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PHARMACOLOGICAL DISPOSITION IN MICE OF O^2:2′-ANHYDRO-1-β-D-ARABINOSYLCYTOSINE 3′-PHOSPHATE, A POTENTIAL ANTILEUKEMIC AGENT*

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Abstract—The observation that the cytidine analog, O^2:2′-anhydro-1-β-D-arabinosylcytosine 3′-phosphate (anhydro-ara-CMP), yields 1-β-D-arabinofuranosylcytosine (ara-C) as a degradation product *in vitro*, prompted us to investigate the metabolism of [^{14}C]-anhydro-ara-CMP in mice. This study includes measurements of excretory rates and appearance of labeled material in the blood stream, distribution of activity in tissue at specified times, and metabolic assay of degradation products within certain mitotically active tissues. Analysis of both urine and faeces indicates that approximately 40 per cent of the labeled material remains within the animals after 24 hr. Measurement of [^{14}C] activity in the blood gave a peak absorption time at 1 hr after intraperitoneal injections. Reasonable levels of activity in the blood were present even after 72 hr. The metabolic assay of liver and spleen tissue taken at 1 hr showed anhydro-ara-C as the major metabolite, with a significant amount of ara-C and its di- and triphosphates also being formed. Cytidine phosphates were also detected. Single doses of 2000 mg/kg and daily doses of 1000 mg/kg for 5 days caused no fatalities among the test animals.

THE ANTILEUKEMIC agent, 1-β-D-arabinofuranosylcytosine (ara-C),§ has shown a relatively high toxicity in animals[1] and man.[2–5] This occurs in spite of its rapid deamination to the biologically inactive 1-β-D-arabinofuranosyluracil (ara-U).[6,7] For this reason, the maintenance of a prolonged action throughout the cell cycle requires frequent administration.

In the preceding paper, we have concluded that derivatives of O^2:2′-anhydro-1-β-D-arabinosylcytosine (anhydro-ara-C) would be ideal nontoxic precursors which would hydrolyze into ara-C *in vivo* at a suitable rate. We have carried out most of our studies on anhydro-ara-CMP because we assumed that the phosphate ester would offer some advantages over the nucleoside. At the time when our program was initiated,[8] the nucleotide was also more readily accessible. Since then, anhydro-ara-C has been tested by the National Cancer Institute staff (ref. No. NSC 129220)

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§ The following abbreviations were used: ara-C, 1-β-D-arabinosylcytosine; ara-U, 1-β-D-arabinosyluracil; ribo-C, 1-β-D-ribosylcytosine; ribo-U, 1-β-D-ribosyluracil; anhydro-ara-C, O^2:2′-anhydro-1-β-D-arabinosylcytosine; anhydro-ara-CMP, the corresponding 3′-phosphate.

and by Hoshi *et al.*,[9] and was found active in several tumor systems. However, there was no substantial difference in the therapeutic usefulness of the nucleoside and nucleotide on a molar basis in the L1210 system.

Our studies *in vitro*[10] have indicated that the conversion of anhydro-ara-CMP involves a rather rapid enzymatic process and a slower chemical hydrolysis (Fig. 1). Using [^{14}C]-anhydro-ara-CMP, we followed the rate of absorption and excretion of the total radioactivity in various tissues over a period of 1 hr to 3 days. We have also analyzed the metabolic products at 1 and 3 hr. The present study suggests that the biological activity of anhydro-ara-CMP is due to its transformation to ara-C and related products.

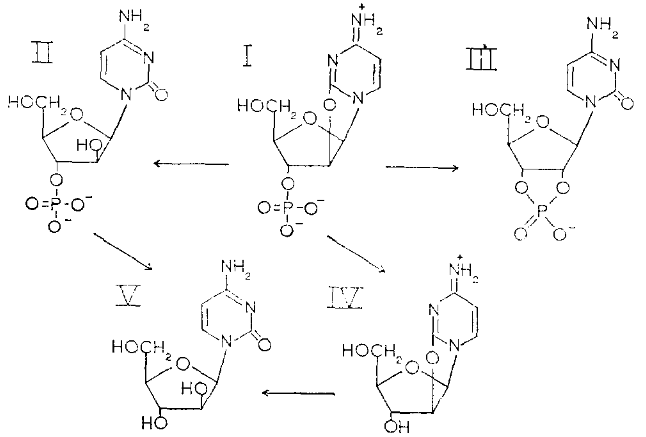

Fig. 1. Presently known metabolism of anhydro-ara-CMP. Abbreviations used include: I, anhydro-ara-CMP; II, 3′-ara-CMP; III, 2′,3′-cyclic CMP; IV, anhydro-ara-C; V, ara-C.

MATERIALS AND METHODS

Animals. Female mice, BDF$_1$ hybrid, weighing 15–20 g were used throughout the experiment. Animals were purchased from Texas Inbred Mice Co. and given food and water *ad lib.*

Radioactive materials, combustion and counting techniques. The starting material, [G-^{14}C]-cytidine-2′(3′) phosphate (sp. act. 170–200 mCi/m-mole) was purchased from International Chemical and Nuclear Corp. Preparation of the [^{14}C]-anhydro-ara-CMP was carried out according to the procedures of Nagyvary.[11] Final conversion to [^{14}C]-anhydro-ara-CMP gave a 75 per cent yield. For sample counting, quenching curves were prepared using [^{14}C]-toluene ($4{\cdot}45 \times 10^5$ dis/min/ml) obtained from New England Nuclear Corp. Radioactive assay of faecal material, blood and tissue was completed according to the oxygen combustion and liquid scintillation procedures of Abou-Donia and Lyman.[12] All samples were counted using the Beckman LS-250 liquid scintillation system. The homogeneous solutions obtained after the combustion of blood, fecal and tissue samples were combined with the mixture consisting of toluene and ethyleneglycol monomethyl ether (2:1, v/v) containing 5 g 2,5-diphenyloxazole (PPO) per liter. The paper sections were directly counted using a mixture consisting of 0·4% PPO–0·05% 1,4-*bis*-2-(4-methyl 5 phenyloxozolyl) benzene in toluene.

Animal dosage of radioactive compound. All animals were given single intraperitoneal injections. The dosage varied from 0·5 to 1·0 μCi (75–80 mg/kg of body wt) for the excretion, blood absorption, and tissue distribution analysis. In order to ensure sufficient activity, 1·75–2·25 μCi (80–100 mg/kg of body wt) was used for the metabolism studies.

Chromatograppy and electrophoresis. The methods of chromatographic and electrophoretic separations had been previously worked out using inactive standards. Ion-exchange chromatography on Dowex 1-X8 and 50 W resins was performed at 4° and monitored with an Isco UV analyzer.

Paper chromatography was carried out on Whatman No. 3 MM paper in the solvent system of *n*-butanol–H_2O (86:14, v/v) for 48–72 hr. The purity of nucleotide fractions was analyzed by high voltage electrophoresis, using a Savant flat-plate apparatus with 0·1 M phosphate buffer, pH 7·0. The nucleoside products obtained after enzymatic hydrolysis were separated using 0·1 M sodium-borate buffer, pH 8·5.[13]

Excretory analysis. The urinary excretion studies were carried out using a metabolism chamber from Curtin Scientific Co. The urine was absorbed on Whatman No. 3 MM paper, 10 cm dia., which fit inside the chamber. The paper was replaced and the faeces were collected every hour for 8 hr following the intraperitoneal injection. Additional measurements were taken after 24 and 48 hr. To prevent the animals from shredding the paper, a circular band of sheet metal, 1·5 cm wide fixed into a 10 cm dia., was placed directly above the paper, followed by a precut piece of 1·3 cm wire mesh. The circular paper used to collect the urine and faeces during each time interval was cut into 1 × 2 cm strips after drying. The faeces were collected, dried, ground and prepared for oxygen combustion.

Analysis of [^{14}C] *activity in the blood.* In this experiment, two blood samples were taken from the tail of test animals every hour for the first 4 hr, then every other hour for up to 8 hr. Additional samples were taken at 24, 48 and 72 hr. Blood (20–25 mg/sample) was placed in two preweighed combustion envelopes and weighed immediately to avoid any evaporation. From this point, the procedure of Abou-Donia and Lyman[12] was followed.

Tissue distribution of label. Mice were sacrificed using surgical ether after time intervals of 1, 3, 24 and 48 hr. The tissues analyzed in the experiment were: skin, adipose, liver, spleen, stomach, pancreas, kidney, sternum, bladder, small intestine, large intestine, heart, lungs, muscle, thyroid and spinal cord (2 cm). Each tissue, after careful washing with 0·85 M NaCl to remove residual contamination, was weighed and, where possible, two portions were removed and placed in combustion envelopes.

Tissue preparation and separation of metabolic products. The degradation products of [^{14}C]-anhydro-ara-CMP were identified through a series of isolation and separatory steps. First, the spleen and liver were dissected 1 hr after injection. Each tissue was prepared for metabolite analysis according to the procedure of Saukkonen.[14] To the final tissue extract were added 4 mg ribo-U, ara-U, 2′(3′)-CMP, anhydro-ara-CMP, anhydro-ara-C and 3′-ara-CMP. The tissue extract with carrier metabolites was passed through a 1 × 19·5 cm Dowex 50 (H^+) column. The ultraviolet monitor indicated two characteristic peaks after washing the column with 225 ml distilled H_2O. The first fraction contained ara-U, ribo-U and the di- and triphosphates of ara-C and ara-U, and was eluted by the first 75–100 ml. This was concentrated *in vacuo* and chromatographed on paper using *n*-butanol–H_2O. This fraction was analyzed for ara-U and ribo-U and the total nucleotide di- and triphosphates. The second peak, containing the anhydro-ara-CMP, 2'(3')-CMP and 3'-ara-CMP was eluted with the remaining 125 ml.

A third fraction, containing ara-C and ribo-C nucleosides, was eluted from the column with 225 ml of 2% pyridine. After concentration *in vacuo*, the compounds were also separated by descending paper chromatography with *n*-butanol-H_2O.

The last fraction from the Dowex 50 column contained only anhydro-ara-C and was eluted with 225 ml of 2% pyridium formate, concentrated *in vacuo*, and spotted for counting.

The second fraction from the Dowex 50 column was concentrated and placed on a 5-ml Dowex 1-X8 anion-exchange column (formate form), and eluted with a 0·01–0·05 M formic acid gradient. Ultraviolet monitoring showed several distinct peaks. Anhydro-ara-CMP appeared as the first peak, followed by 3'-ara-CMP and the poorly resolved 2'(3')-CMP. The 2'(3')-CMP and 3'-ara-CMP were combined as one fraction and treated with alkaline phosphatase in 0·1 M Tris–HCl buffer, pH 8·5, for 48 hr at 37°. The resulting nucleosides were separated by borate electrophoresis. The ultraviolet-absorbant spots were cut into uniform sections (1 × 2 cm) and counted by liquid scintillation techniques.

Measurement of di- and triphosphate metabolites. In this experiment, liver and spleen were removed 3 hr after injection and prepared for metabolic analysis as mentioned previously. In addition to the carriers mentioned, 4 mg each of the following markers —5'-ara-CMP, UDP, UTP, CDP and CTP—were added to the tissue extract and passed through a 1·0 × 19·5 cm Dowex 50 (H^+) column. The eluate containing the first H_2O peak was concentrated to a small volumne and placed on a 2 × 5 cm Dowex 1-X8 (formate form) column. The 2'(3')-UMP and CDP markers were eluted with 400 ml of 0·1 M ammonium formate, pH 3·5. The UDP, UTP and CTP were eluted with 400 ml of 0·7 M ammonium formate, pH 3·5. After concentrating, the two peaks were passed through a 2 × 66 cm Dowex 50 (H^+) column and lyophilized. Each peak was then dissolved in Tris–HCl buffer, pH 8·5, and treated with alkaline phosphatase for 120 hr at 37°. The resulting nucleosides were chromatographed in water-saturated *n*-butanol and counted on paper strips. All other metabolites were separated and counted as previously mentioned. The 5'-ara-CMP appeared as a separate peak preceding the 2'(3')-CMP when the same gradient was used as for the separation of the 1-hr sample. The precipitate from the extraction procedure was dried *in vacuo* over P_2O_5, weighed and combusted for counting.

RESULTS

Toxic effect of various dosage schedules. An LD_{100} of ara-C[1] was observed when the drug was administered at a rate of 15 mg/kg every 3 hr until the total amount of 360 mg/kg was reached.

In search of a lethal dose of anhydro-ara-CMP, single injections of 100, 1000 and 2000 mg/kg were administered to each of four test animals. An intermittent dose schedule of 1000 mg/kg, given at 24-hr intervals for 5 days, was also completed. Surprisingly, no fatalities resulted from either of the dose schedules. Morphological and histological studies are currently being pursued.

Excretion of total radioactivity. Excretion of [^{14}C]-label, administered as [^{14}C]-

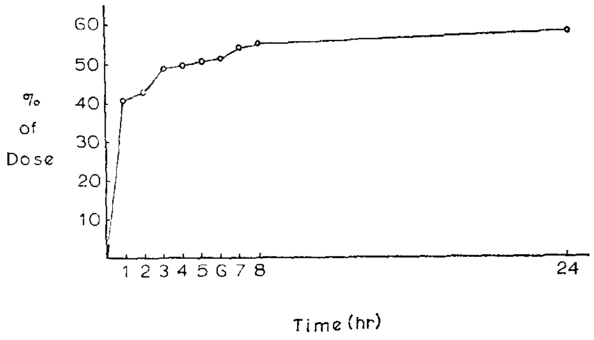

FIG. 2. Cumulative urine excretion as percentage of original dose (0·5–1·0 μCi, 75–80 mg/kg) over a 24-hr period.

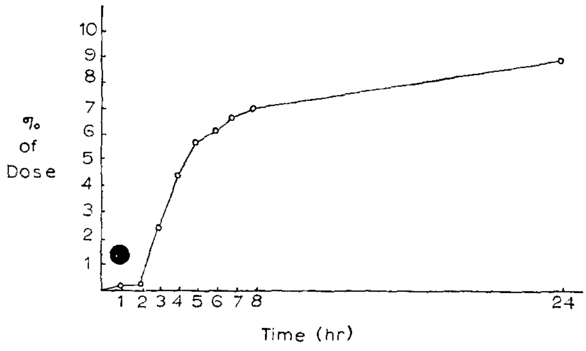

FIG. 3. Cumulative faecal activity of [^{14}C]-anhydro-ara-CMP as percentage of initial dose (0·5–1·0 μCi, 75–80 mg/kg) and function of time for a 24-hr period.

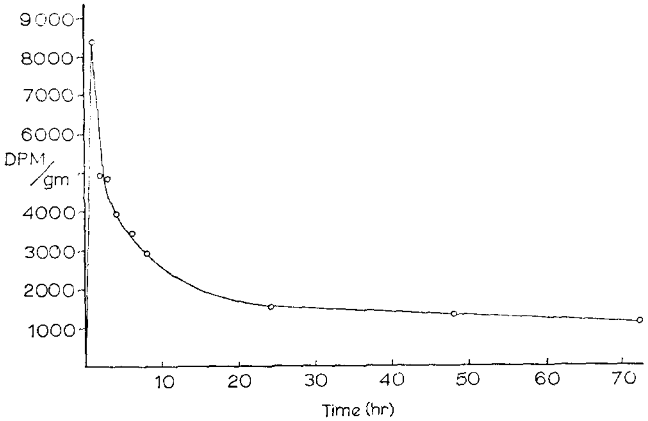

FIG. 4. Activity appearing in the bloodstream after initial dosage of [^{14}C]-anhydro-ara-CMP (0·5–1·0 μCi, 75–80 mg/kg) as a function of time.

anhydro-ara-CMP, was measured for comparison with corresponding excretion rates of ara-C.[1,15] In Fig. 2, it is shown that over a 24-hr period following injection about 58 per cent of the labeled material had appeared in the urine. The activity in the faeces was much lower, only about 9 per cent (Fig. 3).

By both routes of excretion, 40 per cent of the original dosage was expelled in the first hour compared to 50 per cent shown by ara-C.[15] More importantly, the data revealed that only 58 per cent of the administered radioactivity had been excreted in 24 hr compared to more than 85 per cent urinary elimination of ara-C. The faeces contained 9 per cent of the total radioactivity administered as anhydro-ara-CMP, compared to less than 1 per cent for ara-C after 24 hr.

Blood and tissue content of radioactivity. The appearance of labeled material in the bloodstream provides suggestive evidence for the presence of anhydronucleoside in the tissues. The maximum activity was observed after 1 hr, with the level gradually decreasing over the next 72 hr (Fig. 4). The distribution of radioactivity was studied in 19 selected tissues at 1, 3, 24 and 48 hr after injection (Table 1). The sharp decline of radioactivity in most tissues over an extended time period is similar to that observed in blood. As seen in Table 1, there was high ^{14}C activity in the liver, spleen, reproductive organs and small intestine. The sternum showed considerable activity, but cannot be considered a true representation of total bone marrow. The heart and lungs showed a high level of activity, yet those values must be attributed not only to the

TABLE 1. TISSUE DISTRIBUTION OF [^{14}C]-ANHYDRO-ARA-CMP METABOLITES AS A FUNCTION OF TIME

Organs	Radioactivity (dis/min/g × 10^{-3})			
	1 hr	3 hr	24 hr	48 hr
Skin	21·5	25·8	5·2	2·7
Adipose*	14·8	8·7	7·0	1·9
Pancreas*	94·7	73·3	17·5	14·0
Blood	8·9	4·8	1·6	1·4
Liver	57·5	47·1	18·1	15·5
Bladder*	2243·7	1529·8	106·4	11·0
Kidney*	60·7	67·6	16·6	10·0
Spleen*	48·6	53·7	19·5	10·4
Small intestine	88·1	67·4	13·3	6·4
Large intestine	75·1	95·7	14·6	10·0
Muscle*	16·9	26·8	4·6	3·5
Sternum*	30·7	25·2	8·4	3·3
Heart*	68·9	62·0	9·5	4·6
Lungs	129·0	119·2	11·3	4·4
Brain	1·9	2·8	2·2	0·6
Spinal cord*	5·0	5·1	1·9	0·6
Stomach	48·6	21·1	8·2	5·2
Thyroid*	57·9	30·6	12·5	6·8
Reproductive organs	94·7	79·0	18·5	9·6

* Entire organ was combusted; other organs analyzed by taking two aliquots/organ and adjusting to predetermined total weight. Representative times were chosen at 1, 3, 24 and 48 hr. Single i.p. injections of 0·5–1·0 μCi (75–80 mg/kg body wt) were given to each of four mice.

organ, but to a high residual blood level as well. The bladder showed a similarly high radioactivity, but there was some amount of urine within this organ at the time of excision. Values for kidneys must also be carefully interpreted.

In an attempt to account for the total administered radioactivity, the values of all dissected organs (dis/min), as well as the urinary and fecal activity were tabulated and summed. The fractional recovery of label is shown in Table 2. These values do not

Table 2. PERCENTAGE RECOVERY OF INITIAL DOSAGE OF $[^{14}C]$-ANHYDRO-ARA-CMP AFTER 24 hr*

	Radioactivity (dis/min × 10^{-3})			
	Animal 1	Animal 2	Animal 3	Animal 4
Dissected organs	119·4	114·5	128·4	102·8
Urine	717·7	885·1	917·0	698·8
Faeces	127·7	89·7	58·7	277·0
Total radioactivity	964·8	1089·3	1104·0	1078·6
Dosages	1363·7	1324·9	1417·2	1373·7
% Recovery	71	80	78	79

* Recovery based on total activity found in dissected organs, urine and faeces. This percentage is exclusive of excess bone, muscle and adipose tissue remaining in the carcasses. Single i.p. injections of 0·5–1·0 μCi (75–80 mg kg/body wt) were given to each of four host animals.

TABLE 3. RELATIVE AMOUNTS OF METABOLITES OF $[^{14}C]$-ANHYDRO-ARA-CMP ISOLATED FROM LIVER AND SPLEEN 1 hr AFTER SINGLE i.p. DOSE (1·75–2·25 μCi, 80 mg/kg BODY WT)*

	Recovered products in	
Metabolic products	Liver	Spleen
Ribo-U	2·3	3·8
Ara-U	2·6	3·4
Ribo-C	1·3	3·1
Ara-C	2·3	6·4
Anhydro-ara-C	71·6	64·2
Anhydro-ara-CMP	3·5	3·0
3′-Ara-CMP	1·4	2·2
2′(3′)-CMP	2·1	5·0
Nucleotide di- and triphosphates	10·1	3·4

* Soluble metabolites of $[^{14}C]$-anhydro-ara-CMP were extracted at given times by the procedure of Saukkonen[14] and were isolated using a combination of ion-exchange chromatography, descending paper chromatography and high voltage electrophoresis. Samples were counted in a liquid scintillation counter (see Materials and Methods). It was estimated that 60–65 per cent of $[^{14}C]$activity was present in the soluble fraction and 35–49 per cent remained in the residue even after repeated extraction. The metabolites listed in this table represent approximately 90 per cent of the soluble fraction. The numbers in the table indicate relative amounts of these metabolites. The unrecovered amount included 5′-ara-CMP.

account for the carcass consisting of bone, muscle and fatty tissue, nor does it include expired CO_2. A recovery of about 77 per cent was obtained by averaging the four listed values. Allowing 5 per cent error due to injection techniques and 10 per cent error in urine collection, the four values were considered satisfactory.

Metabolic analysis and assay. After careful evaluation of the distribution of radioactivity in tissues as well as the activity appearing in the bloodstream, it was decided to use liver and spleen 1 and 3 hr after injection for analysis of metabolic products. The results of these studies are shown in Tables 3 and 4.

TABLE 4. RELATIVE AMOUNTS OF METABOLITES OF [^{14}C]-ANHYDRO-ARA-CMP ISOLATED FROM LIVER AND SPLEEN 3 hr AFTER SINGLE i.p. DOSE (1·75–2·25 μCi), 80 mg/kg BODY WT)*

Metabolic products	% Recovered products in	
	Liver	Spleen
Ribo-U	<1	<1
Ara-U	<1	<1
Ribo-C	<1	1·2
Ara-C	2·0	5·2
Anhydro-ara-C	18·5	46·3
Anhydro-ara-CMP	1·7	1·2
3′-Ara-CMP	<1	<1
2′(3′)-CMP	<1	<1
5′-Ara-CMP	<1	1·1
Ribo-UMP	<1	<1
Ara-UMP	<1	<1
Ara-CDP	2·2	5·1
Ribo-CDP	1·0	2·7
Ara-CTP	1·0	1·3
Ribo-CTP	<1	<1
Ara-UDP and UTP	1·0	1·5
Ribo-UDP + UTP	<1	1·3
Precipitate	72·5	29·0

* Soluble metabolites of [^{14}C]-anhydro-ara-CMP were extracted at 3 hr by the procedure of Saukkonen[14] and were isolated using a combination of ion-exchange chromatography and high voltage electrophoresis. Samples were counted in a liquid scintillation counter (see Materials and Methods). It was estimated that an average of 50 per cent of [^{14}C]activity was present in the soluble fraction and 50 per cent in the combusted residue even after repeated extraction.

The assay for ara-C and its di- and triphosphate was considered crucial. The liver and spleen, both metabolically active tissues, showed rapid dephosphorylation of the anhydro-ara-CMP to anhydro-ara-C, the positively charged nucleoside, which was present in greatest abundance after 1 and 3 hr in both tissues. The appearance of 3′-ara-CMP is of special interest, since this compound has been shown to be a strong inhibitor of ribonuclease.*

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* D. R. Pollard and J. Nagyvary, presented at the 27th Southwest Regional Meeting of *Am. Chem. Soc.*, Dec. 1, 1971, Abstr. 44.

The data of Table 3 indicate that a rearrangement of anhydro-ara-CMP with the eventual production of 2′(3′)-CMP does take place *in vivo*. This mechanism, first described by Tapiero and Nagyvary,[16] represents a unique property of anhydro-ara-CMP. The 2′- and 3′-CMP isomers were not isolated; only their sum was determined. Due to the rapid hydrolysis of phosphate groups, it is mainly the nucleoside which is incorporated in the tissues from the bloodstream. Additional ribo-C and ribo-U might be present in RNA.

DISCUSSION

Studies *in vitro* on the chemistry of anhydro-ara-CMP, described in the preceding paper,[10] emphasized a potential role in chemotherapy for this compound, since it could possibly serve as a source of ara-C in an organism. The studies *in vivo* reported here show that anhydro-ara-CMP is indeed converted, in part, to ara-C in mouse spleen and liver. Further, analysis of excretion rates and products reveals that anhydro-ara-CMP and anhydro-ara-C have a reasonable metabolic lifetime and are therefore able to act as reservoir for the production of ara-C.

The excretion of total radioactivity after the intraperitoneal administration of [^{14}C]-anhydro-ara-CMP compares favorably with that of ara-C. The retention of ara-C in the body depends probably on its phosphorylation in the cells,[17] but the excretion is complete within 2 days.[15] Although the excretion of anhydro-ara-CMP metabolites is also very rapid during the first 5 hr after injection (Fig. 2), a substantial amount remains for several days. This persistence of radioactivity, especially in the liver, constitutes a puzzle which has not yet been clarified.

The distribution of administered ^{14}C-label in the body would be a useful parameter to compare the precursor with the parent drug. Unfortunately, we do not have for ara-C a broad study on the kinetics of tissue distribution which would be similar to the one presented here. Table 1 summarizes the distribution of radioactivity in 19 selected tissues as a function of time. It presents a picture of considerable accuracy, since all the data were obtained by a reliable radioactive assay, i.e. by measuring $^{14}CO_2$ after combustion. This assumption is also supported by a uniformly high recovery close to 80 per cent, in four mice. Although the values (dis/min) of the mitotically active tissues are quite high, the distribution on the whole is well balanced.

The biological activity of anhydro-ara-C derivatives is obviously due to their ultimate transformation into ara-CDP and ara-CTP, which are considered the crucial intermediates in the ara-C action.[17,18] We have measured all the plausible metabolites of anhydro-ara-CMP in liver and spleen 1 and 3 hr after injection (Tables 3 and 4). Since we did not have the carriers for the arbino di- and triphosphates, their assay is less reliable. The major metabolite is anhydro-ara-C, and thus our results provide some information on what can be expected when this nucleoside is administered directly. The novel aspect of anhydro-ara-CMP metabolism, as studied previously *in vitro*,[16] is the formation of ribo-C phosphates as a function of pH and ionic strength. We have now found that the same process operates also *in vivo* to a small extent. A comparison of data obtained for liver and spleen (Table 3) shows that both the absolute amounts of ribo derivatives and the ribo/arabino ratio are different. It is difficult to assess the extent of the effect small quantities of ribo products might have in

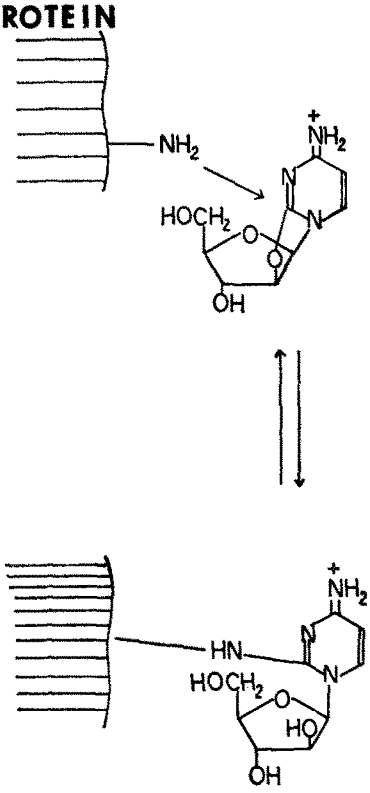

FIG. 5. Model for the possible binding of anhydro-ara-C to proteins.

decreasing the toxicity of ara-C through their reduction to deoxyribonucleotides. It is known that deoxycytidine is able to reverse the toxic effect of ara-C.[19] To some extent, anhydro-ara-CMP seems to possess a built-in mechanism for its own partial detoxification.

We would like to emphasize that the soluble metabolites comprise on an average only 50 per cent of the total radioactivity found in liver and spleen. A substantial amount of label remained in the precipitate even after repeated extraction. The small amount of ribonucleotide which might have been incorporated into RNA cannot account for this finding. We rather assume a direct binding of anhydro-ara-CMP or anhydro-ara-C or of both to proteins through a covalent linkage as depicted in Fig. 5. Since the reaction of ammonia with anhydro-ara-C produces the diaminopyrimidine arabinoside,[20] the free amino groups of proteins might be capable of reacting in a similar fashion. This type of linkage is not stable and the anhydro compound would eventually be liberated. The temporary binding to proteins could, therefore, explain the slow excretion of radioactive label. Work on this novel aspect of anhydro-ara-CMP metabolism will be continued.

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