Incorporation of Bicarbonate-$^{14}$C into Pyrimidines and into Ribonucleic Acid of the Novikoff Ascites Tumor Cell$^{1,2}$

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SUMMARY

The incorporation of bicarbonate-$^{14}$C into pyrimidines of the Novikoff ascites tumor cell has been studied. It was shown that bicarbonate was incorporated into carbamylaspartate and into the C-2 of uracil nucleotides. The incorporation was increased by both glutamine or ammonia; glutamine was the more effective nitrogen donor. Exogenous orotic acid inhibited the formation of uridine monophosphate (UMP) and caused a buildup of labeled carbamylaspartate. The glutamine antagonist, 6-diazo-5-oxo-L-norleucine, inhibited the glutamine-stimulated bicarbonate incorporation into carbamylaspartate.

Bicarbonate-$^{14}$C has been shown to be rapidly incorporated into the ribonucleic acid of the Novikoff ascites cell either in vivo or in vitro. The specific activity of the corresponding liver RNA was approximately 1/2 to 1/5 that of the Novikoff RNA. Over 50% of the label was present in the UMP portion of the Novikoff RNA and less than 30% was present in the combined purine fraction.

The data suggest that an enzyme is present in the intact Novikoff ascites tumor cell that requires glutamine as the nitrogen donor in the formation of carbamyl phosphate. The incorporation of bicarbonate into UMP is consistent with the operation of the orotic acid pathway in the hepatoma.

INTRODUCTION

Carbamyl phosphate, the first intermediate in the de novo pathway for the synthesis of pyrimidines (see Chart 1), may be elaborated by means of several enzyme-catalyzed reactions.

\[
\begin{align*}
\text{(I) } & \text{ATP} + \text{NH}_4^+ + \text{HCO}_3^- \xrightleftharpoons{\text{Mg}^{++}} \text{NH}_2-C-OPO_3H_2 + \text{ADP} \\
\text{(II) } & 2\text{ATP} + \text{NH}_4^+ + \text{HCO}_3^- \xrightleftharpoons{\text{AG, } \text{Mg}^{++}} \text{NH}_2-C-OPO_3H_2 + \text{ADP} \\
\text{(III) } & \text{ATP} + \text{L-glutamine} + \text{HCO}_3^- \xrightleftharpoons{\text{Mg}^{++}} \text{NH}_2-C-OPO_3H_2 + \text{ADP} + \text{L-glutamic acid}
\end{align*}
\]

1 This investigation was supported by grants from the USPHS, National Cancer Institute, (CA06571) and National Science Foundation (GB4339) and by an institutional grant from the American Cancer Society (IN-27-G-Project s 11).

2 Portions of this study have been reported at the American Association for Cancer Research in Denver, Colorado, 1966.

3 Recipient of a Lederle Medical Faculty award.

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The precipitate remaining after removal of the acid-stable extract was treated with 70% ethyl alcohol, 95% ethyl alcohol, and twice with 95% ethyl alcohol-ether (1:3, v/v). The lipid-free precipitate was then suspended in 10% sodium chloride, pH 7.0, and heated in a boiling water bath for 30 min. The precipitate was collected by centrifugation and once again suspended in 10% sodium chloride, pH 7.0, and reheated for 15 min. The supernatant fractions were combined and two volumes of 95% ethyl alcohol were added. The suspension was then stored at −10°C overnight to complete the precipitation of the nucleic acids. The nucleic acid precipitate after collection by centrifugation was dissolved in 0.1 N sodium hydroxide and heated for 1 hr at 80°C to hydrolyze the RNA to its nucleotides (10). The resultant solution was cooled, brought to pH 1.0 and the precipitated DNA was removed by centrifugation. The supernatant fluid containing the RNA-nucleotides was decanted and the optical density at 265 nm was determined. In addition, the radioactivity of an aliquot of this solution was determined by liquid scintillation counting technics.

The RNA nucleotides were neutralized and placed on columns (1 x 10 cm) of Dowex 1-X8 (formate form), 200-400 mesh. The nucleotides were eluted with a continuous gradient from water to 4 N formic acid. Fractions were collected and their optical densities were determined. The individual fractions comprising a single nucleotide were combined, dried under vacuum and redissolved to 5 ml in water, and the specific activities of the nucleotides were determined.

The incorporation of bicarbonate-14C into pyrimidine precursors was measured in the following system: salt buffer, pH 7.4, 7 ml; cellular suspension (50%), 1 ml, added at minus 5 min; sufficient 0.9% NaCl to make a 10-ml volume after the addition of the other ingredients; when present L-glutamine, NH4Cl, glucose, orotic acid, carbamylaspartate, and DON; bicarbonate-14C, 50 μmoles (10 μc), at 0 time. The flasks were shaken for 10 min at 37°C, then were immersed in an ice bath and 2 ml of 4 N HClO4 were added (9).

Separation of Acid-soluble Components. The acid extract of the cells was placed in a boiling water bath for 1 hr, cooled, and neutralized with 4 N NaOH with phenol red as an internal indicator. The neutralized material was then passed through a Dowex 50-X8 (H+ form) column, 200-400 mesh (1 x 6 cm), to remove any amino acids. The column was washed with water, and the washings were combined with the original eluate.

The Dowex 50 eluate was concentrated in vacuo to 5 ml, neutralized, and applied to a Dowex 1-formate-X8 column, 200-400 mesh (1 x 6 cm). The column was eluted as described by Hager and Jones (9). Optical densities at 235, 260, and 280 (A280, A260, and A235) μm and radioactivity of the fractions were determined.

Hydrolysis of UMP and Degradation of Uracil. The UMP fraction isolated from the Dowex 1-formate columns was dried in vacuo and hydrolyzed to the free base with perchloric acid (9). The uracil was purified and degraded according to the method of Hager and Jones (9).

The method of degradation was checked using standard uracil-2-14C (10μmoles containing 4000 dpm). The recovered radioactivity as 14CO2 was 3040 dpm, a recovery of 76%.

Identification of Compounds. Carbamylaspartate, 5-hydantoin acetic acid, UMP, and orotic acid were identified by their elution patterns from Dowex 1-formate columns using pure compounds as standards. Their identity was further verified by

![Diagram](chart.png)

**Chart 1.** The de novo pathway for pyrimidine biosynthesis.

- a, Carbamyl phosphate synthetase; b, aspartate transcarbamylase; c, dihydroorotase; d, dihydroorotic dehydrogenase. ATP, adenosine-5'-triphosphate; AG, α'-acetyl-L-glutamate; L-Asp, L-aspartic acid.

 Obtained from Dr. R. B. Hurlbert of M. D. Anderson Hospital and Tumor Institute and maintained by weekly intraperitoneal injections into male Holtzman rats, 150-170 gm in weight. Seven-day cells were collected by centrifugation, washed with 0.3% sodium chloride, and suspended in one volume of a salt buffer, pH 7.4 (60 mm sodium phosphate buffer, pH 7.4; 40 mm NaCl; 5.0 mm KCl; and 1.3 mm MgCl2). In Vivo Studies. Male Holtzman rats, 150-170 gm in weight, bearing 7-day-old Novikoff ascites cells were used in all experiments. The original hepatoma was obtained from Dr. R. B. Hurlbert of the M. D. Anderson Hospital and Tumor Institute and was maintained by weekly intraperitoneal transplants.

The tumor-bearing rats were injected intraperitoneally with 6 μc of bicarbonate-14C in two doses (3 μc at 0 time and 3 μc 5 min later), and the rats were sacrificed periodically thereafter. The Novikoff cells and ascites fluid were drained from the rats, and the cells were collected by centrifugation at 600 X g for 5 min. The sedimented cells were washed with 0.3% sodium chloride to lyse the erythrocytes, centrifuged to pack the Novikoff cells, and suspended in 1 volume of 1 N perchloric acid. The resulting precipitate was further used for the isolation of RNA. The precipitate remaining after removal of the acid-stable extract was treated with 70% ethyl alcohol, 95% ethyl alcohol, and twice with 95% ethyl alcohol-ether (1:3, v/v). The lipid-free precipitate was then suspended in 10% sodium chloride, pH 7.0, and heated in a boiling water bath for 30 min. The precipitate was collected by centrifugation and once again suspended in 10% sodium chloride, pH 7.0, and reheated for 15 min. The supernatant fractions were combined and two volumes of 95% ethyl alcohol were added. The suspension was then stored at −10°C overnight to complete the precipitation of the nucleic acids. The nucleic acid precipitate after collection by centrifugation was dissolved in 0.1 N sodium hydroxide and heated for 1 hr at 80°C to hydrolyze the RNA to its nucleotides (10). The resultant solution was cooled, brought to pH 1.0 and the precipitated DNA was removed by centrifugation. The supernatant fluid containing the RNA-nucleotides was decanted and the optical density at 265 nm was determined. In addition, the radioactivity of an aliquot of this solution was determined by liquid scintillation counting technics.

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ascend paper chromatography using the following solvents of Fink et al. (5): tert-butyl alcohol:methyl ethyl ketone:formic acid:water (40:30:15:15); isopropyl alcohol:water:HCl (65:18.4:16.6); upper phase from a mixture of sec-butyl alcohol and water; tert-butyl alcohol:methyl ethyl ketone:water:formic acid (44:44:11:0.26).

Radioactivity Analysis. Aliquots of aqueous solutions were transferred to vials containing a scintillation fluid described by Bray (1) and were assayed in a Tri-Carb liquid scintillation spectrometer. When 14CO2 was analyzed, the gas was collected in 1 ml of Hyamine and assayed in a toluene phosphor solution containing 0.5% PPO and 0.03% dimethyl POPOP. The efficiency of counting was determined with toluene-14C as the internal standard and the radioactivity was expressed as disintegrations per min.

RESULTS

In Vivo Incorporation of Bicarbonate-14C. A rapid incorporation of bicarbonate-14C into the RNA of the Novikoff ascites cells occurred (Table 1). The incorporation proceeded linearly for approximately 30 min, reaching a maximum of 365 dpm per mg of RNA at 60 min. The specific activity of the RNA-nucleotides was determined after an incorporation time of 30 min. The specific activity of the purines was 55 dpm/µmole while the specific activity of the uridine monophosphate moiety of RNA was twice this level. Approximately 50% of the radioactivity noted in the RNA was present in the form of UMP and less than 30% was present in the combined purine fraction.

A comparison of the incorporation of the label into liver and Novikoff cells revealed that the liver RNA had a specific activity only ½ to ⅓ that of the Novikoff RNA (Table 1). The peak incorporation of the isotope into liver RNA occurred at 30 min (specific activity of 55 dpm/µg).

In Vitro Incorporation of Bicarbonate-14C into Novikoff RNA. The incorporation of bicarbonate-14C into the RNA of the Novikoff ascites cells in vitro was linear for approximately 120 min (Table 2) at which time the specific activity was 1900 dpm/mg. After 120 min of incubation, considerable label was still present in the acid-stable fraction. The results of nucleotide analysis revealed that most of the label was present in the uridine monophosphate moiety of the RNA (Table 3). The specific activity of the latter was 3-fold that of the purine or cytidine monophosphate fractions.

Separation of Intermediates in the Orotic Acid Pathway. Preliminary experiments with intact Novikoff ascites cells revealed four radioactive peaks eluted from Dowex 1-formate columns (Chart 2). Neither the water eluate which would contain uracil and uridine nor the 0.01 M formate fraction contained significant radioactivity. The 0.2 M formic acid fraction included two peaks, one which corresponded to 5-hydantoin acetic acid (carbamylaspartate was converted to its hydantoin by our isolation procedure) and the second peak has not been identified. Only one peak was eluted with 0.085 M ammonium formate, pH 4.5, which has been identified as UMP (by paper chromatography). Ammonium formate, 0.3 M, pH 4.3, eluted one unknown peak which overlapped standard orotic acid but was not coincident with this substance.

Comparison of Glutamine and Ammonia as Nitrogen Donors. The greatest stimulation in UMP formation from bicarbonate was observed when glutamine was the nitrogen donor (Table 4). A 2-fold increase in UMP formation occurred in the presence of 1 mM glutamine. Ammonia at 5 mM produced a smaller, but significant effect upon UMP formation.

In Chart 3, the effect of these nitrogen donors upon carbamylaspartic formation is demonstrated. Carbamylaspartate, 5 mM, was included in these incubations to stop the reaction at this step. Carbamylaspartate can be readily transported into the Novikoff cell (2). Although the results are not striking, the addition of glutamine does result in a stimulation in labeling. Ammonium ions also enhanced the incorporation of HCO3- but to a lesser extent.

The addition of 20 mM glucose, a possible energy source, had no effect upon UMP formation (Table 4). This held true for cells incubated alone or in the presence of a nitrogen donor.

Effect of Orotic Acid upon Bicarbonate Incorporation into UMP. The presence of 10 mM orotate in the incubation media was attended by a larger amount of label in the car-
Bicarbonate Incorporation in Tumor Pyrimidines and RNA

**Chart 2.** Separation of pyrimidine precursors on Dowex 1-formate. One ml of a cellular suspension in a pH 7.4 salt buffer was incubated with NaH14CO3 (50 μmoles [10 μl]), 10 μmoles glutamine, and 0.9% NaCl to a total volume of 10 ml. A perchloric acid extract was passed through Dowex 50 (H+) to remove the anions and then placed on Dowex 1-formate columns. Dowex 1-formate columns were eluted with water, 0.01 M formic acid, and as shown, 0.2 M formic acid (F.A.), 0.085 ammonium formate (A.F.), and 0.3 M ammonium formate and collected in 10-ml fractions. Standard hydantoin acetic acid (50 μmoles), uridine monophosphate (UMP) (10 μmoles), and orotic acid (10 μmoles) were also added to Dowex columns. The absorbance of UMP and orotic acid were read at 260 and 280 nm (A260 and A280), respectively. Hydantoin acetic acid was determined by the addition of 0.5 ml from each 10-ml fraction to 2.5 ml of 0.1 N NaOH in a 1-cm cuvet and reading the change in absorbance immediately against a similar mixture of eluent and base at 235 nm (Aas) according to the method of Hager and Jones (9). The lower portion of this chart shows the absorption of these standards. The upper tracing indicates the radioactivity of the experimental eluents from the Dowex 1-formate columns.

**Table 4**

<table>
<thead>
<tr>
<th>Additions</th>
<th>μmoles</th>
<th>Acid-stable dpm added to Dowex 1 columns</th>
<th>Percent dpm recovered per fractiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. None</td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Glutamine</td>
<td>10</td>
<td>51,982</td>
<td>15.3 ± 3.8 (8)</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>50</td>
<td>46,103</td>
<td>16.2 ± 3.5 (7)</td>
</tr>
<tr>
<td>B. Glucose</td>
<td>200</td>
<td>51,224</td>
<td>10.4 ± 4.3 (3)</td>
</tr>
<tr>
<td>Glucose</td>
<td>200</td>
<td>40,083</td>
<td>11.9 ± 5.4 (6)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>10</td>
<td>25,338</td>
<td>15.7 ± 8.7 (3)</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Orotic acid</td>
<td>100</td>
<td>21,721</td>
<td>35.6 ± 11.5 (3)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>10</td>
<td>22,624</td>
<td>40.8 ± 11.5 (3)</td>
</tr>
<tr>
<td>Orotic acid</td>
<td>100</td>
<td>15,088</td>
<td>38.6 ± 12.2 (3)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orotic acid</td>
<td>100</td>
<td>16,215</td>
<td>14.2 ± 7.9 (3)</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

See Chart 2 for details of incubation.

a A, B, C, and D represent peaks from Dowex 1-formate columns as shown on Chart 1. A, Carbamyl-aspartate; B, unknown peak eluted with 0.2 M formic acid; C, uridine monophosphate; D, unknown peak eluted with 0.3 M ammonium formate.

b Standard deviation of the mean.

c Number of observations.
bamylaspartate fraction and a lesser amount of radioactivity appearing in UMP (Table 4). In addition, less radioactivity was present in the acid-stable fraction.

**Distribution of Label within the Pyrimidine Ring.** UMP samples isolated from incubations containing 1 mM glutamine were hydrolyzed, and the uracil was purified as described in Methods. Two purified uracil samples containing 880 and 520 dpm, respectively, were degraded to measure the label in C-2 of the ring. The C-2 contained 541 (65%) and 280 (54.5%) dpm, respectively.

As shown in Methods, an authentic sample of uracil-2-14C gave a recovery of 76% of the radioactivity in C-2. A similar recovery has been reported by Hager and Jones (9). Consequently, if an additional 24% is added to the recoveries with the experimental uracil samples, the C-2 contained 84 and 72% of the radioactivity, respectively. Virtually all of the label from H14CO3 was incorporated into this position of the pyrimidine ring.

**Effect of DON upon Carbamylaspartate Formation.** If glutamine is required for the conversion of bicarbonate into UMP, then, a glutamine antagonist should inhibit this incorporation. Accordingly, DON, a potent antagonist of glutamine, was included in the incubation medium with 1 mM glutamine. These results are depicted in Chart 4. A progressively increasing inhibition of carbamylaspartate formation was apparent with increasing concentration of inhibitor.

**DISCUSSION**

The results reported herein indicate that the Novikoff ascites tumor cells contain the necessary complement of enzymes for the incorporation of the small molecular weight precursor, bicarbonate, into the pyrimidine moiety of RNA. The possibility of the production of the pyrimidines or a precursor thereof in the liver and the subsequent transport of the material to the tumor seems quite unlikely for the following reasons. If one compares the specific activities of the RNA of the Novikoff cells and liver from the same animal, it is apparent that the latter possesses a much lower specific activity. In addition, no lag in the labeling of the Novikoff RNA is apparent. Ito and Tatibana (12) have also reported the specific activity of spleen uridine monophosphate to be much higher than the corresponding nucleotide from liver, following the subcutaneous injection of bicarbonate-14C into mice. They have suggested that this nonhepatic tissue is capable of the de novo synthesis of pyrimidines. The existence of a similar pathway in the Ehrlich ascites cells has been suggested by Kusama and Roberts (16) and Hager and Jones (9). Both workers have been unable to detect any carbamyl phosphate synthetase-like activity or show the incorporation of bicarbonate-14C into RNA with ruptured cell preparations. On the other hand, the RNA was very heavily labeled after a short pulse in vivo. Hager and Jones (9) have furthermore demonstrated that the Ehrlich ascites cell can incorporate bicarbonate into carbamylaspartate as well as into UMP.

The results presented here suggest that the incorporation of bicarbonate into carbamylaspartate may proceed via carbamyl phosphate. Attempts to demonstrate carbamyl phosphate synthetase activity in these cells, however, have not been successful either in this laboratory or elsewhere (20). Although carbamylaspartate and UMP are the only intermediates of the orotate pathway that have been identified in the present investigation, the evidence is consistent with the operation of this pathway in these cells. All enzymes of the orotate pathway, except carbamyl phosphate synthetase, are apparently present in this tissue (2, 20).

When orotic acid was added to the incubation system, a depression in the radioactivity of the acid-stable fraction and an increase in the labeling of the carbamylaspartate fraction were
observed. The pile-up of carbamylaspartate certainly is in accord with a feedback mechanism operative at the dihydroorotase step as reported earlier (3). These data also suggest the possibility of a feedback mechanism at the step, bicarbonate into acid-stable material.

It would appear that glutamine is the most effective nitrogen donor for UMP synthesis in the intact Novikoff ascites cell; ammonia at five times the glutamine concentration did not produce full activity. The response, however, with glutamine is not as unambiguous as we would wish. The Novikoff cell itself contains some glutamine which makes difficult the production of an all-or-none effect. Hager and Jones (9) have recently shown that glutamine is an effective nitrogen donor for bicarbonate incorporation into UMP in Ehrlich ascites cells. Perhaps the glutamine-requiring carbamyl phosphate synthetase is operative in these tumor cells.

Further evidence for the glutamine requirement stems from the inhibition of carbamylaspartate formation upon the addition of DON, a glutamine antagonist. DON has been demonstrated to inhibit the glutamine-dependent carbamyl phosphate synthetase in mushrooms (17) and the incorporation of HCO₃⁻ into UMP in Ehrlich ascites cells (9). Studies are currently in progress to extract the enzyme or enzyme system in a soluble form. Attempts, however, to solubilize the enzyme by rupturing the cells by physical means, e.g., blending with glass beads or sonication, or by detergent treatment, e.g., sodium deoxycholate, Triton X100, digitonin, have not proven successful.

REFERENCES

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