma was much more active than host liver in the utilization of the added glucose.

The utilization of glucose by tumor in the present experiment exceeded that observed in a previous experiment (1). The greater utilization may be attributed to the fact that trace levels of glucose-C14 were employed here (less than 0.1 mg/flask), while in the previous experiments substrate levels were used.

In the case of hepatoma, isotope appeared also in glutamate. A group of radioactive spots occupied the region in which citrate is found. A radioactive area near the origin probably represents various phosphorylated derivatives.

A radioactive area just below glucose, termed "glucose artifact,"1 was observed for both host liver and hepatoma. The intensity of this area seems to be a rather constant fraction of the radioactivity of the glucose area.

Whether or not all the isotopic carbon of C14O2 derived from the oxidation of glucose had traversed the tricarboxylic acid cycle in the case of tumor is not known. Recent experiments have indicated that this tumor may participate in reactions of the hexose monophosphate shunt (1).

The high capacity of the tumor to carry out glucose degradation may be responsible for the failure to obtain significant glucose synthesis from pyruvate and other compounds. Glycolytic intermediates derived from such compounds would have to traverse a metabolic gradient to reach the glucose level.

Glucose degradation via the Embden-Meyerhof scheme leads to pyruvate, which may enter the tricarboxylic acid cycle as oxalacetate (44) or as malate (28). A rational explanation may thus be given for the high incorporation of isotope into the di- and tricarboxylic acids when tumor is incubated with a source of labeled 2-carbon fragments.

1 This was previously referred to as "glucose amine" (17) and has not yet been identified.

The increase in pool size of cycle intermediates (cf. 45), resulting from increased glucose breakdown in tumor, could enable greater amounts of isotope to be trapped in the pools than is the case with normal or host liver. Aspartate and glutamate represent alternate pathways for oxalacetate and α-keto-glutarate, respectively, and, in the tumor, an increased level of isotope in these amino acids is probably a reflection of the increase in their pool sizes.

SUMMARY

1. The incorporation of isotope from acetate-1-C14, acetate-2-C14, propionate-1-C14, octanoate-1-C14, pyruvate-2-C14, and from uniformly labeled glucose into respiratory CO2 and various intermediary products was studied with slices of normal mouse liver, host liver, and with the hepatoma C57.

Table 10: Utilization of Uniformly Labeled Glucose by Slices of Hepatoma and Host Liver

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Respiratory CO2 Per cent of incubated counts/min</th>
<th>Ratio of Counts/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatoma</td>
<td>292,000</td>
<td>66</td>
</tr>
<tr>
<td>Host liver</td>
<td>59,000</td>
<td>13</td>
</tr>
</tbody>
</table>

* Average of three chromatograms.
† Average of two chromatograms.
glutamate, and aspartate than did the liver.

5. Normal and host liver incorporated isotope from labeled 2-carbon fragments into glucose, but hepatoma failed to carry out this incorporation to any significant extent. The hepatoma was more active than was host liver, however, in utilization of glucose.

6. Two-carbon fragments from acetate were shown to interact with those from octanoic acid in the hepatoma and to participate in the ketogenic activity of this short-chain fatty acid.

7. Alanine and lactate contained relatively more isotope when acetate-2-C14 was employed instead of acetate-1-C14, with hepatoma. The explanation for this lies in the dynamics of the tricarboxylic acid cycle, the intermediates of which become more heavily labeled with the methyl carbon than with the carboxyl carbon of a 2-carbon fragment.

8. Propionate-2-C14 was converted by the hepatoma (in decreasing order) lactate, alanine, glutamate, aspartate, glutamine, and several unidentified compounds. Little or no isotope was converted to glucose.

9. Presumptive evidence that the tricarboxylic acid cycle operates in the hepatoma was provided by (a) oxidation of labeled 2-carbon fragments to CO2; (b) incorporation of isotope from 2-carbon fragments into compounds of the cycle and to those arising from them by ancillary processes; (c) inhibition by malonate; (d) depression in the oxidation of labeled acetate by short chain fatty acids which are thought to be inhibitors of the tricarboxylic acid cycle; and (e) the pattern of isotope labeling similar to that of liver, in which the cycle is known to operate.

10. The pattern of labeling of various intermediates observed when C14-labeled compounds are incubated with slices of hepatoma differs quantitatively from the pattern observed with host or normal liver. The observations made here on quantitative differences in the hepatoma C954 reflect its neoplastic state, but such differences from the liver tissue do not lend themselves to a clear-cut decision regarding the specific loci for the metabolic derangement(s) associated with neoplasia.

REFERENCES

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FIG. 5.—Autoradiogram: Isotopic compounds derived from propionate-1-C\textsuperscript{14} in presence of mouse hepatoma C\textsubscript{64} slices.

FIG. 6.—Autoradiogram: Isotopic compounds derived from evenly labeled glucose-C\textsuperscript{14} in presence of mouse liver slices.

FIG. 7.—Autoradiogram: Isotopic compounds derived from evenly labeled glucose-C\textsuperscript{14} in presence of mouse hepatoma C\textsubscript{64} slices.
BROWN et al.—Metabolic Pattern of Mouse Hepatoma


32. RATNER, S. Conversion of d-Glutamic Acid to Pyrrolidone-Carboxylic Acid by the Rat. J. Biol. Chem., 188:560-64, 1944.


The Oxidative Metabolic Pattern of Mouse Hepatoma C_{954} as Studied with C^{14}-labeled Acetates, Propionate, Octanoate, and Glucose

G. W. Brown, Jr., J. Katz and I. L. Chaikoff