Substrate Effects on Metabolic Patterns of Pyruvate-2-C\textsuperscript{14} in Tissue Slices*

HARRIS BUSCH,† MORRIS H. GOLDBERG, AND DOLORES C. ANDERSON

(Departments of Medicine, Biochemistry, and Pathology, Yale University School of Medicine, New Haven, Connecticut)

The metabolism of C\textsuperscript{14}-labeled pyruvate in normal and neoplastic tissue has been studied both in vitro (8, 10, 13, 14) and in vivo (3). Although the experiments of the Coris (6) and Warburg (12) indicated a net production of lactate by tumors in vivo and in vitro, the metabolism of trace quantities of C\textsuperscript{14}-labeled pyruvate was not found to be significantly different in tumors and other tissues in vitro (10, 13, 14). In contrast, the “metabolic pattern” for pyruvate-2-C\textsuperscript{14} differed markedly in tumor and nontumor tissues in vivo (3). Within 3 minutes after injection of the tracer, the nontumor tissues contained 30–80 per cent of the total tissue isotope in the amino acids, glutamic acid, alanine, and aspartic acid (3), while the tumors studied contained less than 5 per cent of the total tissue isotope in these amino acid pools. In the nontumor tissues, the peak for the percentage of total tissue isotope in lactate was uniformly found in the first minute after injection of the pyruvate-2-C\textsuperscript{14}, and, thereafter, the isotope in lactate dropped rapidly so that at 8 minutes after injection of the tracer, 3–20 per cent of the total tissue isotope remained in lactate. In the 8-minute interval of the study, the isotope in lactate in the tumors always comprised 50–90 per cent of the total in the tissue.

The present report indicates the results of an initial attempt to elucidate the reasons for the differences noted in the metabolism of pyruvate-2-C\textsuperscript{14}-in tissues in vivo and in vitro. It has been found that increasing concentrations of glucose and glutamic acid in the medium surrounding the slice altered the metabolic pattern for pyruvate-2-C\textsuperscript{14} in the direction of that found in the in vivo experiments.

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† Scholar in Cancer Research of the American Cancer Society; present address: Department of Pharmacology, University of Illinois College of Medicine, Chicago, Illinois.

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MATERIALS AND METHODS

Tissues.—The tumors used in these experiments were the Walker 256 carcinosarcoma, the Jensen sarcoma, and the Guerin uterine carcinoma; before slicing, any visible hemorrhagic or necrotic areas were dissected away. The tumors and other tissues were quickly excised from the tumor-bearing rat following decapitation; they were placed in ice-cold Krebs-Ringer bicarbonate buffer and sliced with the aid of a Stadie-Riggs micromere in the cold room. The wet weights of the slices were determined on a Roller-Smith balance, and they varied from 75 to 150 mg.

Tracer.—The pyruvate-2-C\textsuperscript{14} used initially was obtained by hydrolysis of pyruvamide according to the method of Anker (1). In later experiments, sodium pyruvate-2-C\textsuperscript{14} was obtained from Nuclear Instruments and Chemical Company. There was no significant difference in the results obtained with the different samples of pyruvate-2-C\textsuperscript{14}.

Flask contents.—The main chamber of the Warburg flask contained Krebs-Ringer bicarbonate buffer. The side-arm contained 500,000 counts/min of sodium pyruvate-2-C\textsuperscript{14} in a total of 1 gM of sodium pyruvate, along with the other substrates added. In the test system for any tissue, four flasks were used; one was a control, the second contained 20 gM of glucose in the side-arm, the third, 20 gM of glutamic acid, and the fourth, 20 gM each of glucose and glutamic acid. The concentrations of individual substrates were varied in the course of special experiments. The total volume in the flask was 3.2 cc.

Procedure.—After the slices were added to the main chamber, the flasks were joined to the manometers gassed with 95 per cent oxygen and 5 per cent CO\textsubscript{2} (11) for 10 minutes, and then were incubated in the bath for 10 minutes at 38°C. After this period of equilibration, the contents of the side-arm were added to the main chamber, and the...
flasks were incubated for an additional 20 minutes. This time period was chosen after it was found that utilization of pyruvate and labeling of the metabolites studied were linear up to this time for kidney, liver, and Walker 256 tumor. After the incubation was completed, $\text{C}^{14}\text{O}_2$ was collected from the bicarbonate of the medium: 0.2 ml of 2 N KOH was added to the center well, along with a wick of filter paper, and 0.3 ml of 5 N HClO$_4$ was added to the side-arm; the HClO$_4$ was added to the main chamber after the flask and manometer were rejoined. The open end of the manometer was closed by a rubber stopper to prevent the expulsion of manometer fluid by the evolved CO$_2$. The system was permitted to equilibrate for 2 hours at 38° C.

**Determinations.**—The contents of the flask were chromatographed on anion exchange resins for determination of isotope in alanine, glutamic acid, and aspartic acid (2). Pyruvate-$2-\text{C}^{14}$ remaining was determined as the difference between the total isotope added to the flask and the isotope remaining in solution after quantitative precipitation of pyruvate as the 2,4-dinitrophenyl-hydrazone in the presence of carrier. The $\text{C}^{14}\text{O}_2$ was determined as barium carbonate. All values in this paper are expressed as counts/min/100 mg wet weight of tissue.

**RESULTS**

**Tumors**

Walker 256 carcinoma.—In the unsupplemented Krebs-Ringer bicarbonate medium, the tumor slice transferred 17 per cent of the isotope utilized to $\text{C}^{14}\text{O}_2$, 58 per cent to lactate, and 26 per cent to amino acids (Chart 1). As Chart 1 indicates, glucose markedly altered this distribution of the isotope, inducing an increasing transfer of isotope to lactate as the concentration of glucose was increased. As little as 2 μM of glucose doubled the isotope in lactic acid and suppressed labeling of amino acids and CO$_2$. A concentration of 20 μM (110 mg. per cent) of glucose in the medium resulted in the transfer of 91 per cent of the isotope utilized to lactic acid, 5.2 per cent to amino acids, and 3 per cent to CO$_2$. With a high concentration of glucose in the medium, the tumor slice utilized 133,000 counts/min in comparison with 75,000 counts/min in the control medium. The Walker tumor exhibited no significant change in the metabolic pattern for pyruvate-$2-\text{C}^{14}$ when glutamate was added to the medium. The tumor labeled lactate most extensively when both glucose and glutamate were present in the medium; occasionally, as many as 250,000 counts/min were found in lactate alone under these conditions, in comparison with 120,000 counts/min when glucose alone was present, or 30–50,000 counts/min in the control system.

Chart 2 presents the results of studies designed to test the possibility that simple equilibration of
an increased lactate pool with the pyruvate-2-C\textsuperscript{14} accounted for the increasing labeling of lactate (Chart 1). The data indicate that the isotope in the lactate pool increased more rapidly than did the size of the pool at low concentrations of glucose. At higher concentrations of glucose, there was an increased lactate production without a corresponding increase in isotope entering the lactate pool.

\textbf{Chart 3.}—Effect of increasing concentration of glucose on the specific activity of lactate recovered in experiments with Walker 256 tumor. Values are averages for four experiments.

The changing specific activity of the lactate (Chart 3) suggests that at low concentrations, pyruvate-2-C\textsuperscript{14} serves as a hydrogen acceptor.\textsuperscript{2} At higher concentrations of glucose, the pyruvate formed from nonradioactive glucose is in effective competition with the radioactive pyruvate for hydrogen atoms. In another study on this point (Chart 4), the size of the lactate pool was increased by addition of nonradioactive lactate in concentrations 2–10 times that usually formed in the course of these experiments, i.e., 1–5 \textmu M. Although the tumor transferred more isotope to lactate in the flasks containing more nonradioactive lactate, addition of 5 \textmu M of glucose produced a much greater labeling of the lactate pool despite the fact that the lactate formed was less than 1 \textmu M.

\textbf{Jensen sarcoma.}—As Chart 5 indicates, the over-all pattern for the distribution of the isotope of the utilized pyruvate-2-C\textsuperscript{14} was essentially the same for the Jensen sarcoma as that of the Walker 256 carcinosarcoma. Moreover, the addition of 20 \textmu M of glucose to the medium markedly suppressed the labeling of the amino acids (GL, AL), as well as \textsuperscript{14}O\textsubscript{2} production. At the same time, the isotope in lactate accounted for 88 per cent of the total recovered. In contrast to the Walker and Guerin tumors, the Jensen sarcoma increased the isotope in amino acids, particularly alanine, following the addition of glutamic acid to the medium. The combined effects of glucose and glutamic acid include an increased labeling of both lactate and alanine.
Guerin uterine carcinoma.—This tumor (Chart 6) exhibited an even greater labeling of lactate than did the others studied. In the unsupplemented medium, slices of the Guerin tumor transferred only 5 per cent of the isotope to CO₂, 20 per cent to amino acids, and 75 per cent to lactate.

In the glucose-supplemented system, this tumor transferred 96 per cent of the total isotope to lactate, 1.5 per cent to CO₂, and 2 per cent to amino acids, while the utilization of isotope was increased almost threefold. Very slight changes in either isotope utilization or its pattern of distribution accompanied the addition of glutamate to the medium. With both glutamic acid and glucose in the medium, almost half the isotope in the flask was transferred to lactate.

NONTUMOR TISSUES

Liver.—In the flasks containing unsupplemented Krebs-Ringer bicarbonate buffer, liver slices transferred 10 per cent of the isotope utilized to CO₂, 44 per cent to lactate, and 46 per cent to amino acids (Chart 7). Of the isotope in amino acids, two-thirds was found in alanine. Unlike the tumors, brain, and spleen, addition of glucose did not alter the metabolic pattern for pyruvate-2-¹⁴C in the liver. However, an increasing concentration of glutamate in the medium induced a markedly increased labeling of alanine (Chart 7). Saturating levels of glutamate were reached at 3 mM; at this concentration, 71 per cent of the total isotope added to the flask was found in alanine, and only traces were found in glutamate or aspartate.² Along with the increased labeling of amino acids there was a decreased formation of C⁴O₂ from 10 to 1 per cent of the total isotope utilized while the percentage in lactate decreased from 44 to 4.3 per cent or 40,000 to 15,000 counts/min.

Kidney.—The kidney differed from the other tissues studied in the extensive labeling of CO₂. In the unsupplemented medium (Chart 8), the kidney transferred 42 per cent of the isotope utilized

² The yield suggests a method for biosynthesis of alanine-2-¹⁴C from pyruvate-2-¹⁴C.
to C\textsuperscript{14}O\textsubscript{2}, i.e., a total of 49,000 counts/min. Under these conditions, the kidney slice also transferred 21 per cent of the isotope utilized to lactate and 27 per cent to amino acids, while 10 per cent of the isotope appeared in the neutral effluent. Addition of glucose did not alter this metabolic pattern for pyruvate-2-C\textsuperscript{14} in the kidney, as was the case for liver. On the other hand (Chart 6), a progressive increase of isotope in glutamate was induced by an increasing concentration of glutamate in the medium, at the expense of isotope in C\textsuperscript{14}O\textsubscript{2} and lactate. Flasks containing 40 \mu M of glutamate contained 43 per cent of the total isotope utilized in glutamate in comparison with 13 per cent in the control flasks; the percentages of isotope in lactate and CO\textsubscript{2} dropped from 21 per cent and 42 per cent, respectively, in the control flasks to 15 and 18 per cent, respectively, in the medium supplemented with 40 \mu M of glutamate. Unlike the liver and the tumors, the kidney did not utilize more labeled pyruvate as the metabolic pattern changed.

**Brain.**—In the unsupplemented medium, the brain transferred 12.5 per cent of the isotope utilized to CO\textsubscript{2}, 42 per cent to glutamate, 17 per cent to aspartate, 9 per cent to alanine, and 20 per cent to lactate (Chart 9). Addition of glucose resulted in an increase in labeling of lactate without much change in the total isotope appearing in amino acids. Glutamate addition resulted in a marked increase of the isotope in alanine and appearance of a new peak containing an unstable compound which has not yet been identified. Simultaneous addition of both glucose and glutamate to the medium containing brain slices resulted in an increase in the labeling of both alanine and lactate; under these conditions, the isotope in amino acids was 57 per cent of that accounted for, compared with 68 per cent in the control flasks, while the isotope in lactate increased from 20 per cent in the control system to 41 per cent.

**Spleen.**—Of the nontumor tissues, the spleen most resembled the tumors in distribution of the isotope of pyruvate-2-C\textsuperscript{14} (Chart 10). In the unsupplemented medium, the spleen transferred 18 per cent of the isotope to CO\textsubscript{2}, 39 per cent to amino acids and 44 per cent to lactic acid. These values differed from those obtained for the tumors in the higher percentage of isotope in amino acids and the lower percentage in lactate. Although the addition of glutamate resulted in a slight increase in the total isotope in CO\textsubscript{2}, it did not produce a significant change in the distribution of the isotope. The addition of glucose to the spleen slices increased the isotope in lactate to a total of 69 per cent of the isotope utilized, but 23 per cent of the isotope persisted in amino acids. In comparison,
similar levels of glucose induced the tumors to transfer an average of 92 per cent of the total isotope utilized to lactate and also resulted in a marked increase in the total isotope utilized, either alone or in the presence of added glutamate; only 4 per cent of the isotope was transferred by the tumors to amino acids in the presence of glucose. When both glucose and glutamate were added to the flask, the spleen transferred 75 per cent of the isotope utilized to lactate, 16 per cent to amino acids, and 7.5 per cent to $^{14}CO_2$.

**DISCUSSION**

The objective of these experiments was the exploration of the possibility that the differences in the *in vivo* and *in vitro* fates of pyruvate-$2-^{14}C$ may have been due to the presence of substrates *in vitro* which could influence the metabolism of pyruvate differently in individual tissues. The *in vitro* studies demonstrated that the principal fate of pyruvate-$2-^{14}C$ was the formation of lactate in the tumors, alanine in the liver, and glutamate in the kidney (3). Addition of glucose and glutamate to the medium produces this same characteristic pattern in slice experiments. A number of differences between the *in vivo* and *in vitro* findings were obtained; the extensive labeling of alanine in the brain slice or the moderate labeling found with the slice of Jensen sarcoma were not noted *in vivo*. Moreover, the similarity of the spleen and the tumors would not have been anticipated from the *in vivo* experiments. It is perhaps of significance from the standpoint of the true extracellular medium surrounding these tissues that both brain and spleen slices exhibited a closer pattern to the *in vivo* picture in the unsupplemented medium.

The mechanism of these effects would appear to vary in individual tissues. In brain and liver, it can be assumed that transaminating activity was responsible for the preponderant labeling of alanine. In the kidney, it would appear that glutamate equilibrated with the citric acid cycle sufficiently rapidly to suppress $^{14}CO_2$ formation (4, 5). The tumors and, to a lesser extent, brain and spleen responded to glucose by increased labeling of lactate; it would appear that the tumor was utilizing pyruvate as an acceptor of hydrogen atoms.

The utility of the amination reactions to the nontumor tissues would seem to be apparent; they could provide amino acids for protein synthesis either for growth or replacement. In the presence of glucose, amino acid formation accounted for 5 per cent or less of the total pyruvate utilized by the tumors, while 90–95 per cent of the isotope entered the lactate pool. It is not possible to determine the role of the excessive glycolysis in the metabolism of the tumor from these experiments, but a lead may be furnished by the experiments in which both glucose and glutamate were added to the medium and resulted in maximal labeling of lactate. The oxidation of both of these compounds is coupled through DPN, and accordingly it would appear that, if glycolysis can maintain a high concentration of reduced DPN (7), oxidation of glutamate to alpha-ketoglutarate could be suppressed along with the associated transamination reactions (9) essential to the oxidation of most amino acids. Thus, the tumors might utilize excess glycolysis to suppress amino acid oxidation and direct their over-all metabolism toward protein synthesis.

**SUMMARY**

1. The metabolic pattern for pyruvate-$2-^{14}C$ has been studied *in vitro* in systems containing slices of Walker 256 carcinosarcoma, Jensen sarcoma, Guerin uterine carcinoma, liver, kidney, spleen, and brain. The systems contained Krebs-Ringer bicarbonate buffer alone or supplemented with glucose, glutamate, or both compounds.

2. Addition of 20 $\mu M$ of glucose to the medium containing tumor slices changed the distribution pattern for the isotope from 14 per cent in $CO_2$, 64 per cent in lactate, and 21 per cent in amino acids in the control system to 92 per cent in lactate, 3 per cent in $CO_2$, and 5 per cent in amino acids.

3. Addition of glucose to kidney and liver slices resulted in no significant changes in the metabolic pattern. However, liver slices transferred as much as 71 per cent of the total isotope in the flask to alanine in the presence of glutamate. Kidney slices increased the labeling of glutamate in the medium containing glutamate, at the expense of $^{14}CO_2$ and lactate.

4. Brain slices transferred 66 per cent of the isotope of pyruvate-$2-^{14}C$ to amino acids in the unsupplemented medium. In the presence of glucose, brain slices transferred a greater percentage of isotope to lactate while the addition of glutamate markedly increased the labeling of alanine.

5. Although the results for spleen resembled those for the tumors the most closely of the nontumor tissues studied, spleen slices transferred a greater percentage of isotope utilized to amino acids in the unsupplemented medium, and a greater percentage of isotope to $CO_2$ and amino acids in the medium supplemented with glucose.

6. In the media supplemented with both glucose and glutamate, the Guerin and Walker tumors and the liver and kidney slices distributed...
the isotope of pyruvate-2-C\textsuperscript{14} in patterns very similar to those found in \textit{in vivo} experiments.

7. These studies indicate the necessity for studying both a broad spectrum of products as well as variations in the medium to assess the relative activities of tissues in the metabolism of a given radioactive substrate.

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Harris Busch, Morris H. Goldberg and Dolores C. Anderson


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