Effect of 5-bis-(2 Chloroethyl)-Aminouracil on Metabolism of Amino Acids in Normal and Precancerous Rat Liver.* (27929)

WILLIAM T. BURKE (Introduced by L. L. Miller)
Department of Biochemistry, West Virginia University Medical Center, Morgantown, W. Va.

Using the technic of isolated rat liver perfusion, we have accumulated and published extensive data which indicate striking quantitative and qualitative differences in amino acid metabolism between normal livers and livers of rats which had been maintained on 3′ methyl-4-dimethylaminoazobenzene (3′Me DAB)(1). The present studies were designed to study the effects of a nitrogen mustard derivative of uracil on the metabolism of C^{14} labeled amino acids by the perfused liver.

The compound, 5-bis-(2 chloroethyl)-aminouracil, was first synthesized and described in this country by Lyttle and Petering(2,3). A very similar compound (differing only by the presence of a methyl group in the four position) was described as an anti-tumor agent in the Russian literature in 1955 and has been used clinically in the Soviet Union under the name of Dopan(4, 5).

These compounds are of interest because they produce fewer toxic side effects than many other oncotoxic agents and because they combine the possibility of interfering with tumor metabolism and growth by acting both as pyrimidine analogues and as nitrogen mustards. They have shown clinical effectiveness against Hodgkin's Disease, myelogenic leukemia, and reticulosarcoma.

We have investigated the effects of aminouracil mustard on incorporation of L-lysine-6-C^{14}, L-glutamic acid-U-C^{14}, and L-glutamine-U-C^{14} into plasma proteins and liver acid-soluble and acid-insoluble proteins, and the expiration of $C^{14}O_2$ during perfusion of normal livers and livers from 3′Me DAB fed rats.

Details of the technic for isolation and perfusion of the rat liver have been described (1,6,7).

In the present study the rats used were 200-300 g Sprague-Dawley males.† The animals were maintained for 8-10 weeks on a basal diet with or without added carcinogen (0.06% 3′Me DAB).

The 5-bis-(2 chloroethyl)-aminouracil‡ was dissolved in dimethylacetamide (50 mg/ml). 0.2 ml of this solution was added to 20 ml of Ringer's solution and this added to 100 ml of freshly drawn, heparinized (5000 units) rat blood. To this perfusate 5 μc of C^{14} labeled amino acid and 100 mg D-glucose were added.

Liver perfusions were maintained for 4 hours. Oxygen was provided as a mixture of 95% O_2/5%CO_2. The bile duct was cannulated and bile was collected throughout the perfusion.

Liver and plasma proteins were determined by the method of Lowry(8). Plasma proteins were prepared from hourly blood samples by precipitation with 6% trichloroacetic acid, repeated washings with TCA and 3:1 alcohol-ether. The washed, precipitated proteins were suspended in distilled water. Aliquots were plated onto filter paper discs which were placed in counting vials to which was added 12 ml of toluene-scintillator solution (100 mg of 1, 4-bis-2-(5-phenyloxazolyl)-benzene and 4 g of 2, 5-diphenyloxazole per liter of toluene). The samples were then counted in a Packard Tri-Carb liquid scintillation counter.

At the end of the perfusion the livers were homogenized in distilled water (20 ml/10 g liver) and the samples were dialyzed in the cold. To the liver homogenate an equal volume of 0.5N HCl was added. The acid-soluble liver proteins were extracted by gentle agitation in the cold for 30 minutes. The material was centrifuged for 30 minutes at 1500 rpm. The precipitate of HCl-insoluble liver protein was washed with 6% TCA then with 3:1 alcohol-ether, dried, suspended in dis-

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TABLE I. Effect of Uracil Mustard on Distribution of Carbon-14 from Lysine, Glutamic Acid, and Glutamine.

(Values in brackets indicate No. of experiments)
(Values in parentheses are ranges)*

	Normal liver		3'Me DAB liver	
	No addition	Uracil mustard	No addition	Uracil mustard
A. Lysine-6-C^{14}	[6]	[7]	[5]	[6]
Plasma proteins – 2 hr	.9 (.6- 1.0)	.8 (.6- .9)	1.9 (1.7- 2.2)	1.8 (1.6- 2.9)
" " – 4 "	3.0 (2.7- 3.2)	3.0 (2.5- 3.3)	9.0 (8.6- 9.2)	9.5 (9.1-10.2)
Total liver proteins	6.0 (5.7- 6.4)	5.1 (4.5- 5.3)	13.5 (12.4-14.2)	6.1 (5.9- 6.3)
Liver acid sol. proteins†	17 (15 -21)	8.9 (8.7- 9.1)	30 (29.8-33.1)	7.0 (6.8- 7.4)
$C^{14}O_2$	53.9 (47.1-56.2)	47.1 (42.0-53.1)	26.2 (26.0-27.3)	27.8 (26.8-28.3)
B. Glutamic acid-P-C^{14}	[5]	[8]	[7]	[5]
Plasma proteins – 2 hr	2.3 (2.1- 2.6)	1.0 (.8- 1.1)	4.0 (3.6- 4.3)	4.3 (4.0- 4.4)
" " – 4 "	5.7 (5.5- 6.0)	5.5 (5.3- 5.9)	8.9 (8.0- 9.2)	8.3 (8.0- 8.8)
Total liver proteins	6.0 (5.5- 6.3)	5.3 (5.0- 6.0)	12.0 (10.0-14.2)	11.6 (10.9-12.2)
Liver acid sol. proteins†	8.0 (7.5- 8.3)	3.1 (2.5- 6.0)	20.0 (18.0-24.0)	11.5 (10.0-14.2)
$C^{14}O_2$	31.3 (31.0-35.0)	29.2 (27.6-31.3)	21.2 (19.1-23.4)	20.6 (18.5-23.2)
C. Glutamine-U-C^{14}	[5]	[7]	[6]	[7]
Plasma proteins – 2 hr	3.5 (3.0- 4.5)	3.7 (3.5- 3.9)	5.2 (4.0- 5.6)	4.8 (3.8- 5.0)
" " – 4 "	7.0 (6.8- 7.4)	8.0 (7.9- 8.1)	12.0 (10.3-13.4)	13.1 (12.0-15.1)
Total Liver Proteins	6.0 (5.3- 7.0)	5.5 (5.1- 6.0)	13.9 (12.7-14.9)	12.5 (11.2-13.4)
Liver acid sol. proteins†	10.6 (9.5-11.2)	5.3 (5.0- 6.2)	25.6 (24.0-27.8)	15.2 (12.8-16.0)
$C^{14}O_2$	47.7 (46.1-48.9)	47.8 (45.7-49.4)	61.0 (60.0-65.1)	58.0 (54.0-60.0)

* Expressed as % of added amino acid carbon-14 radioactivity.
† % of total liver protein incorporation.

tilled water, plated, and counted as described above. The acid-soluble liver proteins were dialyzed against repeated changes of 0.25 N HCl and then against 0.5 N acetic acid overnight in the cold. The resulting solution (about 40 ml) was concentrated by reverse dialysis against carbowax as described by Kohn(9). Aliquots were again placed on filter paper and counted.

In Table I are presented data comparing perfused normal livers with livers undergoing carcinogenesis. The perfusates contained lysine, glutamic acid, or glutamine in the presence or absence of uracil mustard. As we have reported previously the feeding of azo-dye results in an increase in incorporation of C^{14}-labeled amino acid into both liver and plasma proteins(1). These data indicate that the difference between normal and precancerous livers is even more striking when the incorporation into acid-soluble liver proteins is compared.

In the normal livers, addition of the uracil mustard inhibits incorporation of the 3 labeled amino acids studied into the acid-soluble liver proteins but not into the other protein fractions examined. There is no significant effect on amino acid incorporation into plasma protein in either liver type during 4 hour liver perfusion. The livers from 3'Me DAB fed rats are affected similarly. However, the mustard also inhibits incorporation of lysine-C^{14} into total liver proteins in these livers.

Expiration of $C^{14}O_2$ is not affected by addition of uracil mustard. However, there are differences in the extent to which the perfused liver converts lysine, glutamic acid, and glutamine carbon-14 to $C^{14}O_2$. As we have noted previously, the perfused livers from rats fed 3'Me DAB show a decrease of approximately 50% in conversion of L-lysine-6-C^{14} to $C^{14}O_2$ as compared with the perfused normal liver(1). The data show the same trend for glutamic acid C^{14}, a reduction of about 30% in the precancerous livers. In neither case does the uracil mustard show any effect.

The data show also that the glutamine-C^{14} is converted to $C^{14}O_2$ by livers from azo-dye fed rats to an extent about 20% greater than by normal livers. These data are consistent with our previous observations that the production of urea-nitrogen from lysine or glutamic acid nitrogen was decreased greatly in precancerous livers, whereas gluta-

mine could serve equally well as a urea-nitrogen source in either normal livers or livers undergoing azo-dye induced carcinogenesis (10).

These data provide further support for the hypothesis that a mechanism exists in the precancerous liver which depresses the catabolism of amino acids thus providing a necessary, though possibly not a sufficient, cause for the increased protein synthesis found in these livers.

Our observations on the effect of 5-bis-(2 chloroethyl)-aminouracil may be compared with data reported by Busch and co-workers (11). These investigators found a 2- to 4-fold stimulation of L-lysine-U-C^{14} incorporation into acid-soluble nuclear proteins of rat liver 12 hours after administration of the drug. At 6 hours there was a slight increase in amino acid incorporation. On the other hand they found a striking decrease in lysine incorporation into the acid-soluble nuclear proteins of the Walker 256 tumor. There was no effect of uracil mustard on arginine-U-C^{14} incorporation into these proteins until 12 hours after administration. There was then a gradual decrease in incorporation until at 24 hours incorporation had been decreased to about 50% of the initial value (12). The aminouracil mustard had no effect on arginine-C^{14} incorporation into liver acid-soluble proteins. In our investigations we find a marked depression of incorporation of the 3 amino acids studied into the acid-soluble proteins of both normal liver and of livers undergoing azo-dye induced carcinogenesis.

The extensive data on amino acid mustards recently published by Nyhan(13) are compatible with Busch's observations in showing no effect of alanine mustard or phenylalanine mustard on incorporation of alanine-U-C^{14}, phenylalanine-U-C^{14} or methionine-S^{35} into the proteins of the cell fractions of liver although these mustards inhibit amino acid incorporation into cytoplasmic and nuclear proteins of the Walker tumor. The experiments are difficult to compare because of the difference in dosage and the relative time of injection of the drug and the C^{14} amino acid.

Our studies, utilizing the isolated perfused liver, provide an indication of a more direct influence of aminouracil mustard on liver protein metabolism. Such effects may be overshadowed in the intact animal by the interaction of the drug with the tumor or spleen.

Summary. These investigations reemphasize the utility of the isolated rat liver perfusion system for studying metabolic activity. The data obtained show that livers from azo-dye fed rats have an enhanced capacity for synthesis of acid-soluble liver proteins. These findings suggest further that 5-bis-(2 chloroethyl)-aminouracil is capable of inhibiting acid-soluble liver protein synthesis in liver of normal rats or in livers undergoing azo-dye carcinogenesis.

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