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BIOTRANSFORMATION OF 9-β-D-ARABINOFURANOSYLADENINE BY RAT AND HUMAN ERYTHROCYTES

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Abstract—Studies were performed to test whether 9-β-D-arabinofuranosyladenine (ara-A) would accumulate in erythrocytes as a result of phosphorylation to the nucleotide level. When [^{3}H]ara-A was incubated with whole blood from rat, monkey or man for 2 hr at 37°, the drug rapidly equilibrated between erythrocytes and plasma but did not concentrate in the cells. Incubation of [^{3}H]ara-A with rat and human erythrocyte lysates for 2 hr followed by chromatographic analysis showed that 2–5 per cent of ara-A was converted to nucleotides. In contrast, 10–35 per cent of [^{14}C]adenosine was converted to adenine nucleotides under the same conditions. Incubation of [^{3}H]ara-A with human erythrocyte lysates for 18 hr resulted in a conversion of approx. 40 per cent of the labeled drug to nucleotides. Additional chromatography revealed, however, that the nucleotide fraction contained almost no arabinosyl nucleotides. Rather, 90 per cent of the label in the nucleotide fraction was identified as IMP. These results indicate that only a minor amount, if any, of ara-A was phosphorylated by erythrocyte enzymes to yield arabinosyl nucleotides. An alternative pathway converted much of the labeled drug to ribosyl nucleotides via the deamination of ara-A to ara-hypoxanthine, cleavage to hypoxanthine and conversion of the free hypoxanthine to IMP.

MANY nucleoside antibiotics require conversion to the corresponding 5′-phosphates to express antineoplastic or antiviral activity. Conversion to the nucleotide can occur in either host tissues, tumor cells or viruses.[1,2] Phosphorylation by host tissues will produce a compound active against not only the tumor or virus, but also against proliferating host cells. Ara-A‡ is a nucleoside antibiotic having broad spectrum activity against DNA viruses[3] but with low toxicity to experimental animals[4] and man.[5] The low toxicity probably is related to the rapid deamination of ara-A to the less active ara-Hx and its subsequent phosphorolysis to hypoxanthine.[6,7] Since the 5′-triphosphate of ara-A appears to be the active form,[8] the low toxicity also might be due to an inability of normal animal tissues to phosphorylate the drug.

Mammalian erythrocytes commonly phosphorylate fraudulent nucleosides resulting in a preferential uptake because the nucleoside is converted to the more ionized

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‡ Abbreviations used: ara-A, 9-β-D-arabinofuranosyladenine; ara-AMP, ara-ADP and ara-ATP, the corresponding 5′-mono-, di- and triphosphates of ara-A; ara-Hx, 9-β-D-arabinofuranosylhypoxanthine; ara-IMP, the corresponding 5′-monophosphate of ara-Hx; Ade, adenine; Hyp, hypoxanthine; PEI, polyethyleneimine.

and, therefore, less diffusable nucleotide. Drugs which are phosphorylated in this manner include arabinosyl cytosine,[9] 7-deaza-adenosine,[10] and ribosyl 6-methylthiopurine.[11] It appeared that ara-A also could be phosphorylated in this way because the amount of label in erythrocytes rose slowly over several days after the administration of the labeled drug to several species of laboratory animals.[6] The studies *in vitro* reported here were undertaken to completely examine the uptake and phosphorylation of ara-A by rat and human erythrocyte preparations.

EXPERIMENTAL

Materials. Hypoxanthine, adenine and adenosine were purchased from CalBiochem., San Diego, California; inosine, IMP, ITP, AMP, ATP and dATP from Sigma Chemical Co., St. Louis, Missouri; ara-Hx from Pfanstiehl Laboratories, Waukegan, Illinois; 9-β-D-arabinofuranosyl-[2-^{3}H]adenine (670 mCi/m-mole) and [8-^{14}C]adenosine (53 mCi/m-mole) from New England Nuclear Corp., Boston, Massachusetts; ara-ADP and ara-ATP from Terra Marine Bioresearch, La Jolla, California; ara-A was a gift of Dr. H. Machamer, Parke, Davis & Co., Detroit, Michigan and ara-IMP and ara-AMP were gifts of Dr. R. L. Tolman, ICN Nucleic Acid Research Institute, Irvine, California. PEI-cellulose plates were obtained from Brinkmann Instruments, Inc., Des Plaines, Illinois.

Uptake methods. Blood was obtained from male, albino, Sprague–Dawley rats by cardiac puncture after stunning the animal by a blow on the head. Blood from male, Rhesus monkeys was drawn from a saphenous vein. Blood from a normal, human male donor was obtained from the left cephalic vein. Blood was drawn into sterile, heparinized syringes and cooled immediately to 0–4°. For uptake experiments, blood was incubated directly with [^{3}H]ara-A for up to 4 hr at 37°. All incubations were performed in duplicate. Cells were sedimented by centrifugation at 5600 *g* for 10 min, and plasma and cells combusted in a Packard model 300 sample oxidizer. Radioactivity was assayed in a Packard model 3320 liquid scintillation spectrometer and dis./min were determined by the external standard method.

Lysate preparation and incubation. For preparation of erythrocyte lysates, blood was centrifuged at 1600 *g* for 10 min at 0° and buffy coat cells and plasma were removed. All subsequent procedures were performed at 0–4°. Cells were washed three times by resuspension in an equal vol. 0·9% NaCl and hemolyzed by repeated rapid freezing in liquid nitrogen and thawing. After centrifugation at 6500 *g* for 10 min, the supernatant was dialyzed against 300 vol. 1 mM Tris buffer (pH 7·4) for 2 hr and either used immediately or frozen at −80° and used within 2 months.

Preparations from rat blood gave dialysates containing 8–12 mg/ml of protein as determined by the method of Lowry *et al.*,[12] whereas preparations from human blood contained approx. 150 mg/ml of protein. The difference in protein concn arose because the centrifugation step before dialysis did not pellet cell debris from human erythrocytes.

Phosphorylation of ara-A and adenosine was examined using the conditions described by Lerner and Rubinstein.[13] Incubations were performed at 25° and contained 3 mg (rat) or 16 mg (human) protein, 2·5 mM $MgCl_2$, 2·5 mM ATP and 19 μM [^{14}C]adenosine or [^{3}H]ara-A in 1·0 ml of 50 mM phosphate buffer (pH 7·0). Aliquots were removed at the indicated times, mixed with an equal vol. 95% ethanol

at −20° and protein was precipitated overnight at −20°. All incubations with ara-A and adenosine were performed twice. In a preliminary experiment, the use of stoichiometric quantities of [^{14}C]adenosine and ATP resulted in a reduced yield of [^{14}C]ATP but increased amounts of labeled ADP and AMP.

Chromatographic analyses. Aliquots of the ethanol-containing supernatants were mixed with solutions of known reference compounds and characterized by TLC. Purines and nucleosides were determined as described previously.[14] Thus, separation of arabinosyl nucleosides and purines was accomplished on Silica gel plates using chloroform–methanol–3% acetic acid (3:2:1, v/v, lower phase) as the solvent. Ribosyl nucleosides and purines were chromatographed on Silica gel in a solvent consisting of 17 parts of chloroform–methanol–3% ammonia (3:2:1, v/v, lower phase) and three parts methanol. Nucleotides were not resolved in these systems and remained at the origin. Individual nucleotides were analyzed on PEI-cellulose plates using 2 N formic acid–2 M LiCl (1:1, v/v) as described by Randerath and Randerath.[15] Prior development with water in the second dimension removed purines and nucleosides and permitted the complete resolution of ATP, ITP, ADP, IDP, AMP and IMP. AMP and IMP were differentiated from corresponding arabinosyl nucleotides by chromatography on PEI-cellulose plates in boric acid-saturated 1 M LiCl (pH 4·5)–ethanol (1:1, v/v) according to Schrecker *et al.*[16] Under these conditions, IMP, AMP and all di- and triphosphates remained near the origin ($R_f \simeq 0{\cdot}1$), while corresponding deoxyribosyl and arabinosyl nucleoside monophosphates migrated with R_f values around 0·3 (Table 1). Examination of developed plates under u.v. light (254 nm) revealed the reference compounds which appeared as dark spots. Radioactive metabolites were detected by cutting or scraping 2- or 5-mm sections of the gel into counting vials, extracting with 2 ml of a solution of 0·1 N HCl in methanol, adding 13 ml of a scintillation soln containing 3 g 2-(-4-*tert*-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole/l. toluene and counting as indicated above. This procedure resulted in the quantitative recovery and detection of all compounds spotted on the plates. Coincidence of labeled and reference compounds in unambiguous systems was taken as indicating identity.

RESULTS AND DISCUSSION

Whole rat, monkey or human blood was incubated with 100 μM [^{3}H]ara-A for 2 hr at 37° as a preliminary test of whether ara-A was phosphorylated by erythrocytes. There was no accumulation of the drug by erythrocytes beyond an initial equilibration of label between cells and plasma, i.e. the same number of dis./min were detected/unit vol. of each. Equilibration occurred during the first 1–3 min of incubation probably as a result of simple or facilitated diffusion.[17] Similar results were obtained after a 30-min incubation of rat or human blood with ara-A concn of 0·1–100 μM. Labeled drug readily back diffused into the medium upon resuspension of cells in an equal vol. 0·9% NaCl. Four or five such washes completely removed the label from cells, indicating that little phosphorylation had occurred. In contrast, 7-deaza-adenosine[10] and ribosyl-6-methylthiopurine[11] were reported to be completely cleared from plasma into erythrocytes during a similar 2-hr incubation. Label from these compounds was not washed from the cells because the nucleosides were converted to the mono-, di- or triphosphate level by erythrocyte enzymes.

In order to determine directly whether ara-A was phosphorylated by erythrocyte enzymes, lysates were prepared and incubated utilizing conditions detailed by Lerner and Rubinstein.[13] These authors demonstrated the presence of adenosine kinase (EC 2.7.1.20) in human erythrocytes. Miech and Santos[18] had previously demonstrated the presence of adenosine kinase in rat erythrocytes. As positive controls, rat and human erythrocyte lysates also were incubated with [^{14}C]adenosine. After incubation, deproteinized aliquots were analyzed for purines, nucleosides and total nucleotides using Silica gel chromatography systems and for individual nucleotides using PEI-cellulose chromatography. These experiments revealed the appearance of [^{14}C]ATP (Fig. 1) which is consistent with the phosphorylation of [^{14}C]adenosine by adenosine kinase and the subsequent conversion of AMP to the triphosphate.[13] The data of Fig. 1 also show the degradation of adenosine to inosine and hypoxanthine followed by the reutilization of hypoxanthine to give IMP. The low amounts of adenosine at zero time were attributed to adenosine deaminase activity which occurred at 0–4° before the first aliquot was removed. The phosphorylation and degradation of adenosine observed here is in accord with other reports of adenosine metabolism by erythrocyte lysates[13] and platelet-rich plasma.[19]

When [^{3}H]ara-A was incubated under identical conditions with rat erythrocyte lysates, merely 2 per cent of the labeled drug was converted to nucleotides during a 2-hr incubation. Analysis on PEI-cellulose showed that most of the labeled nucleotides chromatographed with IMP and AMP, but the low amounts discouraged additional characterization. This is noteworthy because little nucleotide formation occurred despite the long half-life of ara-A in rat erythrocyte lysates (Fig. 2a). Deamination of ara-A also was observed in human erythrocyte lysates (Fig. 2b) but the

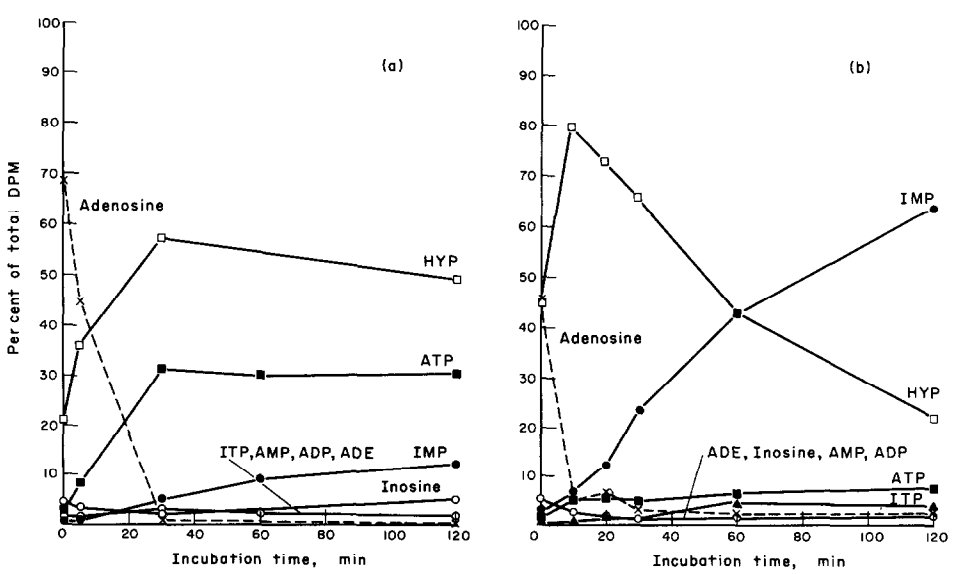

FIG. 1. Metabolism of adenosine by erythrocyte lysates. Lysates from rat (a) and human (b) erythrocytes were incubated with 19 μM [^{14}C]adenosine, 2·5 mM Mg^{2+} and 2·5 mM ATP. Aliquots were removed at the indicated times, deproteinized and analyzed by TLC. Silica gel layers were spotted with 4600–8300 dis./min for the determination of nucleosides; PEI-cellulose layers were spotted with 3600–7400 dis./min for the quantitation of nucleotides.

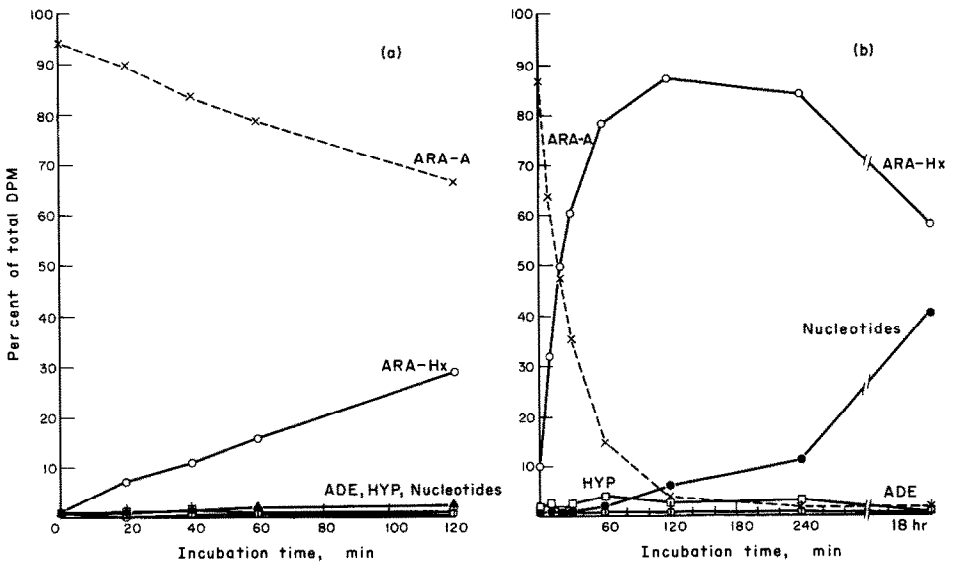

FIG. 2. Metabolism of ara-A by erythrocyte lysates. Lysates from rat (a) and human (b) erythrocytes were incubated with 19 μM [^{3}H]ara-A, 2·5 mM Mg^{2+} and 2·5 mM ATP. Aliquots were removed at the indicated times, deproteinized and analyzed by TLC. Silica gel layers were spotted with 40,000–80,000 dis./min for nucleoside determinations; PEI-cellulose layers were spotted with 60,000–100,000 dis./min for nucleotide quantitation.

half-life was considerably shorter. The difference might be attributed to greater amounts of adenosine deaminase (EC 3.5.4.4) in the human erythrocyte lysates. This does not appear likely, however, as little difference was noted in the deamination rate of adenosine (Fig. 1). The observations agree with those of Koshiura and LePage[20] who found that rat and human erythrocytes deaminated adenosine at a similar rate, but human erythrocytes deaminated ara-A at a greater rate than rat erythrocytes.

Somewhat greater amounts of nucleotides were detected in 2-hr incubations of human erythrocyte lysates with [^{3}H]ara-A, but the rate of nucleotide synthesis was less than 10 per cent of that seen with adenosine. Extended incubation times and chromatography on Silica gel showed that after 4 hr 11 per cent, and after 18 hr 40 per cent of the labeled ara-A had been converted to nucleotides (Fig. 2b). These nucleotide fractions were analyzed additionally by chromatography on PEI-cellulose plates. Chromatography in the formate solvent (Table 1) showed that after 4- and 18-hr incubations most of the labeled nucleotides chromatographed coincident with IMP. Minor amounts of label also were associated with AMP, ADP, ATP, IDP and ITP. Arabinosyl nucleotides also migrated with these ribosyl nucleotides in the formate solvent; therefore, the borate-containing solvent was used to resolve the two sets of nucleotides. In this system nearly all nucleotide radioactivity chromatographed with a R_f value $<0{\cdot}13$, while ara-IMP and ara-AMP chromatographed with R_f values $>0{\cdot}20$ (Table 1). Thus the label associated with the inosine mononucleotides in the formate system represents IMP, not ara-IMP. Roughly 10 per cent of the nucleotides might be arabinosyl nucleotides because the borate-containing solvent would not resolve ATP, ADP, ITP and IDP from the corresponding arabinosyl nucleotides. This seems highly unlikely, however, as little ara-AMP was detected.

These conclusions also are supported by the kinetic data depicted in Fig. 2b, i.e. a precursor–product relationship exists between ara-Hx, not ara-A, and the nucleotides which were formed. Glycosidic cleavage of ara-Hx by purine nucleoside phosphorylase (EC 2.4.2.1) and reutilization of the free hypoxanthine by hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) to give IMP seems to be a likely pathway. The presence of active forms of these two enzymes in the lysate preparations was confirmed by the kinetics of hypoxanthine and IMP formation from adenosine (Fig. 1b). The formation of adenine nucleotides from IMP could follow during the more lengthy incubation period with ara-A via the purine ribonucleotide interconversion pathway (e.g. see Henderson and Paterson).[21] The detection of very limited amounts of adenine nucleotides agrees with the observations of Lowy *et al.*[22] that human erythrocytes have little capacity for the conversion of IMP to AMP.

The present results also are consistent with the statement of Ilan *et al.*[23] that normal mouse blood could not phosphorylate ara-A. In addition, they found that only 12–18 per cent of ara-A accumulated by liver from normal or *Plasmodium berghi*-infected mice was converted to ara-AMP. Brink and LePage[24] observed nearly equal amounts of labeled nucleosides and nucleotides in the liver when [8-^{14}C]ara-A was administered to mice. However, the nucleotides detected by both these groups were characterized by chromatography on Dowex-1 which would not distinguish between ribosyl and arabinosyl nucleotides. More recently LePage[25] found that label from [8-^{14}C]ara-A and [8-^{14}C]ara-Hx was incorporated into nucleotides and nucleic acids of TA3 cells. Most of the label from both nucleosides was incorporated as AMP, not ara-AMP, indicating that glycosidic cleavage had occurred followed by

TABLE 1. CHARACTERIZATION OF LABELED NUCLEOTIDES FROM HUMAN ERYTHROCYTE LYSATES INCUBATED WITH [^{3}H]ARA-A*

	PEI-cellulose chromatography in: Formate solvent			Borate solvent		
		% label migrating with nucleotide			% label migrating with nucleotide	
Nucleotide	R_f	4 hr	18 hr	R_f	4 hr	18 hr
ITP	0·20	6	4	0·00		
ATP	0·34			0·00		
ara-ATP	0·32	3	2	0·02		
dATP	0·34			0·02		
IDP	0·58	<1	1	0·00	95†	96†
ADP	0·65			0·00		
ara-ADP	0·65	<1	<1	0·08		
dADP				0·10		
IMP	0·74			0·05		
ara-IMP	0·74	86	90	0·21	1	1
AMP	0·89			0·13	2	1
ara-AMP	0·88	3	1	0·31		
dAMP				0·33	1‡	1·5‡

* Deproteinized aliquots from the incubation described in Fig. 2B were chromatographed on PEI-cellulose plates and developed in the listed solvents as described in the Experimental section. Approximately 60,000 and 140,000 dis./min were chromatographed in the formate and borate solvents respectively.

† Sum of label chromatographing with R_f <0·10.

‡ Sum of label chromatographing with ara-AMP and dAMP.

a reutilization of the labeled purine. Apparently TA3 cells possess an active purine ribonucleotide interconversion pathway so that IMP formed from hypoxanthine gives AMP as the final product. In human erythrocytes, this pathway is relatively inactive[22] so that IMP accumulates.

The lack of ara-A phosphorylation noted in this study also is consistent with studies which noted that ara-A is a poor substrate for partially purified adenosine kinase. Schnebli *et al.*[26] noted that the amount of ara-A phosphorylated by the kinase from an established line of human epidermoid carcinoma cells (HEp-2) was <7 per cent of the amount of adenosine phosphorylated. Lindberg *et al.*[27] found that the rabbit liver enzyme had a K_m for ara-A 1000-fold greater than that for adenosine.

Although ara-A exhibits at least one activity as the nucleoside, a tRNA methylase inhibitor,[28] conversion to the triphosphate has been assumed to be the requisite step in the formation of the active drug.[8] The apparent paucity of ara-A phosphorylation by the limited number of normal tissues examined raises the possibility that the lack of toxicity to normal animals[4] and man[5] may be related to the lack of conversion of ara-A to ara-ATP by host animals.

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