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ON THE MECHANISM OF STORAGE OF ANHYDRO-araC

WAYNE H. SCHRIER, ROBERT H. HAYASHIKAWA* and JOSEPH NAGYVARY†
Department of Biochemistry and Biophysics, Texas A & M University, College Station, TX 77843, U.S.A.

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Abstract—Anhydro-araC, a precursor of araC, exhibits an unusual tissue distribution and unexpected toxicities which require an interpretation. In order to provide an explanation of these phenomena, we studied the nature of binding of anhydro-araC to some proteins and acid mucopolysaccharides *in vitro* using equilibrium dialysis and potentiometric titration. The results indicate significant electrostatic binding of the cationic anhydro-araC to all polyanionic compounds. The physiological significance of these findings was established in mice with the help of ^{14}C-labeled drug. A portion of the radioactivity (15–30 per cent) could not be extracted with water but with $CaCl_2$. An analysis of this fraction showed predominantly unchanged anhydro-araC and ionic metabolites. Both storage and toxicity of anhydro-araC seem to be a result of interference with the different cation exchanger biopolymers found in the mast cells and the "ground substance."

An increasing volume of literature deals with the development of the araC† analog, anhydro-araC, as a chemotherapeutic agent [1]. Although anhydro-araC is merely a chemical precursor of araC, it exhibits a broader spectrum of anti-tumor activity [2] and has the advantage of a more convenient dose regimen [3]. Biochemical studies have established that anhydro-araC is not taken up by the cells [4], and that it is not a substrate of deaminase [5] and kinase enzymes [6]. These findings are not surprising in view of the chemical structure of anhydro-araC as it was revealed by X-ray crystallography [7], but the implications of structure have escaped the attention of pharmacologists [2, 5, 6, 8]. The two most important structural features are the presence of an immonium group (in place of a neutral amino group in araC) and the fixation of the primary hydroxyl group in a chemically unreactive configuration. Such a structure obviously cannot be deaminated or phosphorylated. In order to avoid the pitfalls associated with the use of cations, previous research in our laboratory has centered on the properties of anhydro-araC 3′-phosphate [9], but this compound, like most administered nucleoside monophosphates [10], is rapidly dephosphorylated *in vivo* [11]. Thus the conclusions made about the excretion and metabolism of the ^{14}C-labeled nucleotide [12] were indicative of the behavior of anhydro-araC itself. We noted, as did subsequently other authors [8, 13, 14] who worked directly with the nucleoside, that anhydro-araC is retained in the body for a prolonged period of time and it is accumulated selectively in some tissues. New toxicities observed during the phase I clinical trial [15] of anhydro-araC point to some hitherto unrecognized mechanisms and consequences of storage.

In this paper, we address ourselves to an unexplored aspect of anhydro-araC storage, i.e. the ionic binding of the cationic drug to polyanionic matrices.

METHODS

Chondroitin sulfate (Nutritional Biochemical Co.) was dialyzed against Na-EDTA, pH 7, and distilled water. Sodium heparinate, choline chloride and sucrose were grade I preparations of Sigma Biochemicals, Inc. Anhydro-araC formate was prepared in our laboratory [12] by dephosphorylation of the nucleotide, followed by ion exchange chromatography on Dowex 50 resin; sp. act. was 0.5 μCi/mg. Analytical techniques were those described by Fòcke *et al.* [12]. Bovine serum albumin, β-lactoglobulin A and calf thymus histone H2a were gifts from Dr. C. N. Pace.

Binding studies in vitro. The measurement of anhydro-araC binding to chondroitin sulfate and heparin followed the procedures of Simard and Friedman [16]. Ten-ml portions of a 0.002 M solution of the polyaminoglycan were titrated with 0.2 M anhydro-araC chloride, choline chloride, and sucrose. The free Na^+ was measured using a Radiometer G502Na sodium electrode–K401 calomel electrode system connected to a Radiometer pH meter 26.

Equilibrium dialysis was carried out in 1-ml Plexiglas cells in 5 mM Na-phosphate buffer, pH 7.0, for 48 hr at 4°. The following solute concentrations were used: 0.227 mM bovine β-lactoglobulin A, 0.390 mM bovine serum albumin, 0.106 and 0.227 mM histone H2a, and 3.1 mM anhydro-araC. Concentrations were determined by a Cary 15 spectrometer using the values, in the above order, $E^{1\%}_{278} = 9.1$, $E^{1\%}_{280} = 5.8$, $E^{1\%}_{276} = 3.21$ and $\epsilon_{263} = 10{,}400$.

Storage of anhydro-araC in vivo. Three male Swiss albino mice (20 g) were given intraperitoneal injections of 1.0, 1.5 and 2.5 μCi of anhydro-araC formate, respectively, dissolved in 0.2 ml of distilled water. Three hr later the mice were sacrificed and seven selected organs were removed. The entire tissue sample was homogenized in distilled water with an

* Abbreviations used in the text are: araC, 1-β-D-arabinofuranosylcytosine; and anhydro-araC, O^2:2′-anhydro-1-β-D-arabinofuranosylcytosine.

† Portions of this research were taken from the Ph.D. Dissertation of the late Dr. R. H. Hayashikawa, Texas A & M University, 1973.

Table 1. Binding of anhydro-araC to various proteins

Proteins	Protein concn (mM)	Net effective charge at pH 7.0	$\bar{\nu}$
Bovine β-lactoglobulin A*	0.229	−12	1.05
Bovine serum albumin†	0.390	−8	0.40
Calf thymus histone H2a‡	0.108	+16	0.00
Calf thymus histone H2a	0.227	+16	0.00

* Ref. 17.
† Ref. 18.
‡ Ref. 19.

Inframo tissue homogenizer model No. RZR64, final volume being 2 ml, except for the liver. The entire liver was homogenized in distilled water to a total volume of 4 ml. The homogenates were placed in dialysis tubings and dialyzed against two 20-ml changes of water at 5° for 24 hr, followed by two 20-ml changes of 0.01 M $CaCl_2$ for 24 hr. The dialysates were freeze-dried and dissolved in 1 ml of deionized water. Aliquots of 0.2 ml were mixed with 1 ml of NCS tissue solubilizer for 4 hr at 50°, followed by 10 ml of Bray's mixture and 1 ml of glacial acetic acid. The samples were prepared in triplicate and counted on a Beckman LS250 scintillation counter.

RESULTS

The nature of binding of anhydro-araC to extracellular macromolecules was studied *in vitro* using equilibrium dialysis and potentiometric titration. The ratios of total bound anhydro-araC to the protein ($\bar{\nu}$, Table 1) [17–19] were determined by calculation of the amount of free anhydro-araC and subtracting this value from the total amount added initially and dividing this difference by the total amount of protein added. Contrary to he hypothesis of Focke *et al.* [12], the linkage to three selected proteins was of ionic nature, showing no evidence of covalent linkage. The greatest extent of binding was observed with the most anionic protein, β-lactoglobulin A, while the cationic histone H2a [20] exhibited no binding at all.

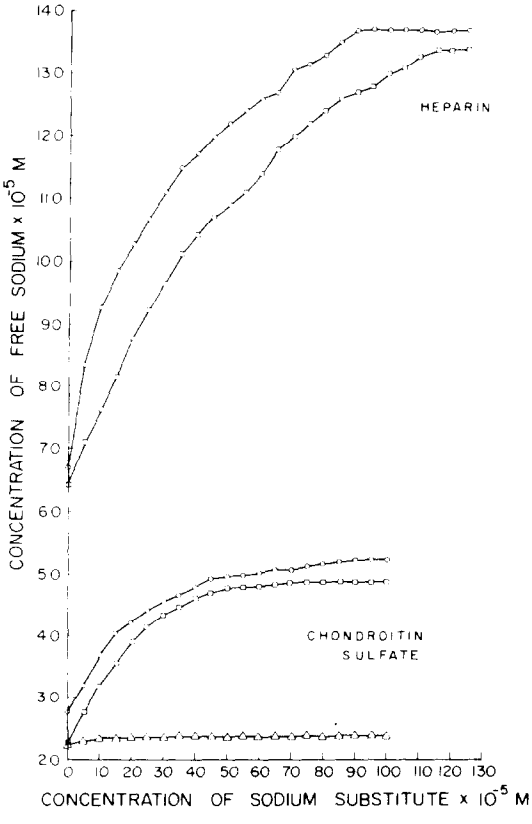

Fig. 1. Binding of anhydro-araC to acid mucopolysaccharides. The Na-salts of heparin and chondroitin sulfate were titrated with anhydro-araC (—□—□—), choline chloride (—○—○—) and sucrose (—△—△—) using a Radiometer apparatus with G502Na and K401 calomel electrodes.

If the storage of anhydro-araC is due to electrostatic forces, the more strongly polyanionic extracellular macromolecules, heparin and chondroitin sulfates, should be directly involved and provide a good model *in vitro*. These polyanions exhibit a large Donnan effect and could be best studied with an ion-specific electrode. The electrostatic binding of a cation was accompanied by the release of free Na^+ which was measured. Choline chloride served as a standard which was used in similar experiments by Simard and Friedman [16]. We found comparable binding of the two organic cations (Fig. 1), which is indicative of primarily coulombic interactions with only a minor contribution resulting from the molecular structure. These results obtained *in vitro* do not necessarily imply that similar lack of specificity exists *in vivo*.

We designed a qualitative study for demonstrating the contribution of coulombic interactions in the storage of anhydro-araC in several tissues. In this approach, homogenates of seven tissues were first dialyzed against distilled water to remove all non-bound drug and its metabolites after the injection of [^{14}C]-anhydro-arac formate. Subsequent dialysis against 0.01 M $CaCl_2$ released a substantial amount of radioactivity in all tissues tested (Table 2), and only traces remained in the suspension. The percentage of total radioactivity found in the $CaCl_2$ dialysates showed some variations according to tissue and dosage, but it remained mainly in the range of 15–30 per cent (Table 3). The absolute values of these electrostatically bound compounds per g of tissue were the highest in the submaxillary gland and sternum. The most important components of both dialysates were identified with suitable carriers using a Savant Instruments, Inc., high voltage paper electrophoresis (Table 4). The main component was anhydro-araC, with araC and its phosphate also present. Calcium is known to be efficient in displacing monovalent cations [6, 21], and the $CaCl_2$ fraction had indeed a high proportion of charged molecules relative to araC. The presence of araC in this fraction may be a result of a continuous hydrolysis [22] of the precursor anhydro-araC. Accordingly, the actual extent of ionic binding may exceed the one suggested by the data in Tables 2 and 3. It has not been the purpose of this preliminary study to provide quantitative data on cation–polyanion interactions which are notoriously difficult to quantitate under conditions *in vivo* [23, 24].

DISCUSSION

In our previous paper [12], we remarked on the difficulty of extracting anhydro-araC from certain tissues, and we felt that this observation could provide

Table 2. Distribution of free and bound [^{14}C]anhydro-araC in various tissue

Tissue	[^{14}C]anhydro-araC formate administered (1.0 μCi)				[^{14}C]anhydro-araC formate administered (1.5 μCi)				[^{14}C]anhydro-araC formate administered (2.5 μCi)			
	Total cpm H_2O	cpm/g Tissue	Total cpm Ca^{2+}	cpm/g Tissue	Total cpm (H_2O)	cpm/g Tissue	Total cpm Ca^{2+}	cpm/g Tissue	Total cpm H_2O	cpm/g Tissue	Total cpm Ca^{2+}	cpm/g Tissue
Submaxillary gland	28,030	121,289	4,940	21,376	43,690	216,073	10,065	49,777	79,845	482,845	16,330	104,345
Sternum	890	38,362	380	16,379	6,360	120,000	2,585	48,774	4,535	59,671	540	7,105
Liver	13,480	7,201	2,730	1,458	16,170	7,340	3,025	1,373	65,215	45,386	12,595	8,765
Pancreas	5,770	14,615	1,370	3,470	2,500	12,327	1,360	6,706	12,105	111,401	2,520	23,077
Spleen	2,320	15,283	630	4,150	11,705	25,490	3,875	8,439	22,760	91,885	4,000	16,149
Lungs	2,890	11,320	950	3,721	5,785	34,997	1,240	7,502	22,365	110,608	3,705	18,373
Reproductive organs	72,310	74,677	3,440	3,552	138,475	124,148	19,520	17,560	210,885	527,608	48,830	122,167

* Testes.

Table 3. Percentage of total radioactivity in the H_2O and Ca^{2+} dialysates*

Tissue	Dialysates	[^{14}C]anhydro-araC formate administered (μCi) 1.0	1.5	2.5
Submaxillary	H_2O	85.0	81.3	83.2
	Ca^{2+}	15.0	18.7	17.8
Sternum	H_2O	70.1	71.1	89.8
	Ca^{2+}	29.9	28.9	10.2
Liver	H_2O	83.2	84.2	98.7
	Ca^{2+}	16.8	15.8	10.3
Pancreas	H_2O	80.8	64.8	83.1
	Ca^{2+}	19.2	35.2	16.9
Spleen	H_2O	78.6	72.3	85.0
	Ca^{2+}	21.4	27.7	15.0
Lungs	H_2O	79.4	82.3	87.2
	Ca^{2+}	20.6	17.7	12.8
Reproductive organs	H_2O	84.4	87.6	84.6
	Ca^{2+}	15.6	12.4	15.4

* Most of the left-over suspensions were checked for residual radioactivity. They were found to contain a maximum of 3 per cent of the total.

a clue to the mode of storage as well. We proposed that anhydro-araC could react with the amino groups of proteins in a reversible fashion. However, the data presented here make it unlikely that the binding of anhydro-araC to proteins would be the major contributing factor in the retention of this drug. If a covalent linkage had indeed existed, anhydro-araC binding would have been highest in the most basic protein, histone H2a. However, it gave negative results.

Our study has focused on the neglected aspect of anhydro-araC research, i.e. the consideration of the ionic nature of this nucleoside analog. Since the human body contains several kilograms of acid mucopolysaccharides in its "ground substance" and mast cells [25], one can expect a substantial contribution of ion-exchanger type binding to the observed retention of anhydro-araC. It is likely that the anhydro-araC cation successfully competes with other monovalent ions for the available binding sites along the sulfate and carboxylate groups, and this binding could be reinforced by hydrogen bonding. The equilibrium to the free, unbound anhydro-araC should be determined by the respective concentrations but there is a great deviation from equilibrium due to fast excretion and slow kinetics of ion diffusion in the colloidal systems. This is expected to yield a biphasic excretion which was observed first by Focke *et al.* [12], and refined by Ho *et al.* [14], Liss and Neil [18], and Hoshi *et al.* [13]. Our results and interpretation are also in accord with the peculiar tissue distribution of anhydro-araC as best evidenced by autoradiography [8, 26]. The distribution seems to follow the tissue level of mucopolysaccharides [27] as it is particularly high in the submaxillary gland, reproductive organs and connective tissue. Unfortunately, the methods suitable to isolate chondroitin sulfate and heparin from the tissues [27] would dissociate the putative anhydro-araC complex.

Table 4. Percentage of labeled anhydro-araC and its metabolites after fractionation by high voltage paper electrophoresis

Dialysis fraction	Anhydro-araC	Nucleosides	Nucleotides
H_2O	75.9	22.8	1.3
Ca^{2+}	70.8	5.8	23.4

Precedents are abundant in the literature which suggest that other, perhaps all, organic bases and biological amines bind to polyaminoglycans. The examples of histamine [23] and tubocurarine [24] are the most relevant. The injection of a massive concentration of cations as is the case with anhydro-araC may cause the release of several endogenous amines. The toxicity observed in the course of phase I trials [15] in humans but not in rodents may lie in the difference in the amount of mast cells between the two species. Schneyer and Galbraith [28] suggested that the toxicity is due to the release of catecholamines and can be blocked by propranolol.

It follows from our findings that the administration of anhydro-araC as its heparinate salts could have some advantage in reducing toxicity. Further studies of zwitter-ionic derivatives of anhydro-araC, as originally proposed [29, 30], seem to be warranted. Also, caution must be exercised in studies on the metabolism of anhydro-araC in blood to avoid the use of heparin and anion precipitates.

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