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STUDIES ON DRUG RESISTANCE—I

DISTRIBUTION OF 1-β-D-ARABINOFURANOSYLCYTOSINE, CYTIDINE AND DEOXYCYTIDINE IN MICE BEARING ARA-C-SENSITIVE AND -RESISTANT P815 NEOPLASMS

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Abstract—A study of the physiological distribution of tritiated-1-β-D-arabinofuranosylcytosine (^{3}H-ara-C) in mice, carrying bilateral implants of ara-C-sensitive and -resistant tumors, indicated a selective, specific and prolonged accumulation of radioactivity in the sensitive tumor compared with the resistant tumor, liver, kidneys, spleen, lungs, blood, small intestines and brain of the host. Although the patterns of the brain and sensitive tumor tissues were similar, the brain accumulated much less radioactivity. The main difference of uptake of ara-C in the sensitive tumor compared with the resistant tumor was the presence of a large amount of ara-C nucleotide in the sensitive tumor and its negligible content in the resistant tumor. Deoxycytidine (CdR)-derived radioactivity was present, but not protracted, in all the above mentioned tissues. ^{3}H-cytidine (^{3}H-CR) was taken up equally in the sensitive and resistant tumors. In the liver its level was high and long-lasting. The relative values of uptake in sensitive and resistant tumors were 6–8:1 for ara-C; 2–4:1 for CdR; and 1:1 for CR 48 hr after injection. Determination *in vitro* of ara-C, CR and CdR deamination by tissue homogenates indicated no sizeable amounts of such nucleoside deaminases in the liver and two tumors. On the other hand, high levels of these substrates were deaminated by the kidney tissue. Of several naturally occurring CR- and CdR-nucleotides and ara-CMP, only deoxycytidine-5′-diphosphate (dCDP) and deoxycytidine-5′-triphosphate (dCTP) were comparably deaminated by both tumors.

Liver, kidney and both tumor tissues *in vitro* phosphorylated CR to the same degree. Ara-C and CdR were phosphorylated readily in the sensitive tumor and to a lesser extent in the liver; the kidney and resistant tumor showed only minor kinase activity.

Cytidine-5′-diphosphate-reductase activity was about equal in both tumors.

SINCE the first report on the synthesis of 1-β-D-arabinofuranosylcytosine, hydrochloride (ara-C) (NSC-63878)‡ by Walwick *et al.*,[2] numerous reports on its experimental[2–4] and clinical effects[5–7] and on its mechanism of action[8–10] have appeared. In 1966 Smith[11] reviewed in detail the aspects of the metabolism of this compound. Distribution, metabolic and excretion studies have been done in normal

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‡ The following abbreviations are used: ara-C, 1-β-D-arabinofuranosylcytosine, hydrochloride; ara-CMP, 1-β-D-arabinofuranosylcytidine-5′-monophosphate; ara-CDP, 1-β-D-arabinofuranosylcytidine-5′-diphosphate; ara-CTP, 1-β-D-arabinofuranosylcytidine-5′-triphosphate; CR, cytidine; CMP, cytidine-5′-monophosphate; CDP, cytidine-5′-diphosphate; CTP, cytidine-5′-triphosphate; dCMP, deoxycytidine-5′-monophosphate; dCDP, deoxycytidine-5′-diphosphate; dCTP, deoxycytidine-5′-triphosphate; POP, 2,5-diphenyl-oxazole; POPOP, 1,4-*bis*-(5-phenyloxazolyl-2)-benzene.

animals[12, 13] and some cancer patients.[14, 15] The present report compares the distribution of ara-C, cytidine (CR), deoxycytidine (CdR) and deaminase(s)-, kinase(s)- and CDP-reductase levels, in mice bearing bilateral transplants of P815 neoplasms sensitive and resistant to ara-C. Futher insight into the mechanism of action of ara-C, and into the development of resistance to ara-C, has been obtained. A preliminary communication appeared elsewhere.[16]

MATERIALS AND METHODS

Tritiated-1-β-D-arabinofuranosylcytosine (labeled in the 5 and 6 positions with most of the activity in the 5 position) (^{3}H-ara-C), purchased from the Schwarz Bioresearch Inc. and supplied by the N.I.H.*, was about 97 per cent pure when evaluated by paper chromatography. Ara-C-3′-phosphate and ara-C-5′-phosphate were gifts of the Upjohn Co. Uniformly tritiated-deoxycytidine (^{3}H-CdR) and cytidine (^{3}H-CR) were purchased from New England Nuclear Corp. Their radiochemical purity evaluated by paper chromatography was above 98·5 and 98 per cent respectively. The nucleosides were diluted with the corresponding unlabeled materials and dissolved for injection in physiological saline to a final concentration of 0·8 mg/ml (6·25 μc/mg). Male and female BDF_1 mice, weighing 18–23 g, purchased from Millerton Research Farm, Millerton, N.Y., were inoculated s.c. in the right abdominal region with 10^6 P815 cells and in the left with 10^6 ara-C-resistant P815 cells (P815/ara-C).† The mice, which were maintained on Purina laboratory chow and water *ad libitum*, were used for the experiments 8–10 days later. Groups of three to five mice were injected i.p. with the labeled nucleosides at a dose of 20 mg/kg.

At indicated time intervals, the animals were sacrificed by cervical dislocation. Blood samples were collected by heart puncture and the tissues were removed and prepared as previously described.[17] Radioactivity of the tissues and blood was evaluated according to the dry combustion technique of Kalberer and Rutschmann.[18] The concentration of the radioactivity was expressed by *F*-values:

$$F = \frac{\text{Specific activity (cpm/g wet wt.)} \times \text{body weight (g)}}{\text{Total cpm administered}}$$

The intracellular distribution of radioactivity after injection of labeled ara-C, CdR and CR was evaluated by homogenizing tumors and tissues of each eight to ten animals and extracting the homogenate according to the Schneider method[19] with either 5% trichloroacetic acid (TCA) or 2 N perchloric acid (PCA). Aliquots of the extracts were either combusted[18] or directly added to Diatol for counting in a Packard Tri-Carb scintillation spectrophotometer.

Paper chromatography was performed by system A: *n*-propanol, tetrahydrofurfuryl-alcohol, citrate buffer at pH 5·66 (20:10:10);[20] or system B: isopropanol, concentrated

* The authors are indebted to Dr. R. R. Engel of the National Institutes of Health, Bethesda, Md., for this material. The radioactive compound had been synthesized for the N.I.H. by the Monsanto Research Corp., Dayton, Ohio. We are grateful to Dr. W. J. Wechter of the Upjohn Co., Kalamazoo, Mich. for reference samples of ara-C-3′-phosphate and ara-C-5′-phosphate.

† J. H. Burchenal and W. Kreis, unpublished results. Ara-C at a dose of 25 mg/kg i.p. showed an increase of life span of more than 200 per cent in mice bearing the P815 tumor. This is comparable to the efficiency of the compound against the ascites form of the P815 neoplasm.[3, 4] When administered to mice implanted with the sensitive and resistant tumors simultaneously, or the resistant tumor alone, the drug does not increase the survival time. The mean survival time of mice carrying either the sensitive or resistant tumor is comparable. Ara-C-sensitive and -resistant cell lines were supplied by Drs. Dorris J. Hutchison and J. H. Burchenal.

HCl, water (340:85:72).[21] The location and quantitation of radioactive spots were effected as described earlier.[22]

Enzyme assays. Eight to 10 days after tumor transplantations the selected tissues were removed, homogenized immediately in a Potter homogenizer and processed according to the conditions specified below. Protein concentrations were determined on the supernatants by the method of Lowry *et al.*[23]

Deaminase. Tissues were homogenized with a 9-fold (w/v) modified Krebs–Ringer buffer at pH 7·4 supplemented with 100 μg each of penicillin and streptomycin per ml.[24, 25] The homogenate was then centrifuged at 2000 rpm for 10 min. The supernatant was used as a crude enzyme source. The reaction mixture for the nucleoside deaminase evaluation contained 4 μmoles substrate (ara-C, CdR or CR), 0·2 ml tissue homogenate and 0·3 ml Krebs–Ringer buffer at pH 7·4. The final volume was 0·6 ml. After incubation for 1 hr at 37°, the reaction was stopped by adding 1·0 ml cold 5% TCA solution. After chilling in ice the protein was removed by centrifugation at 2000 rpm for 10 min. Aliquots of 0·5 ml of the supernatants were applied to standard Conway diffusion dishes (Brunswick Laboratories, St. Louis, Mo.). The amount of ammonia released was determined by following the method of Roth *et al.*[26] For the nucleotide deaminase, the substrate concentration of 0·5 μmole per tube was used; the degree of deamination was evaluated spectrophotometrically, as reported earlier.[22]

Kinase. The tissues were homogenized with a 9-fold (w/v) 0·25 M sucrose solution and centrifuged at 100,000 *g* for 1 hr in a Spinco model L centrifuge. The supernatant was removed carefully avoiding the fat layer. The procedures of Bollum and Potter[27] and Bresnick and Kark; ala[28] were adapted for the studies. The reaction mixture contained 3 μmoles of adenosine triphosphate, 2·5 μmoles of 3-phosphoglyceric acid, 3 μmoles of magnesium chloride, 20 μmoles of Tris buffer, pH 8·0, 3·3 mμmoles of tritium-labeled nucleoside (ara-C, CdR, CR) and 0·02 ml of the supernatant of the tissue homogenate, all in a final volume of 0·18 ml. After incubation for 30 min, 1 hr and 2 hr at 37°, the reaction was stopped by immersing the test tubes in an ice bath. Samples of 50 μl were removed and applied to DEAE-cellulose paper (2 × 2 cm). The papers were immersed consecutively into 0·001 M ammonium formate, water, 0·001 M ammonium formate and water to wash the unreacted nucleoside from the DEAE-cellulose paper, and were finally immersed in 95% ethanol. After drying at room temperature, the papers were counted in 10 ml of a toluene-scintillation mixture (4% PPO and 0·1% POPOP in toluene) in the scintillation spectrophotometer.

Reductase. Tissues were homogenized with twice the volume (w/v) of Tris buffer at pH 8·0. The homogenate was centrifuged at 30,000 *g* for 1 hr in the Spinco model L centrifuge. The supernatant (excluding the fat layer) was used as an enzyme source. The reductase activity was evaluated according to the technique of Moore and Hurlbert.[29] However, no ferric chloride was used in the reaction mixture. In a typical determination, 5·0 μmoles of ATP, 10·0 μmoles of $MgCl_2$, 15·0 μmoles of dithiothreitol, 30·0 μmoles of Tris buffer, pH 8·0, 1·0 μmole CDP-^{14}C and 1·0 ml of the above supernatant were combined. The final volume was 1·8 ml. The reaction was performed at room temperature with continual agitation. At intervals of 0, 15 and 30 min, 0·5-ml samples were removed and added to 0·4 μmole of dCMP. The reaction was stopped by adding cold $HClO_4$ to a final concentration of 0·5 M. Protein, removed by centrifugation, was washed. The combined supernatants were heated for 30 min to convert the tri- and the diphosphates to the monophosphates. Perchlorate was

removed as potassium perchlorate. The supernatant was chromatographed on Dowex-50 as described by Reichard.[30] The corresponding CMP and dCMP fractions were combined and evaporated in a high vacuum .Their radioactivity was evaluated in Diatol by liquid scintillation analysis.

RESULTS

Results after injection of ^{3}H-ara-C are shown in Fig. 1. *F*-values decreased exponentially in the liver, kidney, spleen, lung, resistant tumor and blood. In the blood, ^{3}H-ara-C-derived radioactivity had a short half-life (about 1 hr). The small intestines deviated slightly from the general pattern, most likely because a minor amount of radioactive material was excreted with the bile into the duodenum.[12] In the brain tissues there was only a slight, short-lived accumulation of ara-C or its metabolites.

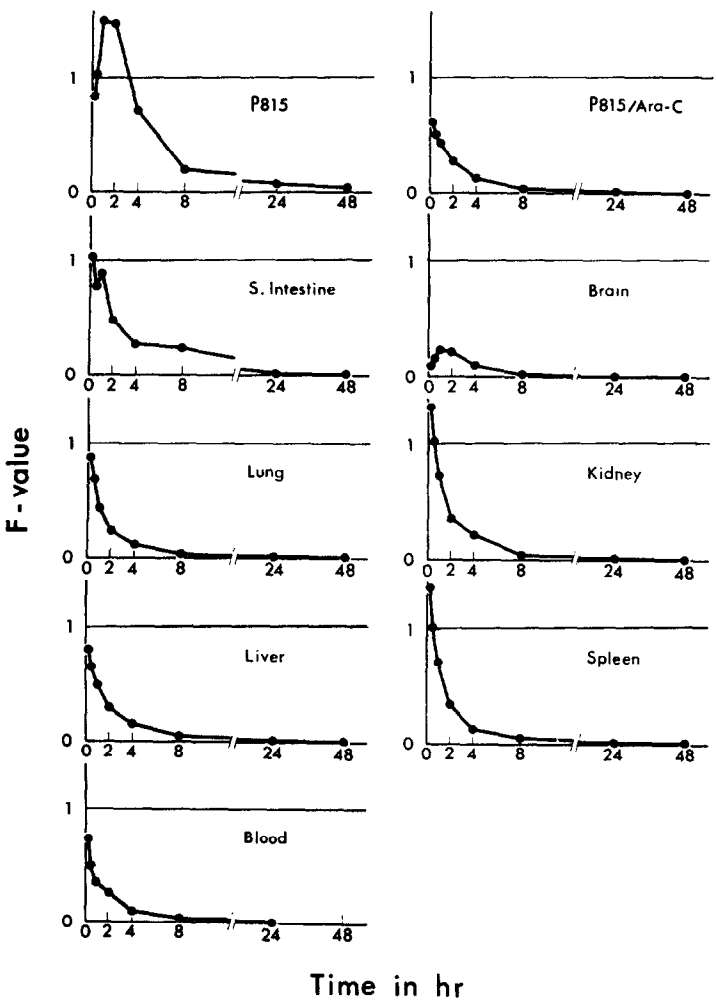

FIG. 1. Distribution of radioactivity after a single i.p. injection of 20 mg/kg of ^{3}H-ara-C into tumor-bearing mice.

Most striking was the selective and protracted accumulation of ^{3}H-ara-C and its metabolites in the sensitive tumor. It was especially marked when the uptake of ^{3}H-ara-C was compared with ^{3}H-CR and ^{3}H-CdR as demonstrated in Fig. 2. The accumulation of labeled CR in the liver was protracted and long lasting and the *F*-

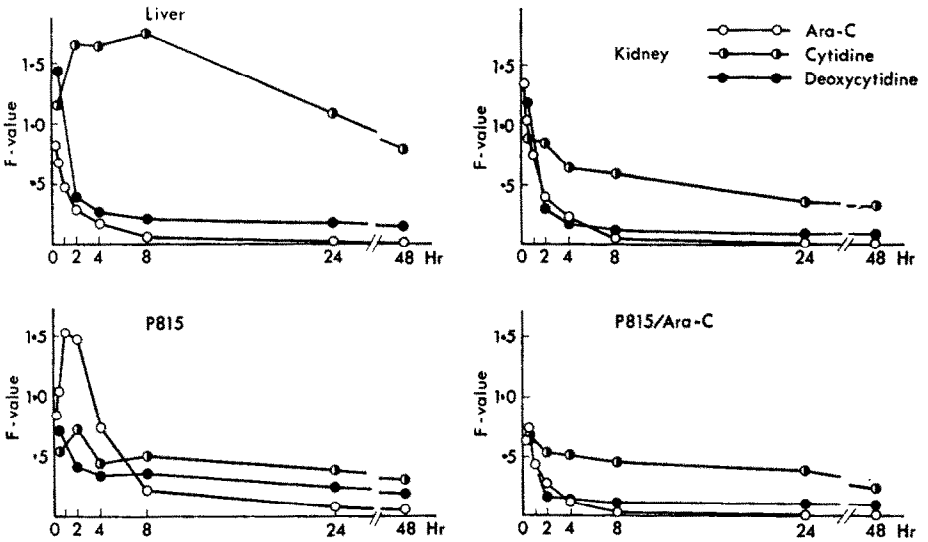

FIG. 2. Distribution of radioactivity after a single i.p. injection of 20 mg/kg of ^{3}H-CR, ^{3}H-CdR or ^{3}H-ara-C into tumor-bearing mice.

values were relatively high. In fact the *F*-values were higher in the liver, kidney, sensitive and resistant tumors after administration of ^{3}H-CR than after ^{3}H-ara-C and ^{3}H-CdR (with the exception of the selective accumulation of ara-C in the sensitive tumor in the first 8 hr after injection). Figure 3 shows that the *F*-values for ^{3}H-ara-C were 6- to 8-fold higher in the sensitive tumor than in the resistant tumor. ^{3}H-CdR and its metabolites also showed a preference for the sensitive tumor but to a lesser degree (2–4). The ratio of the *F*-values of ^{3}H-CR for the two tumors remained close to 1 during the entire experiment.

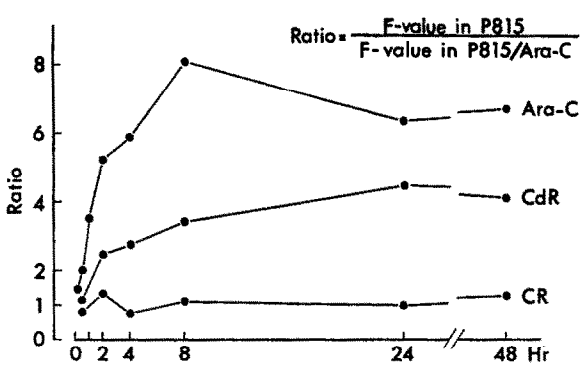

FIG. 3. Ratio of *F*-values, 0 to 48 hr after injection, of ^{3}H-ara-C, ^{3}H-CdR or ^{3}H-CR.

When the uptake and distribution of the radioactivity of ara-C in nontumor bearing animals were compared after i.v. and i.p. administration, it was found that 30 min after injection the respective *F*-values were the same, which indicated fast, complete resorption of the compound.

Intracellular distribution studies of the liver, kidney and two tumors 2 and 24 hr after single injections of ^{3}H-ara-C revealed (Table 1) that the greatest amount of

TABLE 1. INTRACELLULAR DISTRIBUTION OF RADIOACTIVITY AFTER A SINGLE i.p. INJECTION OF ^{3}H-ARA-C TO TUMOR-BEARING MICE*

	Liver		Kidneys		P815		P815/ara-C	
	2 hr†	24 hr†	2 hr†	24 hr†	2 hr†	24 hr†	2 hr†	24 hr†
Acid-soluble fraction	15,794‡	157‡	10,770‡	313‡	59,695‡	392‡	9,139‡	227‡
Lipid fraction	194	133	134	91	88	114	67	59
RNA fraction	168	173	112	88	53	159	95	99
DNA fraction	50	61	32	33	118	291	59	102
Protein fraction	121	83	106	69	55	66	32	38
Total	16,327	607	11,154	594	60,009	1,022	9,392	525

* Evaluated by combustion technique.
† Time after injection of 20 mg/kg, 1 mc/kg of ^{3}H-ara-C.
‡ Each number is expressed by the specific activity (cpm/g tissue).

radioactivity was in the acid-soluble fractions (from 96·6 per cent in the kidneys to 99·5 per cent in the sensitive tumor at 2 hr). The counts in the other fractions were insignificant, although there was a slight indication of incorporation of ara-C, ara-C derivatives or breakdown products, in the RNA and DNA of both tumors, more particularly in the DNA of the sensitive tumor. However, the counts were so low that the nucleic acids were not analyzed. More detailed studies are necessary for conclusive evidence on the incorporation into nucleic acids. By 24 hr after injection of ^{3}H-ara-C, total radioactivity decreased significantly. Only 3·7, 5·3, 1·7 and 5·6 per cent of the radioactivity seen 2 hr following injection was present in the liver, kidneys, sensitive and resistant tumors respectively. This loss was mainly due to a reduction of radioactivity of the acid-soluble fractions. The other fractions showed no significant absolute change except for the slight increase of radioactivity in the RNA and DNA of the sensitive tumor and in the DNA of the resistant tumor.

Paper chromatography of the acid-soluble materials extracted 2 hr after injection of ^{3}H-ara-C revealed (Fig. 4) a relatively large amount of presumably ara-CTP and ara-CDP in the sensitive tumor (60 per cent of the total activity) and, by contrast, a low amount in the resistant tumor (about 14 per cent). In the sensitive tumor only 19·2 per cent of the total activity was ara-C and 18·4 per cent 1-β-D-arabinofuranosyluracil (ara-U). In the resistant tumor these relative values were 31·2 and 51·0 per cent). When these relative percentages were multiplied by the *F*-values determined in the distribution experiment, the calculated specific accumulation values gave an accurate picture of the differences of the absolute amounts of ara-C, their mono-

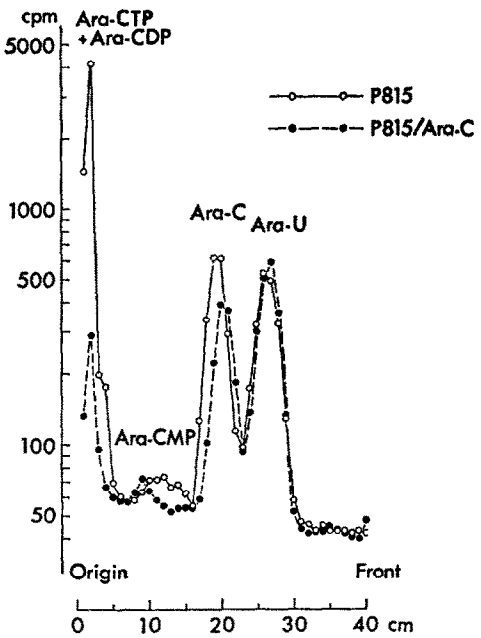

FIG. 4. Paper chromatography (system A) of an aliquot of the acid-soluble fraction of sensitive- and ara-C-resistant tumors, extracted with PCA 2 hr after a single i.p. injection of ^{3}H-ara-C. Ordinate: cpm of each segment of the paper chromatogram. Abscissa: distance from origin in cm.

TABLE 2. ACCUMULATION VALUES OF ARA-C, ARA-U, ARA-CMP, ARA-CDP AND ARA-CTP IN ARA-C-SENSITIVE AND -RESISTANT TUMORS 2 hr AFTER A SINGLE INJECTION OF ^{3}H-ARA-C TO TUMOR-BEARING MICE*

Fraction	Tumor	
	P815 (AV)	P815/ara-C (AV)
Ara-CDP and ara-CTP	0·90	0·04
Ara-CMP	0·03	0·01
Ara-C	0·29	0·09
Ara-U	0·27	0·15

* Accumulation values (AV) calculated by multiplication of the *F*-values by the relative percentage derived from the paper chromatogram.

phosphate, and di- and triphosphates (Table 2). The accumulation of ara-C and ara-CMP was about three times greater in the sensitive than in the resistant tumor, whereas the combined value for their di- and triphosphates was about 22 times greater in the sensitive tumor. This ratio for ara-U was about 2.

When the acid-soluble fractions were extracted with TCA instead of PCA and not immediately chromatographed, only trace amounts of radioactivity were found in the region of the di- and triphosphates; instead, ara-C-monophosphate, identified on the paper chromatogram and spectrophotometrically, was comparable quantitatively to the di- and triphosphates of the PCA extract. Paper chromatograms of TCA

extracts of liver 2 hr after a single injection of ^{3}H-ara-C revealed a pattern similar to that of the sensitive tumor, although in the liver the ara-C nucleotide portion was relatively small. Paper chromatograms of the kidney TCA extract revealed no ara-C nucleotide, but compared with the resistant tumor a relatively large amount of ara-C.

The intracellular distribution studies (Table 3) indicated that 2 hr after a single

TABLE 3. INTRACELLULAR DISTRIBUTION OF RADIOACTIVITY AFTER SINGLE i.p. INJECTIONS OF ^{3}H-ARA-C, ^{3}H-CR OR ^{3}H-CdR*

Distribution	Injected substance											
	^{3}H-Ara-C†				^{3}H-CR‡				^{3}H-CdR‡			
	% Total radioactivity				% Total radioactivity				% Total radioactivity			
	P815		P815/ara-C		P815		P815/ara-C		P815		P815/ara-C	
	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr
Acid-soluble fraction	99·4	38·3	97·3	43·2	65·2	10·6	67·0	9·0	41·8	3·7	81·0	17·1
Lipid fraction	0·2	11·1	0·7	11·2	4·4	3·4	5·1	6·5	2·3	6·0	5·2	24·1
RNA fraction	0·1	15·6	1·0	18·9	22·4	58·5	22·0	59·9	5·6	2·7	4·1	10·9
DNA fraction	0·2	28·5	0·6	19·4	7·0	22·0	4·9	17·0	46·0	83·5	7·9	40·2
Protein fraction	0·1	6·5	0·4	7·3	1·0	5·5	0·9	7·6	4·2	4·1	1·8	7·7
F-values†	1·49	0·08	0·29	0·01	0·71	0·36	0·52	0·37	0·40	0·24	0·16	0·10

* For procedure see text.
† Evaluated by combustion of specimens.
‡ Evaluated by direct addition of specimens to Diatol.

injection of ^{3}H-CR the bulk of the radioactivity was in the acid-soluble portions of the sensitive and resistant tumors. Relatively large amounts of ^{3}H-CR were incorporated in the RNA fractions (22·4 and 22·0 per cent of the radioactivity) in both tumors 2 hr after injection and the amounts increased considerably 24 hr after injection (58·5 and 59·9 per cent) with concomitant loss of radioactivity in the acid-soluble pool and considerable decrease in the *F*-values. Radioactivity in the DNA fractions also increased, although not as extensively as in the RNA fractions. None of these extracts were subjected to paper chromatography.

The studies of intracellular distribution of ^{3}H-CdR (Table 3) revealed that the large percentages of radioactivity present in the acid-soluble extracts 2 hr after injection decreased considerably within 24 hr. No further analysis was performed on these extracts.

When the relative intracellular distribution patterns of CR, CdR and ara-C in the sensitive tumor were compared, the most striking difference 2 hr after injection of the labeled compounds was the slight amount of ara-C-derived radioactivity in the DNA and RNA fractions (0·2 and 0·1 per cent) when CR-derived radioactivity was high in RNA (22·4 per cent) and CdR-derived radioactivity was high in DNA (46 per cent). These differences existed to a lesser degree 24 hr after injection. In the resistant tumors the results were similar but not as marked.

Enzymes of special significance in the metabolism and mode of action of ara-C are the nucleoside and nucleotide deaminases, kinase(s) and the cytidine-, mono-,

di- and triphosphate reductase(s). Their patterns were evaluated in the supernatants of homogenates of the two tumors and in some instances in other tissues as well.

Since it had been shown that deamination is likely to be important in the detoxification of ara-C,[15,22,25] the evaluations of ara-C, CR and CdR deamination in four tissues were compared (Table 4). Only in the kidney were the enzyme levels high,

TABLE 4. NUCLEOSIDE DEAMINASE ACTIVITIES IN LIVER, KIDNEY AND IN THE ARA-C SENSITIVE AND -RESISTANT TUMORS*

Tissue	Cytidine (μmoles/mg/hr)	Deoxycytidine (μmoles/mg/hr)	Ara-C (μmoles/mg/hr)
Liver	0†	0	0
Kidney	0·67	0·29	0·28
P815	0	0	0
P815/ara-C	0	0	0

* For procedure see text.
† Not detectable with the Conway-technique used.

which data confirm those reported by Camiener and Smith[25] and our laboratories;[29] the amounts of CdR and ara-C-deamination were comparable, and less than half the amount of CR deamination. Significantly, the sensitive and resistant tumors and liver did not have measurable nucleoside deaminase activities, confirming partly our previous findings in L1210 leukemia of mice.[22]

The deamination of nucleotides could only be tested with the cytidine-, deoxycytidine mono-, di- and triphosphates and ara-CMP. Data in Table 5 demonstrate

TABLE 5. NUCLEOTIDE DEAMINASE ACTIVITY IN ARA-C-SENSITIVE AND -RESISTANT TUMORS*

	P815 (μmoles/mg/hr)	P815/ara-C (μmoles/mg/hr)
Ara-CMP	0	0
CMP	0†	0
CDP	0	0
CTP	trace	0
dCMP	trace	0
dCDP	0·023	0·017
dCTP	0·070	0·069

* For procedure see text.
† Not detectable with the spectrophotometric assay used.

that only dCTP and dCDP, to a lesser degree, were deaminated and that in both tumors the relative amounts were similar. The only possible deamination of ara-C by the tumor tissues probably takes place at the level of the di- and the triphosphates. These findings are in contrast to the ones reported by Camiener[31] for human liver homogenates as enzyme source for CR- and CdR- nucleotide- *and* nucleoside deaminases. Whether this is an important difference between tumor tissue and normal

tissue or due to the species difference will have to be evaluated in further experiments.

A summary of the kinase(s) for the phosphorylation of CR, CdR and ara-C is shown in Fig. 5. When the same tissue homogenates were used for the three substrates investigated, a similar amount of CR was phosphorylated by all four tissues. Ara-C and CdR, however, were phosphorylated readily and to a sizeable degree by the

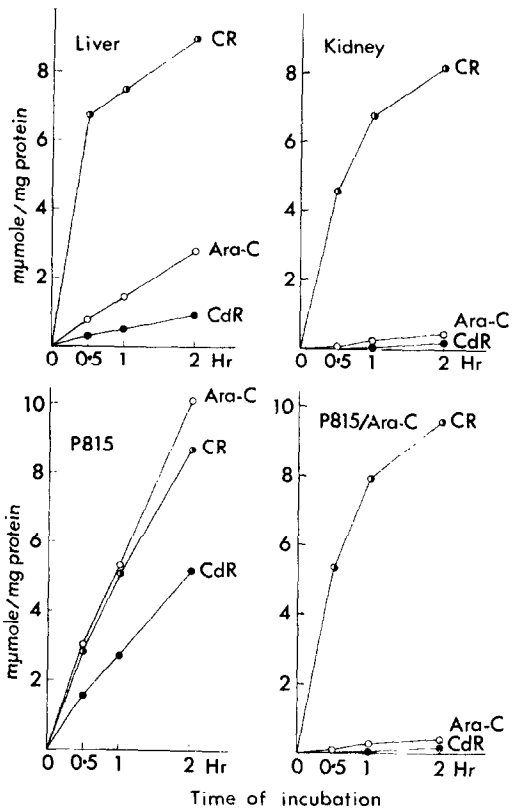

FIG. 5. Nucleoside-kinase activities in liver, kidney, ara-C-sensitive and -resistant tumors. Ordinate: mμmole of substrate phosphorylated per mg protein of crude enzyme preparation. For procedure see text.

sensitive tumor cells, considerably less by liver tissue, and poorly by the resistant tumour cells and kidney tissues. The phosphorylation of ara-C in the sensitive and resistant tumors was more than 20:1, and of CdR also more than 20:1.

Competitive experiments for the phosphorylation of ^{3}H-ara-C in the presence of CdR and ^{3}H-CdR in the presence of ara-C were performed *in vitro* with the homogenate of the sensitive tumor. The difference of affinity for what is probably a common kinase was 50 (CdR):1(ara-C).

The CDP-reductase levels (Fig. 6), studied only in the two tumor tissues, did not differ significantly. The slight increase in the resistant tumor, although consistently seen in different experiments, was too small for speculation.

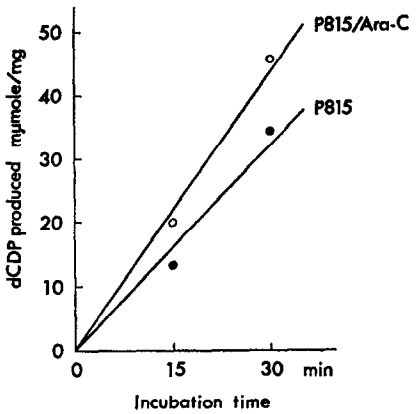

FIG. 6. The dCDP-reductase activity of ara-C-sensitive and -resistant tumors. Ordinate: mμmole dCDP produced from CDP per mg of protein in crude enzyme preparation. Abscissa: incubation time in min. For procedure see text.

DISCUSSION

Resistance to ara-C, easily achieved in experimental tumors and tissue cultures, has been reported by Reins and Johnson,[32] Wodinsky and Kensler,[33] Chu and Fischer,[34] and Dollinger *et al.*[22] To date ara-C-resistant systems have been found to be cross resistant only to 1-β-D-arabinofuranosyl-5-fluorocytosine,[35] a close relative of ara-C. Experimental leukemias resistant to azaserine,[3] 6-mercaptopurine,[3] vinblastine,[4] 4 terephthalanilides,[4] methotrexate,[4] 1,1′-[(methylethane diylidene)dinitrilo]-diguanidine, dihydrochloride hydrate,[4] 5-fluorouracil,[36] and cytoxan,[3] have shown no cross resistance to ara-C.

In the present studies in P815 and P815/ara-C tumor systems, of the nine tissues investigated, only the sensitive tumor showed a strong selective and protracted accumulation of ara-C-derived radioactivity (Fig. 1). The small intestine, which proliferates rapidly, and the brain had but slight and fast disappearance of radioactivity. The other tissues, especially the resistant tumor, immediately and exponentially cleared the radioactivity. By contrast, the distribution of CdR-derived radioactivity rapidly and exponentially decreased in all the tissues. The small intestines, brain and sensitive tumor had small, insignificant irregularities. When we compare the *F*-values of ara-C, CdR- and CR-derived radioactivity in fast proliferating tumor tissues it is obvious that 24 and 48 hr after the injection of ^{3}H-CdR and ^{3}H-ara-C, the ^{3}H-CdR-and ^{3}H-CR-derived radioactivity suggests sizeable incorporation of CdR into the DNA and CR into RNA. This was further evidenced in the intracellular distribution studies for the two tumors (Table 3) by the strong and long lasting accumulation of ^{3}H-CdR-and ^{3}H-CR-derived radioactivity in the two tumors in the DNA and RNA fractions. By comparison, 2 hr after the injection of ^{3}H-ara-C the fractionation of the cell extracts did not yield convincing evidence pertaining to the incorporation of ara-C into the DNA of the two tumors (Tables 1, 3). Most of the radioactivity appeared in the acid-soluble fractions where it remained longer (even 24 hr after the injection) than the ^{3}H-CdR- and ^{3}H-CR-derived radioactivity.

As shown in Fig. 2, there was a marked and long lasting accumulation of CR radioactivity in the liver. This was particularly evident when CR accumulations were

compared in the liver, kidney and two tumors. The intracellular distribution of CR, or its metabolites, indicates a strong but rapid decrease of activity in the acid-soluble fraction and a significant and concomitant increase of radioactivity in the RNA fraction. Thus, as expected, during incorporation of the radioactivity, CR into RNA and CdR into DNA, only insignificant amounts of ara-C-derived radioactivity accumulated in either the DNA or RNA; in fact most of it appeared in, and was rapidly depleted in the acid-soluble fraction, and none significantly accumulated in any other fraction.

The ratios of *F*-values of the two tumors (Fig. 3) confirmed the specific preference of ara-C derivatives for the sensitive tumor. Although less marked, CdR also showed a greater preference for the sensitive than the resistant tumor. CR was equally taken up by both tumors over the period of the experiment. In respect to CR consumption, therefore, both tumors behave the same. The very similar intracellular disposition of CR in both tumors appears to confirm the above observation.

The paper chromatographic analyses of the PCA-extract of the sensitive and resistant tumors 2 hr after injections of ^{3}H-ara-C gave evidence that the major difference is the strong predominance of ara-C-nucleotides in the sensitive tumor. The ratio of the absolute amounts was 22:1. Quantitatively, ara-C and ara-U were low in both tumors. The present results do not yield any information regarding the source of ara-U in the different tissues.

Evaluation of the enzyme levels in the crude homogenates confirmed the conclusion drawn from the distribution and paper chromatographic studies: nucleoside deamination of ara-C, CR and CdR occurs measurably only in kidney homogenate but not in the liver and the two tumors. The deamination of di- and triphosphates of CdR occured to about the same degree in both tumors, but there was no deamination of dCMP, ara-CMP and CDP. CTP was deaminated in trace amounts only by the sensitive tumor. Probably the behaviour of ara-CDP and ara-CTP, not available to us at the time of these experiments, would have been similar to the di- and triphosphates of deoxycytidine.

The kinases for ara-C and CdR had similar patterns in the four tissues investigated. The most striking phenomenon was the marked kinase activity for ara-C in the sensitive tumor. For CdR it was less marked in the sensitive tumour and low in the resistant tumor. The differences in the enzyme activities for CdR in the two tumors were more than 20:1, which is comparable to the differences found for the phosphates of ara-C in both tumors. The high values for CR-kinases in the two tumors, liver and kidney are compatible with the incorporation of CR into the RNA of these tissues. Furthermore, the kinase activities for CdR coincide with the incorporation of CdR into the DNA. There was a noticeable variation for ara-C: the compound was readily phosphorylated in the sensitive tumor and liver tissue; its incorporation into the DNA and RNA was, under the conditions of these experiments, almost nil 2 hr after administration, at which time maximum levels of ara-C-dependent radioactivity were found in the sensitive tumor (Table 3).

The data indicate that the major, and most likely the only, difference between the two tumours is the negligible ara-C kinase activity in the resistant tumor. This finding has been reported for other ara-C resistant tumors by Chu and Fischer,[34] Kessel *et al.*,[37] Schrecker and Urshel,[38] and Kessel.[39] Probably the major impact of ara-C on the cells occurs during its phosphorylation or at the level of the nucleotide. The

affinity of CdR was 50 times greater than ara-C for what is probably an identical kinase. This could explain the often demonstrated reversal effect of CdR for the effect of ara-C. CDP-reductase, which was similar in both tumors, obviously could not be responsible for the development of resistance. Both tumors easily reduce CDP and likely CTP, when available, to dCDP and dCTP, respectively, which assures the necessary precursors for the synthesis of DNA. However, this pathway is not more important in one tumor than the other because CR is phosphorylated abundantly in both tumors and can be converted and incorporated into the DNA of the sensitive and resistant tumors to the same extent.

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