Carbon Dioxide Metabolism in the Jensen and JA Sarcomas in Vitro

THOMAS A. MCCOY, MERLE D. MAXWELL, AND PAUL F. KRUSE, JR.

(Biomedical Division, Samuel Roberts Noble Foundation, Inc., Ardmore, Okla.)

SUMMARY

A requirement for carbon dioxide or bicarbonate has been demonstrated for the growth and survival of the Jensen, JA-1, and JA-2 sarcomas in vitro. When the cells were grown in the presence of CO\textsubscript{2}, isotope was isolated in incorporated aspartic acid, glutamic acid, and proline as well as in the purines and pyrimidines. Although the relative specific activity of the purines and thymine was approximately the same, it was much lower in cytosine and slightly higher in uracil. From this information carbonate-sparing experiments were performed, and uracil partially spared this requirement, whereas the other organic bases were inactive.

Recently, this laboratory reported the isolation of two nutritional variants of the Jensen sarcoma (11). Neither of these variants (JA-1, JA-2) required an exogenous source of asparagine for growth, whereas the parental neoplasm exhibited this requirement. Since this change seemed stable and heritable over 10 and 100 generations in vitro and in vivo, respectively, further studies regarding their metabolism were initiated. The present report describes some aspects of carbon dioxide metabolism with respect to these three neoplasms.

MATERIALS AND METHODS

All tissue culture work was conducted with freshly excised tumors. These techniques have been described previously (15), and the medium employed (unless otherwise stated) was Medium 5a (18). The initial inoculum was $2 \times 10^4$ cells/ml. When a bicarbonate-deficient system under nitrogen was prepared, care was taken to prevent exposure of the substrate and cells to air, and the flasks were thoroughly gassed with nitrogen. Media changes and gassing were conducted daily to prevent accumulation of metabolic carbon dioxide in the system. Cell counts were made in a hemocytometer with trypan blue used as a vital stain, and duplicate counts were made on each T-15 flask. All treatments were prepared in duplicate, and all experiments were repeated 3 times.

In radiometric studies the cells were cultured in T-60 flasks in Medium 4a (Medium 5a minus aspartic acid, glutamic acid, alanine, proline, and hydroxyproline). At the end of 48 hours the medium was changed, the cells in two flasks were counted to determine "cell take," and the remaining flasks were given fresh Medium 4a containing sodium carbonate-C\textsubscript{14}. Following an additional incubation period of 48 hours, the cells were harvested for analysis.

Cell protein was prepared as described previously (14). The amino acids were separated on Amberlite CG-120 according to the method of Moore et al. (16). Seven-tenths ml. of each fraction (1.60 ml. volume) was used for amino-nitrogen analysis (17), and 0.8 ml. was dried and assayed in a windowless gas-flow (methane) counter.

Sodium nucleates were isolated according to the method of Tyner et al. (23) and were hydrolyzed at 95°C in 70 per cent perchloric acid for 90 minutes (11). Purine and pyrimidine bases were recovered after passage of the hydrolysate through a $3 \times 0.6$ cm. column of charcoal (21). The eluate was evaporated, and the organic bases were separated by descending paper chromatography with an isopropanol:water:hydrochloric acid solvent system (27). Bases were located, and the specific activity was determined by the method of LePage (10).

RESULTS

The growth response of the Jensen, JA-1, and JA-2 cells to tris(hydroxymethyl)aminomethane (Tris) buffer can be seen in Table 1. When the gas
phase in the flask was air a slight increase in cells was evident, whereas, in an atmosphere of nitrogen, the viable cell population decreased. This decrease was more pronounced as the time of the experiment was increased, and the cells became rounded and granular. Comparable experiments with a bicarbonate buffer and a gas phase composed of 8 per cent carbon dioxide in air or 8 per

### TABLE 1

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Gas Phase</th>
<th>Jensen</th>
<th>JA-1</th>
<th>JA-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris†</td>
<td>Air</td>
<td>1.48</td>
<td>1.11</td>
<td>1.47</td>
</tr>
<tr>
<td></td>
<td>N₂</td>
<td>0.53</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>8% CO₂ in air</td>
<td>6.63</td>
<td>10.1</td>
<td>9.84</td>
</tr>
<tr>
<td></td>
<td>8% CO₂</td>
<td>4.32</td>
<td>5.03</td>
<td>4.75</td>
</tr>
<tr>
<td></td>
<td>92% N₂</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Growth period = 6 days. Initial inoculum = 1.
† Earle's balanced salt solution with bicarbonate replaced by 5 mM tris(hydroxymethyl)aminomethane. The pH was adjusted to 7.4 with hydrochloric acid. The amino acids, vitamins, etc., were those of Medium 5a (18).

cent carbon dioxide in nitrogen yielded a seven- to tenfold and four- to fivefold increase, respectively, in cell population. From this, it was evident that carbon dioxide or bicarbonate was essential for growth and survival of these three neoplasms.

Cells were cultured in the presence of sodium carbonate-C\textsubscript{14}, and the incorporated amino acids were assayed for radioactivity. Radioisotope was isolated in aspartic acid, glutamic acid, and proline. Of these three compounds, aspartic acid contained the highest specific activity, but none of the other amino acids exhibited a detectable isotope content under the conditions described.

The contribution of carbon dioxide to the nucleic acid bases was then studied, and the results are shown in Table 2. It can be seen that carbon dioxide contributed to all organic bases but not to the same degree. The relative specific activity of adenine and guanine was essentially the same. Cytosine was much lower in relative specific activity, whereas thymine was approximately the same as the purines. The relative specific activity of uracil appeared to be higher in two of the three tumors studied, and in repeat experiments the relative specific activity of uracil was found to be even higher in all three tumors.

In view of these results, investigations were initiated to determine whether any of the organic bases could spare the carbon dioxide requirement of the cells. Adenine and guanine were toxic at levels above 0.5 mM, whereas thymine appeared to be innocuous at all levels tested (0.1–10 mM). Cytosine appeared to show slight activity in some cases, but it was not consistent. With Tris buffer, increasing amounts of uracil definitely stimulated cell survival and growth (Table 3). When the bicarbonate buffer was employed, uracil had essentially no effect on cell proliferation. Thus it was shown that one function of carbon dioxide could be partially replaced by the addition of uracil to the substrate.

### DISCUSSION

Much of the early work regarding carbon dioxide and growth in tissue culture dealt with gas-sing cultures, which primarily demonstrated that carbon dioxide was toxic in high concentrations. For example, Mottram (18, 19) reported complete inhibition of outgrowth of rat kidney, rat fibroblasts, and Jensen sarcoma explants when the partial pressure of carbon dioxide exceeded 400 mm. over the culture. On the other hand, Harris (6, 7) reported bicarbonate was essential for outgrowth of chick heart fibroblast cultures, but this essentially could be reversed by controlling the pH of the substrate.

### TABLE 2

<table>
<thead>
<tr>
<th>Organic Base</th>
<th>RSA* of Neoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Jensen</td>
</tr>
<tr>
<td>Adenine</td>
<td>10.0</td>
</tr>
<tr>
<td>Guanine</td>
<td>10.3</td>
</tr>
<tr>
<td>Cytosine</td>
<td>3.8</td>
</tr>
<tr>
<td>Uracil</td>
<td>15.6</td>
</tr>
<tr>
<td>Thymine</td>
<td>11.8</td>
</tr>
</tbody>
</table>

* Relative specific activity where activity of adenine = 10.0. Relative specific activity was used, since the specific activities of any one compound varied among experiments with different neoplasms. These unavoidable differences were probably due to the differences in growth in individual experiments as well as to many other factors. For example, specific activity of adenine was: Jensen = 650 counts/min/μmole; JA-1 = 910 counts/min/μmole; and JA-2 = 2030 counts/min/μmole.

Eagle (2), using the mouse fibroblast (cloned strain L) and strain HeLa, reported that, when bicarbonate was replaced by 5 mM Tris buffer in the medium, rapid acidification of the medium occurred within 12 hours and the cells grew at a normal rate. Consequently, he classified bicarbo-
nate as nonessential for the growth of the mouse fibroblast and the HeLa cell. Working with six strains of fibroblasts from human, mouse, and rabbit origin, Swim and Parker (22) used a phosphite buffer in lieu of the conventional bicarbonate system. A requirement for bicarbonate was noted, provided the necks of the flasks were covered with loosely fitting metal caps. If the flasks were stoppered tightly, cell proliferation occurred, suggesting that the cells could provide sufficient carbon dioxide for growth. These results could explain why Eagle (2) was unsuccessful in demonstrating a bicarbonate requirement in his system.

Geyer and Chang (4) studied the essentiality of bicarbonate in some carefully controlled experiments with human conjunctiva cells and the HeLa cell. In the absence of bicarbonate, no net increase in cells occurred, but growth would resume by the addition of this anion even after the cells were exposed to a bicarbonate-deficient system for 14 days. They concluded that the cell viability was high but the absence of bicarbonate depleted some factor(s) concerned with cell multiplication.

In later work, Geyer and Neimark (5) prepared a cell extract which would promote the growth of carbon dioxide-deficient cells. This extract had no growth-stimulatory action on control cultures, and an extract from carbon dioxide-deficient cells caused a reduction in the population of cells cultured in the absence of bicarbonate. As a result, they concluded that one of the first effects of carbon dioxide depletion may be the inhibition of cell multiplication even though the cells retained the ability to oxidize glucose-1-\(^{14}\)C and acetate-1-\(^{14}\)C.

In the presently reported studies, carbon dioxide was demonstrated to be required for growth of the Jensen, JA-1, and JA-2 sarcomas, and this inhibition could be partially spared by the addition of uracil. Interestingly enough, adenine, guanine, cytosine, and thymine were not active. It should be pointed out that Swim and Parker (22) could obtain some growth (one-half to one-third that of control cultures) when the bicarbonate-deficient medium was supplemented with whole serum and undialyzed embryo extract. Therefore, it is entirely possible that these undialyzed materials, as well as the cell extract of Geyer and Neimark (5), contained sufficient uracil or some active nucleosides or nucleotides which could stimulate the process of cellular multiplication.

The contribution of C\(^{14}\)O\(_2\) to incorporated aspartic acid, glutamic acid, and proline was not surprising, since the intimate relation of carbon dioxide and Krebs cycle intermediates is well known in other biological systems (9, 20, 24–26). It was interesting, however, to find that aspartic acid was labeled relatively much more than glutamic acid and proline. This result furnished additional evidence that tumors can carboxylate pyruvate directly to yield oxalacetate and, hence, aspartic acid. This facet of tumor metabolism has been discussed by Freedman and Graff (3). The relatively high degree of labeling isolated in uracil and thymine when compared with that in cytosine deserves some consideration. If the contribution of carbon dioxide is via carbamyl phosphate, then most of the isotope would be found in carbon 2 of uracil, and this has been demonstrated in the intact rat (8). Further C\(^{14}\)O\(_2\) experiments have demonstrated that the specific activity of uridylic acid and cytidylic acid was the same in \textit{E. coli} (1).
Under these reported conditions, the relative specific activity of uracil and cytosine should have been similar, provided the principal source of cytidine nucleotides was from uridine nucleotides. Thus, it appears that the relation between carbon dioxide and the pyrimidines should prove a fruitful field of investigation in tumor cell cultures.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Mr. Eugene Conway, Mr. Wilbur Whittle, Mr. Gerald Orr, Mr. Earl Schoolar, and Mr. James Nash for technical assistance.

ADDENDUM

Since this manuscript was submitted, Chang, Liepins, and Margolish (Proc. Soc. Exper. Biol. & Med., 106:149-52, 1961) reported that ribosides plus oxaloacetic acid could substitute for the CO₂ requirement in HeLa and conjunctival cells. The free organic bases, however, were ineffective.

REFERENCES

Carbon Dioxide Metabolism in the Jensen and JA Sarcomas

in Vitro

Thomas A. McCoy, Merle D. Maxwell and Paul F. Kruse, Jr.