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The Relation between the Functions of 9-β-D-Arabinofuranosyladenine as Inactivator and Substrate of S-Adenosylhomocysteine Hydrolase[1]

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ABSTRACT

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9-β-D-Arabinofuranosyladenine (ara-A) was converted to adenine in the presence of S-adenosylhomocysteine hydrolase (EC 3.3.1.1.) and to adenine and S-[5′-(9-arabinofuranosyladenyl)]-L-homocysteine (ara-AHcy) when the incubation mixture contained L-homocysteine. The formation of adenine proceeded until 3.3 mol of adenine was formed per mol of enzyme, and at this point the enzyme was totally inactivated. In the presence of homocysteine, the rate of ara-AHcy formation was about half the rate of adenine formation. The association of the conversion of ara-A to adenine with the inactivation process was further demonstrated by the kinetics of these processes and by the observation that in the presence of homocysteine both inactivation of the enzyme and formation of adenine were reduced by 30%, *i.e.*, by a factor corresponding to the synthesis of ara-AHcy. Inactivation of the enzyme was associated with reduction of enzyme bound NAD^+. Adenine or the substance liberating adenine was tightly bound to the enzyme, whereas ara-AHcy was dissociable. These data suggest that inactivation, adenine formation and reduction of NAD^+ result from an abortive catalytic cycle, whereas enzyme entering a complete catalytic cycle leading to formation of ara-AHcy is not inactivated. The fact that the inactivation and the enzyme catalysis occur simultaneously at about equal rates offers an opportunity to demonstrate the competing relationship between these two processes.

Investigation of the biochemical and biological properties of the antibiotic ara-A (Cass, 1979) has been stimulated by the development of potent adenosine deaminase inhibitors like 2′-deoxycoformycin (Woo *et al.*, 1974) and erythro-9-(2-hydroxy-3-nonyl)-adenine (Schaeffer and Schwender, 1974), inhibiting the metabolic conversion of ara-A to the inactive metabolite ara-Hx (Brink and LePage, 1964a,b; Cohen, 1966; Cory *et al.*, 1965). These inhibitors potentiate the oncostatic effects of ara-A in intact cells (Borondy *et al.*, 1977; Cass and Au-Yeung, 1976; Plunkett and Cohen, 1975) and *in vivo* (Lee *et al.*, 1977; LePage *et al.*, 1976; Plunkett *et al.*, 1979; Schabel *et al.*, 1976).

The biological effects of ara-A have been attributed to its conversion to ara-ATP, which is an inhibitor of ribonucleotide reductase (Moore and Cohen, 1967) and DNA polymerases (Furth and Cohen, 1967, 1968; LePage, 1978) and its incorporation into nascent DNA may inhibit the replication of DNA (Tsang Lee *et al.*, 1980). A nucleotide-independent mode of action of ara-A is suggested by the findings that this nucleoside is a potent inhibitor of AdoHcy hydrolase from rat liver (Trewyn and Kerr, 1976) and irreversibly inactivates this enzyme from human lymphocytes and placenta (Hershfield, 1979; Hershfield *et al.*, 1979) and mouse liver (Helland and Ueland, 1981). The kinetics of the inactivation process suggest that ara-A is an active site-directed agent (Helland and Ueland, 1981; Hershfield, 1979; Hershfield *et al.*, 1979). ara-AMP and ara-ATP are both inhibitors and inactivators of the mouse liver enzyme, but are far less potent in this respect than their congener, ara-A (Helland and Ueland, 1981). These data obtained from studies with isolated enzymes are in agreement with the finding that ara-A elevates the level of AdoHcy in intact mouse lymphocytes (Zimmermann *et al.*, 1980). Thus, increasing evidence is accumulating in favor of the possibility that some effects of ara-A stem from blocking degradation of AdoHcy, a product from and a potent inhibitor of S-adenosylmethionine-dependent transmethylation reactions (Cantoni and Chiang, 1980) as originally proposed by Trewyn and Kerr (1976).

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ABBREVIATIONS: ara-A, 9-β-D-arabinofuranosyladenine; ara-Hx, 9-βD-arabinofuranosylhypoxanthine; ara-ATP, 9-β-D-arabinofuranosyladenine 5′-triphosphate; AdoHcy, S-adenosylhomocysteine; ara-AHcy, S-[5-(9-arabinofuranosyladenyl)]-L-homocysteine; Ado, adenosine; Hcy, DL-homocysteine; ADA, adenosine deaminase; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.

Our present knowledge of the metabolism of ara-A, its mechanism of action and its possible interference with the metabolism of AdoHcy, are summarized in figure 1.

The interaction of ara-A with AdoHcy hydrolase and the metabolic conversions of ara-A in the presence of the enzyme, is the subject of the present work. The main conclusions derived from data in this report are as follows. 1) Inactivation of the enzyme by ara-A is associated with conversion of ara-A to adenine and reduction of enzyme-bound NAD. These events result from an abortive catalytic cycle. 2) In the presence of homocysteine, synthesis of ara-AHcy from ara-A proceeds at a rate comparable to that of the inactivation process, and the enzyme entering a complete catalytic cycle is not inactivated. 3) The inactivation and the enzyme catalysis are competing processes.

Materials and Methods

Chemicals. Ado, AdoHcy, adenine, Hcy (DL-homocysteine), ADA (type I from calf intestinal mucosa), AMP, ADP and Hepes were purchased from Sigma Chemical Company, St. Louis, MO and ara-A was kindly provided by the Developmental Therapeutics Program, Chemotherapy, National Cancer Institute, Bethesda, MD. Polyethyleneimine-impregnated thin-layer sheets (0.25 mm) on glass plates (20 × 20 cm) were prepared as described by Randerath and Randerath (1967). [2,8-^{3}H]Adenine-β-D-arabinoside (22 Ci/mol) was obtained from the Radiochemical Centre, Amersham, England.

High-pressure liquid chromatography. Samples were deproteinized by heating to 100°C or by mixing with perchloric acid followed by neutralization, as described elsewhere (Ueland and Sæbø, 1979c). Precipitate was removed by centrifugation. A Spectra Physics model SP 8000B liquid chromatograph and a model SP 8300 UV detector were used. Isocratic elution (2 ml/min) was carried out at ambient temperature (23°C). The absorbance at 254 nm was recorded and in some experiments a fraction collector was coupled in series after the UV monitor. Fractions of 0.66 ml were collected.

Thin-layer chromatography. This was performed on polyethyleneimine-impregnated cellulose thin-layer sheets (0.25 mm). The chromatograms were developed in isobutanol-ethanol-water (2:1:1). Adenine (R_f = 0.61), ara-A (R_f = 0.52), ara-Hx (R_f = 0.35) and ara-AHcy (R_f = 0.15) were separated in this system.

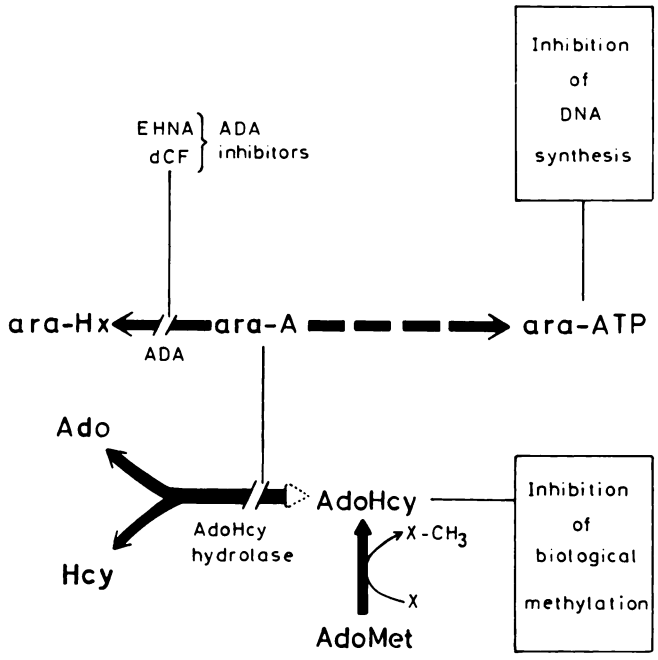


Fig. 1. Metabolism and mode of action of ara-A. EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; dCF, 2′-deoxycoformycin; AdoMet, S-adenosylmethionine.

Purification of AdoHcy hydrolase from mouse liver. The enzyme was purified to apparent homogeneity by a slight modification (Ueland, 1978) of a purification procedure published elsewhere (Ueland and Døskeland, 1977).

Preparation of [2,8-^{3}H]ara-AHcy. This compound was synthesized enzymatically from [^{3}H]ara-A as follows. [^{3}H]ara-A (200 μM) and Hcy (3 mM) were incubated for 30 min at 37°C in the presence of AdoHcy hydrolase (4 mg/ml) from mouse liver. The incubation buffer was 15 mM Hepes (pH 7.0) containing 0.2% bovine serum albumin, 150 mM KCl, 5 mM magnesium acetate and 2 mM dithiothreitol (buffer A). The reaction was stopped by heating for 5 min at 100°C and the denatured protein was removed by centrifugation. Samples of 100μl from the supernatants were subjected to high-pressure liquid chromotography on a C_{18}-μBondapak column using 8% methanol in water as solvent. The fractions corresponding to a UV absorbing peak eluting at a higher retention time than ara-A and adenine, and which cochromatographed with radioactive material, were collected, pooled, lyophilized and [^{3}H]ara-AHcy was resuspended in distilled water to a final concentration of 51 μM.

Assay for AdoHcy synthase activity. This was performed by a radiochemical method described elsewhere (Ueland and Sæbø, 1979a). The incubation mixture contained enzyme, Ado (100 μM) and Hcy (3 mM) in buffer A. The temperature was 37°C.

Assay for the conversion of ara-A to adenine and ara-AHcy. [^{3}H]ara-A was incubated at 37°C in the presence of AdoHcy hydrolase (in buffer A). The incubation mixture was supplemented with Hcy when indicated. The reaction was terminated by adding samples of 25 μl into 30 μl of 0.8 N perchloric acid. The acid was neutralized and the solution subjected to thin-layer chromatography as described previously (Ueland and Sæbø, 1979a). The radioactivity residing in the separate spots was determined by liquid scintillation counting.

Spectrophotometry. Enzyme (1.3 mg/ml) and ara-A (100 μM) in 15 mM Hepes (pH 7.0) containing 5 mM magnesium acetate, 150 mM KCl and 2 mM dithiothreitol (buffer B) were placed in one cell of a two-compartment cuvette (light path, 0.493 cm × 2). The other cell contained buffer B. The reference cuvette contained enzyme in buffer B in one cell and ara-A in buffer B in the other. The reaction was started by the addition of ara-A to the enzyme. Optical density was continuously recorded at 330 nm using a Cary model 219 recording spectrophotometer. The difference spectrum between 450 and 280 nm was recorded after the increase in absorbance had nearly come to a halt.

Determination of protein. Protein was determined by measuring the absorbance at 280 nm using a specific extinction coefficient ($E^{1\%}_{280\ nm}$) of 13.0 for the enzyme (Ueland *et al.* 1978).

Results

Formation of ara-AHcy and adenine from ara-A. [^{3}H]ara-A, incubated in the presence of AdoHcy hydrolase, was converted to a substance which was identified as adenine by reverse-phase (fig. 2) and thin-layer chromatography. When the incubation mixture was supplemented with homocysteine, an additional radioactive peak was detected in the chromatogram. The formation of this radioactive compound from [^{3}H]ara-A was dependent on the presence of ara-A, homocysteine and enzyme (fig. 2) and was not observed when the enzyme was denatured by heating before incubation (data not shown). The isolated compound was a substrate for AdoHcy hydrolase in the hydrolytic direction (see below) and cochromatographed with AdoHcy on polyethyleneimine cellulose thin-layer plates. Based on these data, it was concluded that the radioactive compound was [^{3}H]ara-AHcy. The amount of adenine and ara-AHcy formed increased as a function of the enzyme concentration (data not shown).

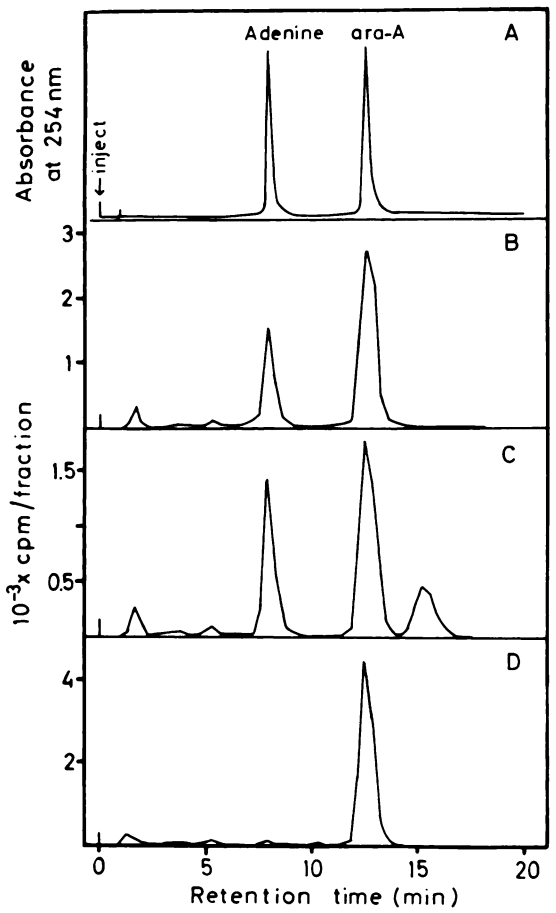


Fig. 2. Separation of ara-A and its metabolites by reverse-phase liquid chromatography. [^{3}H]ara-A (5 μM) was incubated for 30 min in the presence of AdoHcy hydrolase (113 μg/ml) (B), enzyme and 3 mM Hcy (C) or Hcy (3 mM) without enzyme (D). Deproteinized samples (50 μl) from the incubation mixture were analyzed by high-pressure liquid chromagraphy on a C_{18} μBondapak column using 10 mM acetate buffer (pH 5.5) containing 8% methanol as solvent. Fractions of the effluent were collected and radioactivity was determined by liquid scintillation counting. A shows the elution profiles of standards.

Time course for the formation of ara-AHcy and adenine. The progress curves for the formation of ara-AHcy and adenine were characterized by an initial linear phase after which a plateau was observed. Short time elapsed before the curves levelled off at high concentration of ara-A (figs. 3, A and B), consistent with rapid inactivation of the enzyme under this condition (Helland and Ueland, 1981). The conversion of ara-A to adenine paralleled the formation of ara-AHcy in that the ratio [adenine]/[ara-AHcy] was 2:1 and remained constant. The maximal amount of adenine formed was 3.3 mol/mol of enzyme (fig. 3) (MW = 185,000) (Ueland *et al.*, 1978).

Relation between adenine formation and inactivation of the enzyme by ara-A. The inactivation of AdoHcy hydrolase by ara-A showed first order kinetics for the first two half-lives.[2] In the presence of homocysteine, the rate of inactivation was reduced by one-third (fig. 4A). Kinetic treatment of the data for adenine formation showed that the conversion of ara-A to adenine closely followed the inactivation process both in the absence and presence of homocysteine (fig. 4B). Thus, in the presence of homocysteine, the rate of inactivation of the enzyme and formation of adenine was reduced by a factor corresponding to the rate of formation of ara-AHcy.

Reduction of enzyme-bound NAD$^+$. The reduction of enzyme-bound NAD$^+$ during the inactivation of the enzyme by ara-A was followed by recording the increase in the absorbance at 330 nm (Abeles *et al.*, 1980). The change in absorbance followed the inactivation of the enzyme by ara-A. The result obtained at 20°C is shown in figure 5. The same result was obtained at 37°C, except that the rate of inactivation and change in absorbance was higher. At high temperature (37°C), the recording of absorbance was obscured by formation of precipitate after prolonged incubation. The difference spectrum after 40 min of incubation at 20°C was characterized by a shoulder at 320 to 340 nm and a maximum at 300 nm (data not shown).

Separation of free and bound metabolites by gel filtration. Samples from a incubation mixture containing [^{3}H]ara-A and AdoHcy hydrolase were subjected to Sephadex G-25 chromatography after 30 min of incubation. The separate fractions were analyzed by thin-layer chromatography. The elution pro-

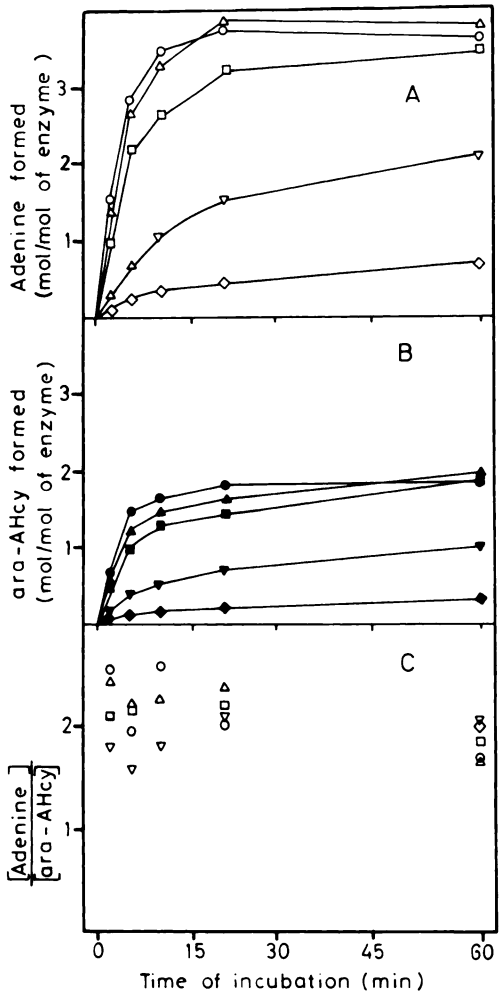


Fig. 3. Progress curves for the formation of adenine and ara-AHcy. The time course of the formation of adenine (A) and ara-AHcy (B) from ara-A in the presence of AdoHcy hydrolase (200 μg/ml) was determined at an initial concentration of 1 μM (◇, ◆), 3 μM (▽, ▼), 10 μM (□, ■), 30 μM (△, ▲) and 100 μM (○, ●) of ara-A. The incubation mixture contained 3 mM Hcy. Panel C shows the ratio obtained when dividing the concentration of adenine by the concentrations of ara-AHcy. The same symbols are used as in panel A.

[2] Deviation from linearity may be explained by consumption of ara-A.

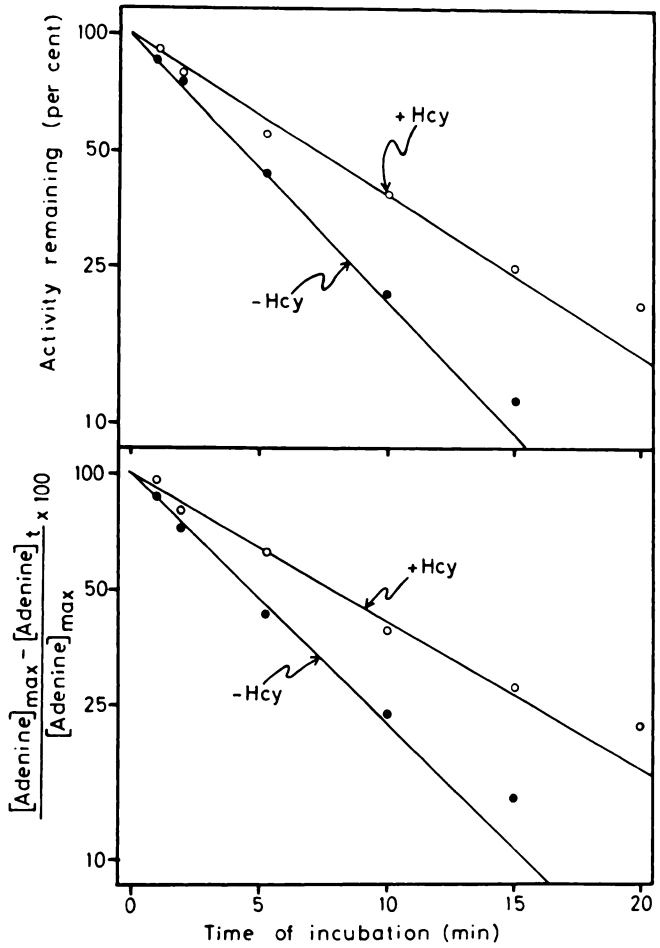


Fig. 4. Kinetics of inactivation of AdoHcy hydrolase and formation of adenine in the absence and presence of homocysteine. AdoHcy hydrolase (100 μg/ml) was preincubated with 5 μM [^{3}H]ara-A in the absence and presence of 3 mM Hcy in buffer A. After various periods of time, aliquots (10 μl) were added to perchloric acid, neutralized and the amount of adenine formed determined by thin-layer chromatography. From the same preincubation mixtures, samples (5 μl) were transferred to the incubation mixtures (300 μl) containing 100 μl of [^{14}C]Ado and 3 mM Hcy and assayed for AdoHcy synthase activity. The incubation time was 2 and 6 min. Panel A shows a semilogarithmic plot for the inactivation of the enzyme in the absence (●) and presence (○) of homocysteine. Panel B shows a semilogarithmic plot for the formation of adenine under the same conditions.

files for ara-A and adenine are shown in figure 6A. The major portion of adenine was eluted in the void volume, showing that adenine was mainly protein-bound and only a small fraction was liberated from the enzyme. Only a small portion of the radioactivity associated with the enzyme was identified as ara-A. Exactly the same results were obtained when the fractions were deproteinized with perchloric acid (as described under "Materials and Methods") or by heating (100°C for 5 min) before chromatographic analysis (data not shown).

In a similar experiment, samples of an incubation mixture containing [^{3}H]ara-A, Hcy and enzyme were subjected to gel filtration. The elution profiles for adenine, ara-A and ara-AHcy are shown in figure 6B. The main portion of adenine appeared in the protein-bound fraction, whereas ara-AHcy was recovered in the free fraction. These data show that ara-AHcy-enzyme complex is characterized by sufficiently low half-life to be nearly completely dissociated before or during chromatography.

Hydrolysis of ara-AHcy. A time-dependent decrease in the concentration of [^{3}H]ara-AHcy was observed when the compound was incubated with AdoHcy hydrolase and excess ADA. The radioactivity was mainly recovered as adenine and only a small fraction as ara-Hx (fig. 7). [^{3}H]ara-AHcy was stable for at least 60 min when incubated under the same conditions in the absence of AdoHcy hydrolase (data not shown). Thus, AdoHcy hydrolase acts upon ara-AHcy by converting this compound to adenine, whereas only small amount of ara-A (deaminated to ara-Hx in the presence of ADA) is formed.

Discussion

Inactivation of AdoHcy hydrolase by ara-A (Helland and Ueland, 1981; Hershfield, 1979) is associated with tight binding of the nucleoside to the enzyme (Helland and Ueland, 1981) and conversion of ara-A to adenine or a substance liberating adenine (fig. 1). The formation of adenine seems to be closely linked to the inactivation process (fig. 4). The amount of adenine formed per mole of enzyme inactivated equals the NAD content of the enzyme (Palmer and Abeles, 1979). These data suggest a similar mechanism of inactivation of AdoHcy hydrolase by ara-A as proposed by Abeles *et al.* (1980) for the inactivation of the enzyme by 2-deoxy-Ado. The hydroxyl group in position 3 of the furanosyl-residue of the nucleoside is oxidized by enzyme bound NAD^+. The 3′-keto-nucleoside spontaneously liberates adenine leading to a irreversible reduction of NAD^+ (fig. 5). This suggestion is in accordance with the findings of Hershfield (1980) showing that inactivation of AdoHcy hydrolase from human placenta is associated with conversion of ara-A to adenine and loss of enzyme-bound NAD^+. A similar mechanism has been proposed for the formation of adenine from Ado in the presence of the mouse liver enzyme (Ueland and Helland, 1980).

In the presence of homocysteine, AdoHcy hydrolase catalyzes the formation of ara-AHcy from ara-A (fig. 2). The rate of formation of adenine plus ara-AHcy in the presence of homocysteine equals the rate of formation of adenine in the absence of homocysteine. Furthermore, both the velocity of the inacti-

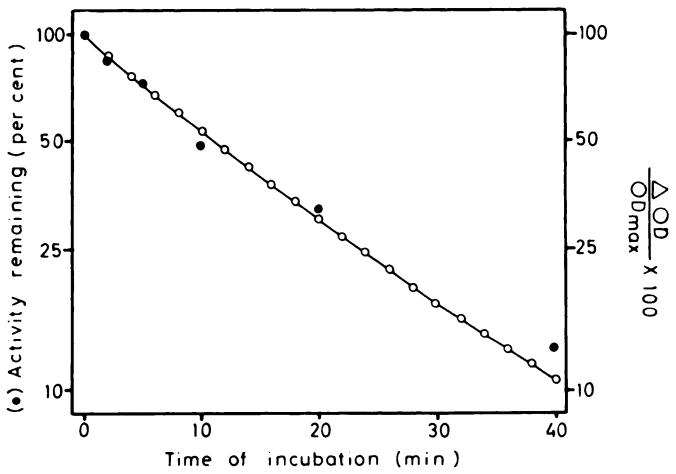


Fig. 5. Relation between inactivation of AdoHcy hydrolase by ara-A and increase in absorbance at 330 nM. Inactivation of the enzyme in the presence of 100 μM ara-A at 20°C was determined as described in legend of figure 4. In a parallel experiment, the increase in absorbance at 330 nm was recorded during inactivation of the enzyme (1,3 mg/ml) by 100 μM ara-A at the same temperature. Δ OD is the difference between the maximal absorbance (OD_{max} = 0,15 adjusted to 1-cm light-path) and the absorbance at each time point (OD_t).

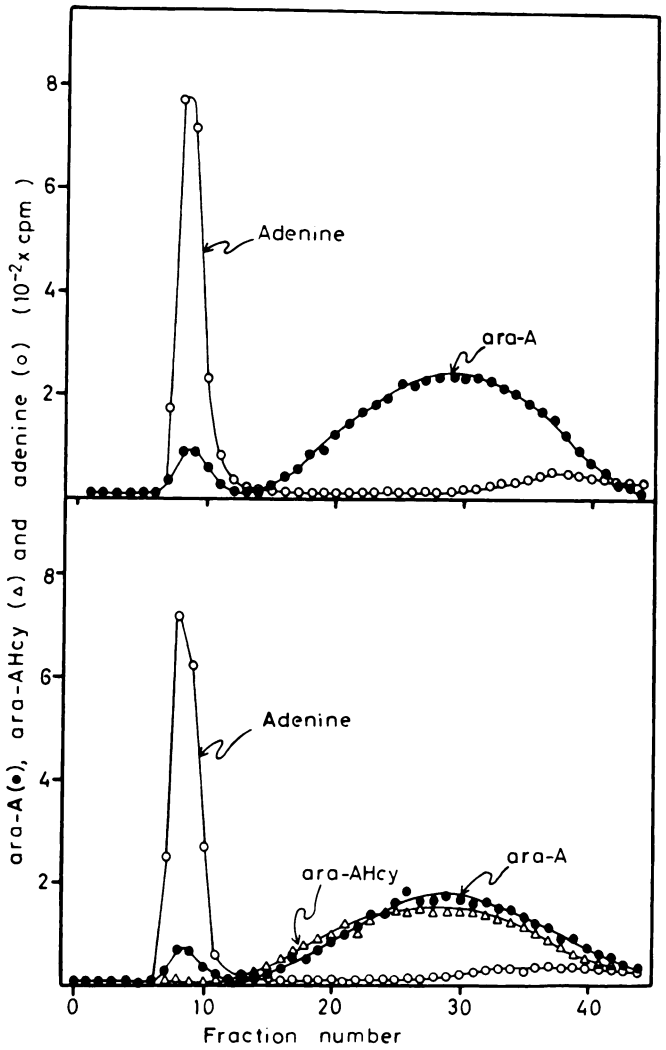


Fig. 6. Separation of free and bound metabolites by Sephadex G-25 chromatography. A, [^{3}H]ara-A (1 μM) was incubated at 37°C with AdoHcy hydrolase (30 μg/ml) in buffer A. After 30 min of incubation, a sample of 50 μl was subjected to chromatography on a Sephadex G-25 column equilibrated with 15 mM Hepes containing 10 mM 2-mercaptoethanol and eluted with the same buffer. Fractions of 144 μl were collected, deproteinized with perchloric acid, neutralized and analyzed by thin-layer chromatography. The elution profile for ara-A and adenine is shown on the figure. Panel B shows the results of a similar experiment. The incubation mixture in this experiment was supplemented with 3 mM Hcy. The elution profiles for ara-A, adenine and ara-AHcy are shown.

vation process and formation of adenine are reduced by one-third when the incubation mixture is supplemented with homocysteine (fig. 4). These observations can be explained by a reaction scheme depicted in figure 8. Formation of the complex between 3′-keto-ara-A and the enzyme (3′-keto-ara-A·E) is characterized by the limitating rate constant k_1. This complex is converted to the inactive enzyme at a rate corresponding to k_i. Alternatively, the complex adds homocysteine and is dissociated into active enzyme and the enzymatic product, ara-AHcy, at a rate corresponding to the pseudo-first-order rate constant, k_2. The relation between these parameters is: $k_1 < k_i \simeq 2k_2$. Thus, the enzyme entering a complete catalytic cycle is not inactivated.

ara-AHcy is converted to adenine in the presence of AdoHcy hydrolase, and only small amount of ara-A are formed (fig. 7). This observation suggests that ara-AHcy interacting with AdoHcy hydrolase enters an abortive catalytic cycle leading to inactivation of the enzyme.

The observation that AdoHcy hydrolase catalyzes the synthesis of ara-AHcy from ara-A and homocysteine is not in agreement with the statement that ara-A is not a substrate of AdoHcy hydrolase (Guranowski *et al.*, 1981; Zimmermann *et al.*, 1980). However, ara-AHcy is a poor substrate for AdoHcy hydrolase and is not formed in quantities exceeding one-third of the number of catalytic sites on the enzyme (see "Results"). Thus, determination of ara-AHcy formation requires concentration of enzyme which is of the same order of magnitude as the concentration of its substrate(s), whereas enzyme activities usually are measured with substrate in excess. Furthermore, the presence of Ado, AdoHcy and other adenine derivates in the intact cell may inhibit the formation of ara-AHcy. This may explain that ara-AHcy is not formed in detectable amounts in cell-free system (Guranowski *et al.*, 1981) or in intact mouse lymphoblasts (Zimmermann *et al.*, 1980).

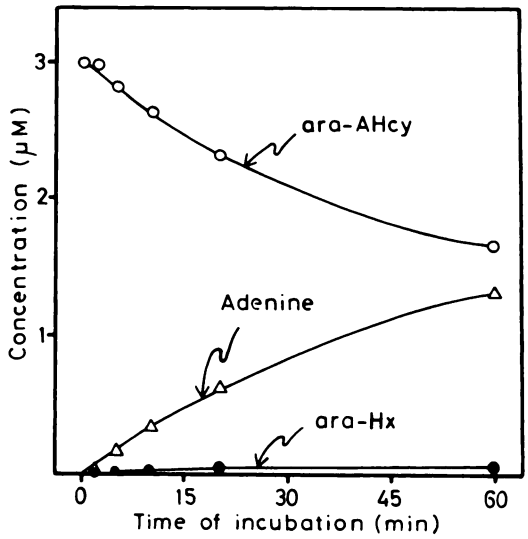
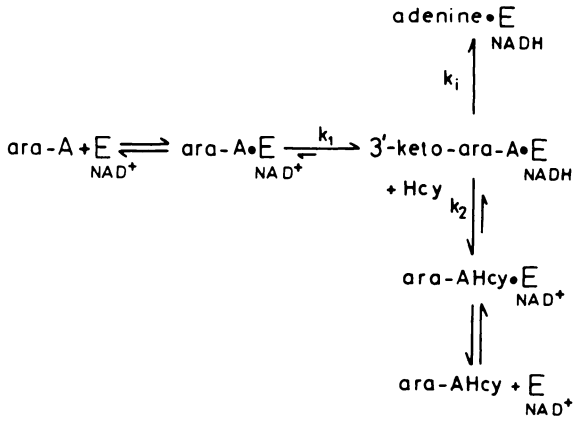


Fig. 7. Hydrolysis of [^{3}H]ara-AHcy. [^{3}H]ara-AHcy (3 μM) was incubated in the presence of AdoHcy hydrolase (2 mg/ml) and ADA (50 U/ml) in buffer A. Temperature was 37°C.



ara-A = 9-β-D-arabinofuranosyladenine
ara-AHcy = S-(5′-(9-arabinofuranosyladenyl))-L-homocysteine
Hcy = L-homocysteine
E = enzyme

Fig. 8. Hypothetical model for the relation between inactivation of AdoHcy hydrolase by ara-A and formation of adenine and ara-AHcy. (Only the steps assumed to be rate limiting are included in the figure.)

Although the cellular level of AdoHcy hydrolase is high in mammalian liver and some other tissues (Kajander and Raina, 1981; Ueland and Sæbø, 1979b), only limited quantities of ara-AHcy are expected to be formed in intact cells. It therefore seems unlikely that ara-A exerts some of its biological effects through ara-AHcy-mediated inhibition of cellular methylation. Furthermore, ara-AHcy is a weak inhibitor of transmethylases tested so far (Borchardt and Wu, 1975). However, the observation that homocysteine modulates the effect of ara-A on AdoHcy hydrolase should stimulate studies of the possible influence of homocysteine on the cellular response to ara-A.

In conclusion, analogs of Ado may function as inactivators, inhibitors or substrates of AdoHcy hydrolase (Cantoni *et al.*, 1979; Chiang *et al.*, 1981; Hershfield *et al.*, 1979; Zimmermann *et al.*, 1980). In the presence of ara-A, the inactivation and the enzyme catalysis occur simultaneously at about equal rates. This offers an opportunity to demonstrate the competing relationship between these two processes. Studies with intact cells are required to determine the possible implications of these findings for the effects of ara-A *in vivo*.

Acknowledgments

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