Carbon Dioxide Incorporation into the Uracil of Mouse Liver and Ehrlich Ascites Tumor Cells*

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$NaHC^{14}O_3$ solution was injected intraperitoneally into mice bearing the Ehrlich ascites tumor, and uracil was isolated from the RNA of the ascites cells and from the acid-soluble fractions of both ascites cells and liver. The activity of the C-2 carbon of uracil was measured by a new micromethod usable down to 5 μmoles of uracil and was compared to the activity of the other carbon atoms. The percentage of total activity found in the C-2 of the uracil of the ascites cells (90%) was much greater than that in liver (50%). Although attempts to demonstrate the presence of carbamyl phosphate synthetase activity in preparations from the Ehrlich ascites cells were unsuccessful, the results are consistent with a primary fixation of CO_2 into carbamyl phosphate or some other carbamyl donor which participates subsequently in pyrimidine biosynthesis in these cells. It is possible that in the tumors some compound other than carbamyl phosphate is the carbamyl donor or that the carbamyl phosphate used *in vivo* for the synthesis of uracil may be formed in some other tissue, possibly the liver, and then made available to the tumor cells for biosynthetic purposes.

Biosynthesis of RNA takes place rapidly in Ehrlich ascites cells, and the enzymes related to RNA synthesis are very active in these cells in comparison with other tissues (Calva *et al.*, 1959; Reichard and Skold, 1957; Skold, 1960). At the time this work was initiated there were no reports of the detection of carbamyl phosphate synthetase activity, the first step of pyrimidine biosynthesis, in cancer cells. We attempted to measure the activity of this enzyme in Ehrlich cells by methods previously applied to liver and intestine (Hall *et al.*, 1960), but failed to detect any activity in various types of extracts of these cells. During the course of this work it was reported that neither carbamyl phosphate synthetase nor ornithine transcarbamylase activities could be detected in Ehrlich ascites tumor cells but that high levels of aspartate transcarbamylase were found (Jones *et al.*, 1961). We then changed to a study of the *in vivo* incorporation of $C^{14}O_2$ into the C-2 position of the uracil of the ascites cell RNA and of the acid-soluble nucleotides of the ascites cells and the livers of the tumor-bearing animals in order to get further insight into this problem.

Hitherto, approximately 200 μmoles of uracil has been required for the degradative procedures in the estimation of radioactivity of the C-2 carbon of uracil (Heinrich and Wilson, 1950). In the present work the procedure has been made more sensitive, so that the minimum amount required is about 5 μmoles.

EXPERIMENTAL

Animals.—Swiss mice bearing 7- to 10-day-old Ehrlich tumors were used for the experiments.

Determination of Radioactivity.—Measured amounts of aqueous samples were pipetted into Tricarb vials and dried under a stream of warm air. One ml of 1 M Hyamine 10-X in methanol[1] and 15 ml of 100% toluene counting solution were added.[2] Radioactivity was assayed in a Tricarb Liquid Scintillation Spectrometer (Model 314 X) for several periods of 10 minutes each at −8°. In the case of CO_2, the gas was liberated by acidification and absorbed directly by 1 ml of Hyamine solution in an apparatus similar to that described by Sisken *et al.* (1961).

Preparation of RNA Hydrolysate.—In each experiment, two mice were injected intraperitoneally with 0.5 ml of $NaHC^{14}O_3$ solution containing either 5 μc or 25 μc (solution prepared from original material of approximately 10 mc/mmole obtained from California Corporation for Biochemical Research) and the mice were sacrificed 5 minutes, 15 minutes, or 1, 3, 6, or 24 hours after injection. Ascites fluid was drained from the mice and the cells were collected by centrifugation, then twice washed with cold 0.85% NaCl solution. An equal volume of 1 N perchloric acid was added to the tumor cells, the mixture was stirred and centrifuged, and the precipitate was washed twice with 0.3 N perchloric acid. The supernatant fluids were combined as the "acid-soluble fraction." The precipitate was used for the preparation of a KOH hydrolysate of the cell RNA according to Tyner *et al.* (1953). The KOH solution was neutralized with perchloric acid and the $KClO_4$ centrifuged. The supernatant fluid was called the "RNA hydrolysate." Livers were removed from the same mice, after the ascites fluid was drained, and homogenized with 1 N perchloric acid in a Waring Blendor. The homogenate was treated the same as the ascites cells and separated into the same two fractions.

In the *in vitro* experiments, 2 ml of Ehrlich ascites fluid and cells (obtained 9 days after inoculation) containing a small amount of heparin was placed in a small flask and the mouth was closed tightly with a rubber stopper. Five-tenths ml $NaHC^{14}O_3$ (25 μc) solution was injected through the rubber stopper. This flask was incubated for 15 or 30 minutes at 37° and the contents were poured into a centrifuge tube. Two ml of 1 N perchloric acid was added and the precipitate was centrifuged. The procedure thereafter was the same as for the *in vivo* experiments.

Separation of Nucleotides from RNA Hydrolysate.—The neutralized RNA hydrolysate again was made alkaline by addition of ammonium hydroxide solution and run through a 1 × 5 cm column of Dowex 1 X8 (formate form, 200–400 mesh), followed by 50 ml of water. CMP, AMP, and GMP were eluted separately

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[1] Hyamine 10-X is *p*-(diisobutyl-cresoxyethoxyethyl)-dimethylbenzylammonium hydroxide.

[2] 1000 ml of toluene contains 3.0 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP).

by a modification of the method of Cohn and Volkin (1951). The solvents used were 0.02 M formic acid, 0.15 M formic acid, 0.01 M formic acid + 0.075 M ammonium formate, and 0.2 M formic acid + 0.2 M ammonium formate.

The effluent was collected in 10-ml fractions and the optical density of each fraction was determined at 260 mμ with a Beckman Model DU spectrophotometer. The several fractions having significant optical density at 260 mμ were assayed for their radioactivity.

Separation of Acid-Soluble Components.—For assay of amino acids and other carboxylic acids, the acid-soluble fraction from liver was neutralized with 1 N KOH and kept in a refrigerator overnight, and the precipitate of $KClO_4$ was centrifuged off. The neutralized supernatant was passed by gravity flow through Dowex 1 X10 (formate form, 200–400 mesh, 1 × 15 cm). The adsorbed substances were eluted by gradient elution with formic acid (Hurlbert *et al.*, 1954). The fractions with high radioactivity were rechromatographed on Dowex 1 X10 with ammonium formate (Hurlbert *et al.*, 1954) and then identified by paper chromatography.

For the isolation of purine and pyrimidine compounds, the acid solution obtained as described above was heated in a boiling water bath for 1 hour and passed through an activated charcoal[3] column, 1 × 5 cm (Tsuboi and Price, 1959). The column was washed with 15 ml of water and 15 ml of 0.01 N ammonia solution. The adsorbed substances were eluted with water–ethanol–concentrated NH_4OH (2:2:1) and the eluate was dried under a stream of warm air. The residue was dissolved in 20 ml of water and the solution was run through a column of Dowex 1 X8 (formate form, 200–400 mesh, 1 × 6 cm).

The purine derivatives, hydrolyzed by the acid to adenine and guanine, and all pyrimidine derivatives, changed to their monophosphates, were successively eluted by water, by 0.01 M and 0.2 M formic acid, and finally by 0.01 + 0.075 M ammonium formate solution. Only UMP appeared in the last solution.

Hydrolysis of UMP to Uracil.—The UMP fractions isolated from the RNA or the acid-soluble fraction were dried at 100°. The residue was dissolved in a small amount of 12 M perchloric acid, quantitatively transferred to a glass test tube with a stopper, and heated in a boiling water bath for 1 hour (Marshak and Vogel, 1951). The hydrolyzed solution was diluted with 5 volumes of distilled water and the charcoal column treatment was repeated. The eluted bases were dried, dissolved in several ml of water, and passed through a Dowex 50 X8 column (H^+ form, 1 × 2 cm), which was then washed with 10 ml of water. The effluent and washings, containing the pure uracil derived from the UMP, were combined and decomposed as described below.

Decomposition of Uracil.—At first, uracil was decomposed by the method of Heinrich and Wilson (1950), except that the amount of the uracil in the sample was less (5 μmoles) than used by them (200 μmoles). Uracil was decomposed to CO_2 and oxaluric acid by $KMnO_4$ in acid solution, and the CO_2 (CO_2 fraction) was absorbed in Hyamine solution. Then the oxaluric acid was decomposed by alkali to urea and oxalic acid and the latter was precipitated as calcium oxalate by addition of calcium chloride solution and removed by centrifugation from the supernatant, which contained the urea. However, when the amount of sample was very small, no precipitation occurred. In these cases, either 10 mg of nonradioactive uracil was added to the samples at the start, or 5–10 mg of oxalic acid was added just before this step. The precipitate of calcium oxalate was washed with a small amount of water, dissolved in 2 N sulfuric acid, and decomposed to CO_2 by $KMnO_4$ (oxalic acid fraction). The supernatant solution and washings were combined, and urea was decomposed to CO_2 and NH_3 by urease (urea fraction).

Since recovery of the radioactivity in the uracil by this procedure was only 60–85% (Table II), the method described below was employed.

Improved Method.—Five ml of uracil-containing solution (5 μmole) was dried in a reaction flask and dissolved in 0.5 ml of water and 0.12 ml of 0.5 N sulfuric acid. The flask was connected with a glass U-tube to a Tricarb vial containing 1 ml of Hyamine solution, and the side-arm of the flask was closed with a rubber serum bottle stopper (Sisken *et al.*, 1961). Six-tenths ml of 0.4 N $KMnO_4$ was injected with a needle and syringe through the rubber stopper and the flask was shaken for 3 hours at 37°. After the reaction, the vial and flask were removed from the U-tube (CO_2 fraction). The contents of the flask were filtered and the flask was washed with hot water four times (each 0.5 ml). The filtrate and washings were collected in another flask and 0.15 ml of 2 N NaOH was added; the solution was then heated in boiling water for 15 minutes. After cooling, 1 drop of phenolphthalein solution was added and the solution was neutralized with 1 N acetic acid. The addition was stopped while the color of the solution was still slightly pink. The flask was connected to a new vial and sealed as before, then 0.2 ml of a solution containing 0.3 mg urease per ml (Nutritional Biochemicals Corporation) in 0.1 M Tris buffer, *p*H 8.0, was injected through the rubber stopper. During incubation at 37°, the flask was shaken, and after the second injection of urease (30 minutes later) the shaking was continued for more than 1 hour, after which 0.2 ml of 12 N sulfuric acid was injected. Again the vessel was shaken for 3 hours (urea fraction). A third vial containing 1 ml of Hyamine was connected, and 1 ml of 0.4 N $KMnO_4$ was injected into the flask, which was shaken for 3 hours (oxalic acid fraction). These three vials were used for the determination of radioactivity.

Results and Discussion

Failure to Detect Carbamyl Phosphate Synthetase Activity in Ehrlich Ascites Tumor Cells in vitro.—Several types of cell preparations were examined for carbamyl phosphate synthetase activity, among them the following: the whole ascites fluid from Swiss mice 9 days after transplantation; a suspension of cells in Tris buffer (*p*H 7.5, 0.1 M) after washing with 0.85% NaCl solution; the supernatant fluid obtained from ascites cells after homogenization in a Potter-Elvehjem homogenizer and centrifugation at 0°, 3000 rpm. Since carbamyl phosphate is very labile and there are no methods published by which to determine it directly, the following two methods were employed for detection of synthesis of this substance. (1) If carbamyl phosphate is synthesized and aspartic acid is present in the same reaction mixture, carbamylaspartic acid should appear, since aspartic acid transcarbamylase activity is high in ascites tumor cells (Calva *et al.*, 1959; Jones *et al.*, 1961). Carbamylaspartic acid was measured by the method of Koritz and Cohen (1954). (2) Active ornithine carbamyl transferase was prepared from rat liver (Grisolia, 1955) and this enzyme and ornithine were added to the reaction mixture. If carbamyl phosphate were synthesized, citrulline would be produced.

[3] Barneby-Cheney No. 1654 Activated Carbon, Barneby-Cheney Co., Columbus 19, Ohio.

TABLE I
INCORPORATION OF $C^{14}O_2$ INTO THE RNA OF ASCITES CELLS AND LIVER

Samples		cpm/OD Unit[a]	cpm/μmole in			
Source	Time		UMP	CMP	AMP	GMP
In vitro						
Ascites cells	15 min.	0	—	—	—	—
	30 min.	0	—	—	—	—
In vivo						
Ascites cells	5 min.	1.1				
	15 min.	35	521 (100)[c]	8 (2)	90 (17)	33 (6)
	1 hr.	130	807 (100)	121 (15)	143 (21)	102 (13)
	1 hr.[b]	18				
	3 hr.[b]	50	178 (100)	75 (42)	81 (46)	53 (30)
	6 hr.	226	546 (100)	334 (60)	356 (64)	346 (62)
	48 hr.	206	258 (100)	266 (107)	228 (89)	250 (97)
Liver	6 hr.	1.9				
	48 hr.	1.5				

[a] An optical density unit is defined as an absorbance of 1.0 in the 1-cm cell of the Beckman DU spectrophotometer at 260 mμ. [b] 5 μc of $NaHC^{14}O_3$ was injected in these experiments, 25 μc in the others. [c] Figures in parentheses are the specific activities relative to uracil, times 100.

Citrulline was measured by the method of Archibald (1944). The reaction solutions (5 ml, *p*H 7.5) contained: enzyme solution; 100 μmoles NH_4HCO_3 (or glutamine + $NaHCO_3$, or $(NH_4)_2SO_4$ + $NaHCO_3$); 20 μmoles ATP; 30 μmoles $MgSO_4$; 10 μmoles acetylglutamic acid; and an ATP-regenerating system (phosphenol pyruvate + phosphoenol pyruvate kinase). These mixtures were incubated for 15 or 30 minutes at 37° and $HClO_4$ was added to stop the reaction, after which the expected products were measured by the indicated methods. No carbamylaspartate or citrulline formation was observed in any case. Hence, it may be concluded that carbamyl phosphate was not synthesized by ascites cells *in vitro* under our conditions. After this work was completed a similar finding was reported by Jones *et al.* (1961).

Incorporation of C^{14} from $NaHC^{14}O_3$ into RNA Nucleotides (Table I).—No radioactivity was detected in the RNA nucleotides in freshly removed Ehrlich ascites cells after 15 or 30 minutes' incubation of the whole ascites fluid (heparinized to prevent clotting) with $NaHC^{14}O_3$ (25 μc) *in vitro*. On the other hand, there was incorporation of $C^{14}O_2$ into RNA even at 15 minutes *in vivo*. The UMP moiety was labeled much more rapidly than the other nucleotides, and only 48 hours after the injection of the isotope did the specific activity of the other nucleotides approach that of UMP. In contrast, there was little detectable appearance of the label in liver RNA at the 6- and 48-hour intervals.

Incorporation of C^{14} from $NaHC^{14}O_3$ into Specific Positions of the Uracil of the Acid-Soluble Fraction and RNA.—Since our own experiment and those of others (Jones *et al.*, 1961) failed to show carbamyl phosphate synthetase activity *in vitro* in Ehrlich tumors, it became of interest to see whether the C-2 atom of uracil can be derived to any extent from $C^{14}O_2$ *in vivo*. The C-2 atom of uracil appears as CO_2 after the final urease treatment in our analytical procedure. The efficacy of that portion of the analytical method is demonstrated by the finding of 99% of the total radioactivity of synthetic uracil-2-C^{14} in this fraction and the recovery of 96–97% of the total radioactivity in the sample (Table II). The C-4, C-5, and C-6 atoms of uracil are estimated as the sum of the CO_2 and oxalic acid fractions (Lagerkvist, 1953; Fairley *et al.*, 1953). The adequacy of the modified procedure for these carbons is indicated by the generally good recoveries of the total radioactivity in the isolated samples of uracil (samples 6, 8–10, and 13–15 in Table II). Results for duplicate runs on the same sample also are shown (samples 9 and 10 and 14 and 15).

There was only little incorporation of the $C^{14}O_2$ into the acid-soluble nucleotide fraction of the Ehrlich tumor cells *in vitro*. In the *in vivo* experiments incorporation of $C^{14}O_2$ took place into the C-2 of uracil, both in the acid-soluble fraction and in RNA, to a greater extent than into the other carbons, the radioactivity in this carbon accounting for approximately 90% of the total radioactivity found. In the uracil isolated from the acid-soluble nucleotide fraction of liver the C-2 contained only 49–70% of the total radioactivity, values similar to that in RNA of regenerating liver (Lagerkvist, 1950). In the chromatography of extracts of livers of animals that had received $NaHC^{14}O_3$, relatively high levels of radioactivity were noted in aspartic acid. The latter amino acid could serve as a source of C-4, C-5, and C-6 of uracil, and therefore the proportion of the total radioactivity furnished by C-2 would be reduced. A smaller amount of label entering aspartate from the $NaHC^{14}O_3$ in the tumor cells could account for the higher proportion of the isotope from this source being found in C-2 of the uracil in tumors.

The rapid appearance of radioactivity in C-2 of the uracil of the RNA and of the soluble uracil pool in the tumor cells suggests that the $C^{14}O_2$ may enter rather directly into C-2 and is compatible with the existence of a carbamyl phosphate synthetase pathway in these animals. The simplest assumption which can be made about the discrepancy of the *in vivo* and *in vitro* results is that the methods to date employed for the *in vitro* work have failed to preserve and demonstrate the activity of an extremely sensitive enzyme. It is interesting in this connection that the carbamyl phosphate synthetase of intestine is much more labile than that of liver (Hall *et al.*, 1960). It may also be that some other mechanism, similar to that for the synthetase but requiring other conditions for demonstration, is involved in the CO_2 fixation. Another possibility is that the carbamyl phosphate may be made in the liver, the tissue with by far the highest synthetase activity, and transported in some form to the tumor cells and other tissues *via* the blood stream. The much smaller incorporation of $C^{14}O_2$ into uracil in a short *in vitro* experiment with whole tumor (sample 6, Table II) and the smaller proportion of total radioactivity found in C-2 of uracil than in a comparable *in vivo* experiment (sample 8, Table II) is consistent with such a possibility. The high aspartate transcarbamylase levels in Ehrlich tumors (Calva *et al.*, 1959; Jones *et al.*, 1961) together

TABLE II
DISTRIBUTION OF C^{14} IN THE URACIL MOLECULE

Sample No.	Description	Time	Total Activity (cpm)	C-4, 5, 6		C-2 / Urea (cpm)	C-2 × 100 / C-2 + C-4 + C-5 + C-6 (%)	Recovery of C^{14} in Sample (%)
				CO_2 (cpm)	Oxalic Acid (cpm)			
1[a]	Synthetic uracil-2-C^{14}		9464	13.8	36.8	8977	99	96
2	Same		9464	20.2	38.8	8974	99	96
3	Same		9464	27.2	24.6	9128	99	97
4[b]	Same		9464	40.4	30.0	8018	99	84
5[c]	Same		9464	31.0	16.6	7539	99	80
	In vitro							
6	Ascites cells, acid-soluble fraction	15 min.	17	7.0	2.6	6.0	38	92
7[b]	Same	15 min.	17	4.0	4.2	4.8	37	76
	In vivo							
8	Ascites cells, acid-soluble nucleotide fraction	5 min.	500	35.4	3.2	367	90	81
9	Same	6 hr.	293	22.0	8.4	263	90	100
10	Same	6 hr.	293	22.6	6.2	257	90	97
11[b]	Same	6 hr.	293	18.6	11.2	166	85	67
12[b]	Ascites cells, RNA	3 hr.	270	16.0	3.6	159	88	63
13	Same	6 hr.	70	2.4	6.0	55	87	91
14	Liver, acid-soluble fraction	5 min.	194	66.0	23.8	96	52	96
15	Same	5 min.	175	64.8	24.0	89	49	104
16	Same	6 hr.	247	61.8	15.2	182	70	104
17[b]	Same	6 hr.	247	58.0	41.2	66	40	67

[a] Purchased from Isotope Specialties Co., Inc., 0.6 mc/mM. [b] Precipitation achieved by addition of unlabeled oxalic acid. Method of Heinrich and Wilson (1950). [c] Addition of unlabeled uracil. Method of Heinrich and Wilson (1950).

with the results of the present work are strongly suggestive of the existence of the orotic acid pathway of pyrimidine biosynthesis in these tumor cells. The Ehrlich tumor also possesses high levels of uridine phosphorylase and uridine kinase, enabling the tumor to make UMP directly from uracil (Reichard, 1959). However, it does not appear likely from our results that uracil formed in the liver is a direct precursor of *most* of the uracil in the tumor cells, since the proportion of total radioactivity in the C-2 is so much higher in the uracil of the tumors.

Label from $NaHC^{14}O_3$ is incorporated into the RNA and DNA of cancer cells in tissue culture (McCoy *et al.*, 1961; Chang *et al.*, 1961). From the fact that $C^{14}O_2$ also is incorporated into aspartate (McCoy *et al.*, 1961) and oxalacetate (Chang *et al.*, 1961), it is possible that $C^{14}O_2$ might first be incorporated into aspartic acid, as in liver, after which the carbamyl moiety from citrulline might be transferred to make carbamyl aspartic acid (Smith and Reichard, 1956; Reichard, 1957), which would then be incorporated into RNA *via* the orotic pathway. At the present time it is not possible to compare our results with those obtained under tissue culture conditions employing different tumor cell lines.

REFERENCES

Archibald, R. M. (1944), *J. Biol. Chem. 156*, 121.

Calva, E., Lowenstein, J. M., and Cohen, P. P. (1959), *Cancer Res. 19*, 101.

Chang, R. S., Liepins, H., and Margolish, M. (1961), *Proc. Soc. Exp. Biol. Med. 106*, 149.

Cohn, W. E., and Volkin, E. (1951), *Nature 167*, 483.

Fairley, J. L., Dans, L. L., and Krueckel, B. (1953), *J. Am. Chem. Soc. 75*, 3842.

Grisolia, S. (1955), *Methods in Enzymol. 2*, 350.

Hall, L. M., Johnson, R. C., and Cohen, P. P. (1960), *Biochim. Biophys. Acta 37*, 144.

Heinrich, M. R., and Wilson, D. W. (1950), *J. Biol. Chem. 186*, 447.

Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R. (1954), *J. Biol. Chem. 209*, 23.

Jones, M. E., Anderson, A. D., Anderson, C., and Hodes, S. (1961), *Arch. Biochem. Biophys. 95*, 499.

Koritz, S. B., and Cohen, P. P. (1954), *J. Biol. Chem. 209*, 145.

Lagerkvist, U. (1950), *Acta Chem. Scand. 4*, 1151.

Lagerkvist, U. (1953), *Acta Chem. Scand. 7*, 114.

McCoy, T. A., Maxwell, M. D., and Kruse, P. F., Jr. (1961), *Cancer Res. 21*, 997.

Marshak, A., and Vogel, H. J. (1951), *J. Biol. Chem. 189*, 597.

Reichard, P. (1957), *Acta Chem. Scand. 11*, 523.

Reichard, P. (1959), *in* Proc. IVth Internat. Congr. Biochem., Vienna, 1958, Vol. XIII, Hoffmann-Ostenhof, O., Editor, Pergamon Press, p. 119.

Reichard, P., and Lagerkvist, U. (1953), *Acta Chem. Scand. 7*, 1207.

Reichard, P., and Skold, O. (1957), *Acta Chem. Scand. 11*, 17.

Sisken, B., Sano, K., and Roberts, E. (1961), *J. Biol. Chem. 236*, 503.

Skold, O. (1960), *Biochem. Biophys. Acta 44*, 1.

Smith, L. H., Jr., and Reichard, P. (1956), *Acta Chem. Scand. 10*, 1024.

Tsuboi, K. K., and Price, T. D. (1959), *Arch. Biochem. Biophys. 81*, 223.

Tyner, E. P., Heidelberger, C., and LePage, G. A. (1953), *Cancer Res. 13*, 186.