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A Comparative Study Of The Metabolism Of 1-Beta-D-Arabinofuranosyl Cytosine In Various Animal Species.

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A COMPARATIVE STUDY OF THE METABOLISM OF 1-β-D-ARABINOFURANOSYL CYTOSINE IN VARIOUS ANIMAL SPECIES

by

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A Dissertation

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to tess

in innocence

in pain

in growth

in fullness

of life

of love

of freedom

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LIST OF ABBREVIATIONS

Ara-C	1-β-D-arabinofuranosylcytosine; arabinosyl cytosine
Ara-U	1-β-D-arabinofuranosyluracil; arabinosyl uracil
Ara-T	1-β-D-arabinofuranosylthymine; arabinosyl thymine
H_4U	tetrahydrouridine
Ara-CMP	1-(5-phosphate-β-D-arabinofuranosyl)cytosine
dCR	deoxycytidine
ATP	adenosine triphosphate
GTP	guanosine triphosphate
UTP	uridine triphosphate
TTP	thymidine triphosphate
CTP	cytidine triphosphate
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
Ara-CTP	1-(2,3,5-phosphate-β-D-arabinofuranosyl)cytosine
dCTP	deoxycytidine triphosphate
CDP	deoxycytidine diphosphate
dATP	deoxyadenosine triphosphate
dTTP	deoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
CPM	counts per minute
Tris	tris-(hydroxymethyl)-aminomethane

LIST OF ABBREVIATIONS
(continued)

TLC	thin-layer chromatography
q	every
i.v.	intravenous
i.p.	intraperitoneal
$t^{\frac{1}{2}}$	biological half-life
K_m	enzyme-substrate dissociation constant
V_D	volume of distribution
R_x	treatment
RBC	red blood cell; erythrocyte
WBC	white blood cell; leukocyte
V	maximum velocity
S	substrate concentration
CML	chronic myelocytic leukemia
AML	acute myelocytic leukemia
ALL	acute lymphatic leukemia
CGL	chronic granulocytic leukemia
CLL	chronic lymphatic leukemia

I. INTRODUCTION

The first reported arabinose-containing nucleoside was isolated by Bergman and Feeney (1950) from a Caribbean sponge Cryptolethya crypta. The compound was extracted by alcohol and acetone and, to date, has been found only in this single species of sponge. It was given the trivial name of spongothymidine and later shown chemically to be 1-β-D-arabinofuranosylthymine (ara-T). A second compound later extracted from this same source was identified as 1-β-D-arabinofuranosyluracil (ara-U) (Bergman and Feeney, 1955).

The isolation and identification of the above naturally occurring compounds led to the chemical synthesis of a whole new series of arabinosyl nucleosides. Many of these latter nucleosides have never been found to occur naturally and remain the products of chemical synthesis.

One of these derivatives, 1-β-D-arabinofuranosylcytosine (ara-C), was found to be a promising cancer chemotherapeutic agent. The first published synthesis of the compound was effected from cytidine or its mixed 2'-3'-phosphates by Walwick and co-workers (1959). The conversion of uridine-type nucleosides to cytidine compounds via thiation and amination was later described by Fox and his collaborators (1959) and utilized by Hunter (1963) to

synthesize ara-C.

Evans and his associates (1961) were first to report the antitumor activity of ara-C. These authors found the compound to be active against recently transplanted and established Sarcoma 180, Ehrlich carcinoma, and L1210 leukemia in mice. Preliminary studies by Talley and Vaitkevicius (1963) confirmed this activity in humans with malignant diseases. The latter finding prompted a major undertaking of the preparation of ara-C by The Upjohn Company and the U. S. Cancer Chemotherapy National Service Center.

In less than a decade since the first reported antitumor activity of ara-C, the compound has been extensively investigated in the tumor and virus fields, including studies with cell cultures, bacteria, tissues, and whole animals — including man with neoplastic disease. Although much has been learned concerning the biochemistry, toxicology, pharmacology, and therapeutics of ara-C, its full potential as an anticancer agent is yet to be explored. A search remains for dose scheduling, drug form, and route of administration parameters which yield maximum antitumor effect while minimizing toxicity.

II. REVIEW OF THE LITERATURE

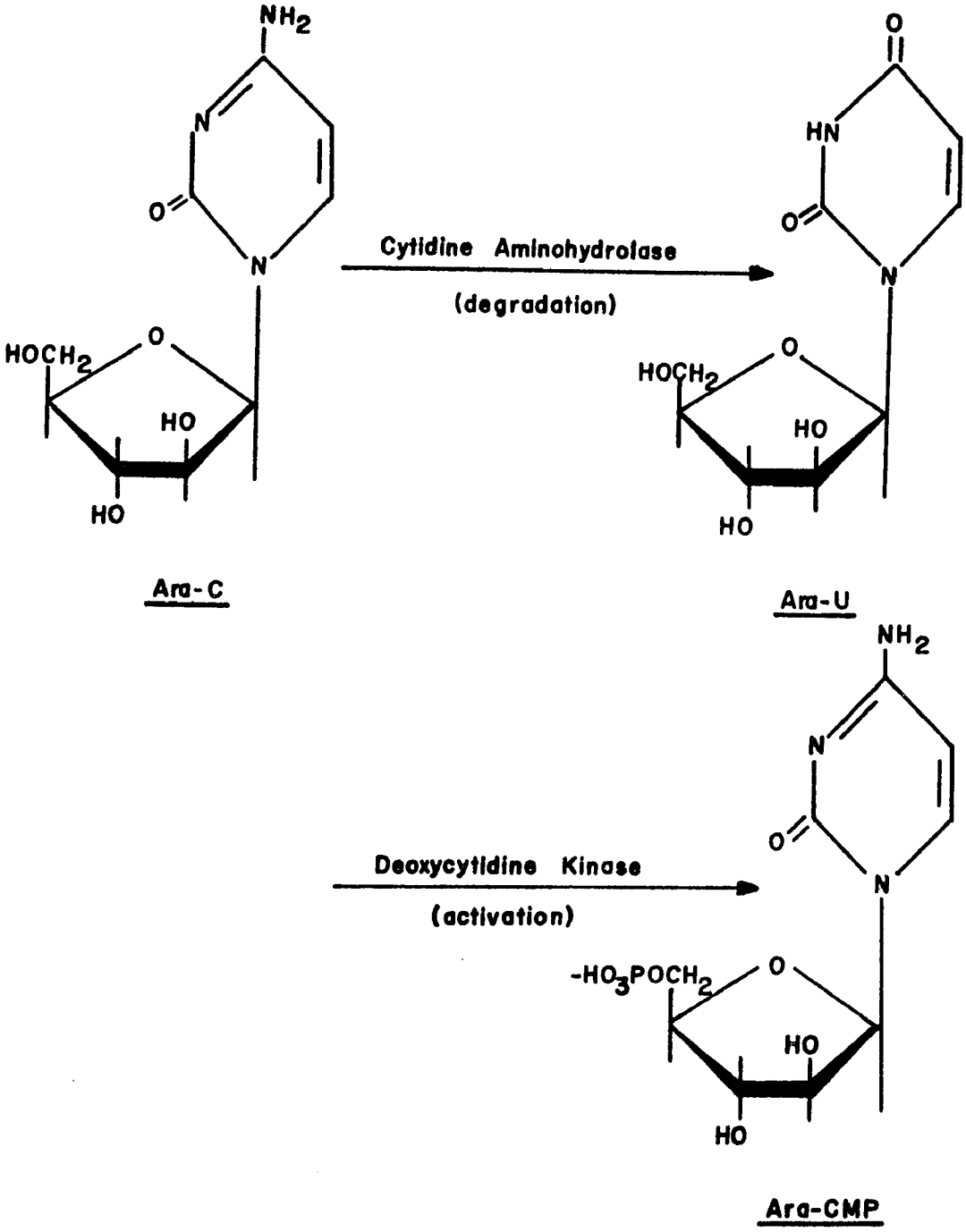
In the last eight years, over two hundred papers concerned with ara-C have appeared in the scientific literature. The work has ranged from methods of synthesis to various clinical approaches to its usage. Between this work of the organic chemist and clinical investigator is that of the biochemist, pharmacologist, toxicologist, virologist, microbiologist, and the numerous investigators of related disciplines.

Due to the large quantity of these reports, the author has limited this review section. Only those reports which deal with metabolic pathways, proposed mechanisms of action, and pharmacological disposition of the compound will be included. These areas were chosen for review since their content has influenced the direction of this investigation. Details of the fate and distribution studies will, for the most part, be withheld until the DISCUSSION section.

Although ara-C is a potent antitumor and antiviral agent at nonlethal levels to the host cells, its activity is limited by its metabolic pathways. Two enzymes directly responsible for the anabolism and catabolism of ara-C are deoxycytidine kinase and cytidine aminohydrolase, respectively (see Figure 1). The following sections constitute a review of studies involving these two enzymes and their

Figure I

Catabolic and Anabolic Pathways Involved in Metabolism of Arabinosyl Cytosine



effect on the cytotoxicity and fate of ara-C in biological systems.

METABOLIC DEGRADATION

Although ara-C has no cytotoxic effect without activation, of major importance is that it not be catabolized before reaching the activation site. The major pathway for the degradation of the compound involves a deamination catalyzed by an enzyme now referred to as cytidine aminohydrolase. Since ara-U has been shown to have none of the cytotoxicity of ara-C (Camiener and Smith, 1965), the extent of deamination is important to the overall effect of the parent compound.

Pizer and Cohen, in 1960, working with pyrimidine-requiring mutants of E. coli (uracil auxotrophs), showed that their uracil requirements could be replaced by either spongouridine (ara-U) or spongocytidine (ara-C). The authors demonstrated that ara-C was deaminated to Ara-U by these organisms. This work was later verified by Slechta (1961), who found that ara-U was further split by E. coli to uracil.

In studies involving human cancer patients (Smith et al., 1959), intravenously administered ara-C, as measured by a biological assay, disappeared very rapidly from the blood. Since the rate of disappearance could not be accounted for by urinary excretion, it was postulated

that the drug was metabolized to some inactive product. Camiener and Smith (1965) later identified this metabolite as ara-U. These authors also completed an extensive study of the tissue distribution of aminohydrolase activity and reported marked quantitative differences among four tissues and twelve animal species. They found the cytidine aminohydrolase enzyme, prepared from human liver, to be soluble, stable, and active within a wide pH range. The enzymatic reaction required no co-factors. Camiener (1967a) later demonstrated that p-chloromercuribenzoate destroyed the enzymatic activity, indicating that reduced sulfhydryl groups were required for activity. The reported K_m value was 1.2×10^{-4}M. Aminohydrolase activity has recently been found in human spleen, liver, kidney, and bone marrow—in that order of decreasing specific activity (Steeper and Steuart, 1970; Hall and Levine, 1967).

Camiener (1967b) determined the various configuration requirements of ara-C as a substrate for this enzyme and reported the following criteria for activity: a 2-keto group, an unsubstituted N-3 ring nitrogen; a C-6 ring carbon; a pentofuranose at pyrimidine position 1; a free 3'-α-hydroxyl group; and a nonphosphorylated 5'-hydroxyl group.

Recently, Tomchick (1968) purified sixtyfold an aminohydrolase enzyme from BDF_1 mouse kidney which was most active with cytidine as substrate and moderately

active with ara-C and deoxycytidine (dCR). As compared to cytidine, the relative deamination rates for dCR and ara-C were 55 and 29 percent, respectively. Values in human liver for the same relative rates were 82 and 74 percent, respectively (Camiener and Smith, 1965). Cytosine nucleotides did not act as a substrate for either enzyme preparation.

In 1963, Creasey purified 107-fold an aminohydrolase enzyme from Swiss mouse kidney. The enzyme preparation had essentially the same substrate requirements as the purified enzymes described above except for a much lower activity toward ara-C. In contradiction to this work, Camiener and Smith (1965) reported an aminohydrolase preparation from the kidney of an Upjohn line of Swiss mice that had a very high activity toward ara-C. These results suggest that kidney aminohydrolase may be different in different strains of mice.

Wisdom and Orsi (1967) purified 200-fold an enzyme from sheep liver with substrate requirements similar to the mouse kidney preparation reported by Creasey.

METABOLIC ACTIVATION

Chu and Fischer, in 1965, reported that the active form of ara-C in L5178Y cells was likely not the nucleoside, but rather a phosphorylated derivative. An L5178Y line resistant to ara-C was derived by the incubation of L5178Y cells (parent strain) with a level of ara-C

sufficient to completely inhibit the reproduction of the sensitive cells. The authors found that suspensions of the drug-resistant cells had an impaired capacity to form phosphorylated derivatives of ara-C. The same inability to phosphorylate the drug was found in a P815/ara-C neoplasm (Uchida and Kreis, 1969), an L1210/ara-C cell line (Schrecker and Urshel, 1967), and rabbit kidney cells infected with herpes virus (Ben-Porat et al., 1967). Rabbit kidney cells infected with herpes virus were more resistant to ara-C than noninfected cells. Though one would expect the rat to be susceptible to ara-C toxicity because of low cytidine aminohydrolase activity, experimental results show the rat very resistant to the compound. This resistance has been attributed to low or absent kinase activity (Camiener and Smith, 1965).

The effect of ara-C on the survival time of L1210 leukemic mice was compared with the active phosphorylated metabolite, 1-β-D-arabinofuranosylcytosine 5'-monophosphate (ara-CMP), by Schrecker and Goldin (1968). The compounds were administered subcutaneously in equimolar daily doses. The nucleotide was similar to the nucleoside in effect when compared in this fashion. The results were not surprising since, as a rule, intact nucleotides are unable to pass through cell membranes, but must first be dephosphorylated at the membrane surface. Ara-CMP would therefore be dephosphorylated to ara-C at the cell membrane

and, if completely dephosphorylated, would be expected to give the same response as ara-C.

Kessel and co-workers (1967) demonstrated that the capacity for nucleotide formation of ara-C by mice carrying various transplantable leukemias correlated with their response to the drug. These authors found that in human cell types the rate of ara-C phosphorylation was greatest in lymphocytes and lower in granulocytes and red cells. Patients with cells showing highest phosphorylation rates were usually markedly drug-sensitive.

Durham and Ives (1969) found dCR kinase primarily in tissues of lymphoid origin and suggested that the relatively high levels of this enzyme in lymphoid neoplasms may account for the clinical effectiveness of ara-C in treatment of acute lymphatic leukemia. Ho (1970) recently correlated antitumor effect of ara-C in mouse tumors with aminohydrolase/kinase ratios.

Momparler and Fischer (1968) purified a kinase enzyme one-hundred-fold from extracts of calf thymus which utilized ara-C and dCR equally as substrate. Cytidine was not a substrate for this enzyme preparation. The enzyme is therefore referred to as deoxycytidine kinase. The K_m value for ara-C was 4.0×10^{-5}M, while that for dCR was 1.4×10^{-5}M. Deoxycytidine mono-, di-, and tri-phosphates were all active inhibitors of the enzyme. Deoxycytidine was a potent competitive inhibitor of ara-C ($K_i = 1.3 \times 10^{-9}$M).

Ara-C was less effective as an inhibitor of dCR (K_i = 3.6 x10^{-5}M). The reaction is dependent on the presence of Mg^{++}, a divalent cation. ATP, GTP, UTP, and TTP all act as phosphate donors. Durham and Ives (1969) and Kessel (1968), who purified a kinase enzyme from L1210 mouse leukemia cells, found similar substrate requirements for their enzyme preparations.

This difference in K_m and K_i for ara-C and dCR apparently explains part of the reversal, by dCR, of ara-C toxicity against vaccinia virus in cell cultures (Renis and Johnson, 1962), murine lymphoblast L5178Y cells (Chu and Fischer, 1962), and three tumor systems in mice (Evans and Mengel, 1964).

PROPOSED MECHANISMS OF ACTION

Ara-C inhibits DNA synthesis in numerous animal cells in tissue culture (Chu and Fischer, 1962; Kim and Eidinoff, 1964) and *E. coli* (Pizer and Cohen, 1960; Slechta, 1961). The drug is widely used at present to produce this effect. The compound does not initially inhibit RNA and protein synthesis in animal cells, which causes considerable enlargement of the cells without division (unbalanced growth) (Cohen, 1966). After prolonged exposure to this agent at inhibitory concentrations, the cells irreversibly lose the power to multiply. Inhibited cells are also observed to suffer chromosome breaks and other abnormalities (Brewen

and Christie, 1967; Heneen and Nichols, 1967).

Highly radioactive ara-C has been reported to be incorporated at low levels into RNA and DNA in L5178Y cells by Chu and Fischer (1965) and in L cells by Silagi (1965). Although no incorporation of ara-CTP into RNA or DNA has been detected with the DNA polymerase of animal cells (Furth and Cohen, 1968) or E. coli (Cardeilhac and Cohen, 1964), it is not clear whether incorporation of the compound does or does not occur.

The effect of ara-C on E. coli is unique, since DNA synthesis in this organism is only inhibited at a concentration one thousand times that needed in animal cells (Lark and Lark, 1964). Ara-CTP does not inhibit the DNA polymerase of this microorganism (Furth and Cohen, 1968). It is conceivable that the mechanism of this inhibition is not comparable to that seen in animal cells.

The major mechanism of action of ara-C was first thought by Chu and Fischer (1962) to be the inhibition of the nucleotide reductase responsible for the formation of dCR derivatives. These authors (1968a) found that in L5178Y cells ara-C inhibited the incorporation of deoxythymidine into DNA due to a deficiency of dCTP. This deficiency was produced by ara-C inhibiiton of CDP reductase. A rescue from ara-C death produced by dCR was attributed to the ability of inhibited cells to form dCTP.

The formation of dCTP was a result of appropriate kinase activity from exogenous dCR (salvage pathway). Moore and Cohen (1967), however, demonstrated that nucleotides of ara-C weakly inhibited a reduction system partially purified from Novikoff ascites tumor cells grown in rats. These authors did not believe this to be the major site of action in tumor cells. Their position was further strengthened by the finding that deoxythymidine inhibits dCR reductase (Whittle, 1966) while exhibiting neither the antileukemic activity of ara-C nor sparing its cytotoxic actions (Grindey et al., 1968).

Ara-C does not inhibit ribonucleotide reduction in rabbit kidney cells but inhibition does occur when a small amount of dCR is added. This effect is thought to be due to an increase in the intracellular pool of dCTP and an ensuing negative feedback inhibition. Ara-C stimulates incorporation of dCR into DNA by stimulating dCR kinase and thereby indirectly increases dCTP (Kaplan et al., 1968). The stimulatory effect of dCR kinase by ara-C was also pointed out by Chu and Fischer (1968b).

Furth and Cohen (1968) found that in both mammalian and bacteria cells ara-C inhibited DNA polymerase but not RNA polymerase. These authors suggested that in these cells ara-CTP is a competitive inhibitor of dCTP in DNA synthesis by mammalian DNA polymerase. Cross resistance

of L1210/ara-C to thymidine also suggests that DNA polymerase might be involved (Bach, 1969).

The proposed mechanisms of action of ara-C are summarized in Figure 2.

IN VIVO METABOLISM

Little has been reported on the *in vivo* metabolism of ara-C by various animal species. This sparcity of work might be attributed to: (1) the limited supply of ara-C up to the last five years; (2) the lack of a sensitive and specific method of assay for the parent compound and its metabolites; and (3) the difficulty involved in the separation of ara-C from its metabolic products.

The work reported to date — pertinent details will be presented in the DISCUSSION — has been limited in number of species of animals included, routes of administration, and doses of ara-C involved. No correlation studies between serum levels, urinary excretion, and tissue distribution of enzyme activities responsible for ara-C metabolism have been reported, nor are there any direct comparisons of the metabolism of the drug by various animal species and man.

The work that has been done includes several studies in mice (Borsa et al., 1969; Dixon and Adamson, 1965), several in humans (Camiener and Smith, 1965; Creasey et al., 1966; Talley et al., 1967) and one in dogs (Dixon and

Figure 2

Proposed Mechanisms of Action of Arabinosyl Cytosine

I. Inhibition of the Biosynthesis of Deoxycytidine Nucleotides

$$CDP \xrightarrow{\text{Ribonucleotide Reductase}} dCDP$$

II. Inhibition of the Biosynthesis of Cytidine Nucleotides

$$dCR \xrightarrow{\text{Deoxycytidine Kinase}} dCMP$$

III. Inhibition of DNA Synthesis

$$\left.\begin{matrix} dATP \\ dTTP \\ dGTP \\ dCTP \end{matrix}\right\} \xrightarrow{\text{DNA Polymerase}} DNA$$

Adamson, 1965). No *in vivo* metabolism studies have been reported in monkeys, rats, or hamsters.

From these scattered studies it is clear that the metabolic pathways of ara-C catabolism, although not qualitatively different from one species to another are quantitatively different. Therefore, in order to select the appropriate animal model for man, it is essential that the metabolic pathways of ara-C in various animals be clearly defined.

III. PLAN AND DEVELOPMENT OF THE INVESTIGATION

Since no complete and systematic comparative metabolism study of ara-C in various animal species has been reported to date, work was initiated to explore the metabolism, fate, and distribution of this compound in the mouse, rat, hamster, dog, and monkey. Serum levels and urinary excretion patterns were determined in the various animal species for ara-C and its metabolites after parenteral and oral administration of tritium-labeled ara-C.

As stated earlier, the effectiveness of ara-C as a cytotoxic agent depends on which pathway is involved in the metabolism of the compound. It may be degraded to ara-U and has no chance for further activation, or it may be metabolized to a phosphorylated derivative of ara-C — the active form of the compound. For this reason, it is important to obtain, in conjunction with the in vivo studies, information concerning the levels and distribution of the enzymatic activities involved in each of these pathways in the various animal species. Therefore tissues (liver, kidney, spleen, small intestine, thymus, and serum) from the various species were assayed for cytidine aminohydrolase and deoxycytidine kinase activity.

It was hoped that the in vivo together with the in

in vitro results would form a more complete study of the metabolism of ara-C in these animals and, therefore, give a more firm basis for possible explanations for the variation in response to ara-C.

Sera of normal and cancer patients were also assayed for cytidine aminohydrolase activity to establish normal ranges for this enzyme in each group.

In order to accomplish the above studies, a thin-layer chromatography procedure for the separation and determination of ara-C and each of its metabolic products was developed. Although the details of the development of the assay will not be reported in this work, the final form of the procedure is described under METHODS.

Studies incorporating the use of tetrahydrouridine (H_4U), a potent *in vitro* inhibitor of the deamination of ara-C (Camiener, 1967), were an important aspect of this work.

A study was designed to determine the effectiveness of the compound as an inhibitor of the *in vivo* metabolism of ara-C. The monkey was chosen as the test species for reasons which will be apparent later. Serum levels and urinary excretion patterns were determined for ara-C and its metabolites in the monkey after intravenous administration of ara-C and H_4U, singly and together.

Toxicity, as determined by gross observation and

changes in biochemical and hematological parameters, was followed up to ten days after each treatment.

In summary, the objectives of this work were as follows:

(1) A comparative study of the in vivo metabolism of ara-C in various animal species;

(2) A study of the distribution of cytidine aminohydrolase and deoxycytidine kinase activities in various animal tissues;

(3) A study of the in vivo effectiveness of H_4U as a cytidine aminohydrolase inhibitor; and

(4) The development of necessary methodology.

IV. MATERIALS

CHEMICALS

Ara-C, as the hydrochloride salt, was supplied by the Cancer Chemotherapy National Service Center through the courtesy of Dr. Robert Engle. Ara-U was purchased from Calbiochem, Los Angeles, California. ^{3}H-labeled ara-C (1.4 c/millimole) was obtained from Schwarz Bio-Research, Inc., Orangeburg, New York. The radiopurity of the compound was stated as 99 percent by the manufacturer and was confirmed in our laboratory by the thin-layer and paper chromatography systems described below. The compound is tritium labeled on the 6-carbon of the pyrimidine ring. ^{3}H-labeled ara-U (6.65 c/millimole) was obtained from New England Nuclear Corporation, Boston, Massachusetts. To provide a weighable stock, 1.0 ml of the labeled ara-C (in ethanol) containing 1.0 mc of radioactivity was mixed with 25 mg of unlabeled drug in 2.0 ml of H_2O. The resulting solution was dried *in vacuo* to yield a fine white powder. The distillation process also removes any volatile radioactivity. The ^{3}H-labeled ara-U was treated identically to ara-C. The labeled stock could be further diluted with unlabeled drug for administration. Tetrahydrouridine (H_4U) was supplied by The Upjohn Company,

Kalamazoo, Michigan. The Bio-Solv Solubilizers, BBS-2 and BBS-3, were purchased from Beckman Instruments, Inc., Fullerton, California.

V. METHODS

IN VIVO STUDIES

The animals utilized in the *in vivo* studies were BDF_1 mice, Sprague-Dawley rats, Golden Syrian hamsters, beagle dogs, and rhesus monkeys (*Macaca mulatta*). All animals were fasted twelve hours before drug administration, whereas water was permitted *ad libitum*.

Ara-C injection solutions were prepared by combining an appropriate amount of tritiated ara-C (prepared as under CHEMICALS) with unlabeled drug dissolved in saline. The solutions were administered either parenterally (intravenously in monkeys and dogs, intraperitoneally in other animals) or orally. Oral dosing of the monkeys was accomplished by use of a catheter (Davol, Robinson One-Eye; Fr 16) connected to a 10-milliliter syringe. Dogs were orally dosed with a stomach tube (Davol; Fr 28), while small animals were dosed by means of a small length of polyethylene tubing connected to a one-milliliter tuberculin syringe.

Urine was collected either by an indwelling catheter for short time intervals or by the use of metabolism cages for the longer time periods. Mouse and hamster urine was collected by catheter only.

Blood data for mice, hamsters, and rats were obtained from pooled blood from six animals.

Control blood and urine were collected prior to drug administration. As blood samples were collected, they were immediately chilled in an ice bath, allowed to clot, centrifuged, and serum was removed. All samples were kept cold or frozen until analyzed.

All excess injection solutions were reserved and assayed by radioisotopic and/or microbiological procedures to verify their concentrations.

Preparation of collection tubes. To vacutainer tubes (7-ml) was added 0.1 ml of a 1-mg/ml solution of tetrahydrouridine. The tubes were heated to dryness in an oven at 110°C for one hour. (Assuming that 5 ml of blood would be added to each tube, the final concentration of H_4U would be approximately $1x10^{-4}$M.)

The effectiveness of the prepared tubes to inhibit the aminohydrolase enzyme was determined by placing 3 milliliters of fresh monkey blood in one of the tubes, incubating with ara-C, and determining the amount of ara-U formed.

It was necessary to use H_4U collection tubes only with the monkey and human in vivo work since no significant cytidine aminohydrolase activity was found in the serum of any of the other animal species.

Total Radioactivity in Serum and Urine

Total radioactivity in serum was determined by adding 0.2 ml of the sample to a 20-ml scintillation counting vial.

Fifteen ml of a scintillation counting solution (14 ml of a standard toluene phosphor solution plus 1 ml of Beckman Bio-Solv Solubilizer BBS-3) was then added to each vial. The standard toluene phosphor solution contained 3.0 grams of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis-2-(5-phenyl-oxazolyl (POPOP) diluted to one liter with toluene. After addition of the scintillation solution, the vials were capped, shaken, and their radioactivity content determined in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3315).

Total radioactivity in urine samples was analyzed in the same manner as serum, with the exception of substituting Beckman Bio-Solv Solubilizer BBS-2 for BBS-3.

Microbiological Assay for Ara-C in Serum

The microbiological assay for serum levels of ara-C utilizes an actinobolin-resistant strain of Streptococcus faecalis and has been described in detail by Pittillo and Hunt (1967). Ara-C can be estimated in the presence of ara-U by this method since the metabolite exhibits none of the cytotoxicity of the parent compound in this system.

Paper Chromatography Assay for Ara-C and Ara-U

The paper chromatography system for the separation of ara-C from ara-U in urine utilized Whatman #1 paper developed for 18 hours in a mixture of butanol saturated water (84:16 v/v).

The urine samples (up to 100 µl) were spotted on paper premarked into channels 4.5 cm wide and developed as descending chromatography with the above solvent system. After development, the individual channels were marked crosswise every centimeter and cut from the paper sheets. The marked strips cut from the channel were then cut into two equal parts and placed into 20-ml scintillation counting vials. One channel usually contains 50 to 55 strips.

To each vial was added one milliliter of water and 10 ml of a special aqueous phosphor solution (80 g napthalene, 8 g PPO, 200 mg POPOP, 110 ml toluene, 110 ml ethylene glycol monoethyl ether, diluted to one liter with p-dioxane). After capping, the vials were shaken to insure complete mixing of the solution. Radioactivity content was determined as described for total radioactivity assays.

The data, punched on tapes, were processed through an IBM #1130 computer to obtain concentration data, R_f values, percent of radioactive components, and a plot of strip number versus percent of total radioactivity per strip.

Serum samples were not assayed by the above system.

IN VITRO STUDIES

Thin-Layer Chromatography of Ara-C, Ara-U, and Associated Nucleotides

Gelman's ITLC-Type SA glass microfiber sheets impregnated with silica gel adsorbents were utilized in the TLC method. The samples (up to 100 µl) were spotted on the media, premarked into channels 2.25 cm wide, and developed in a methanol:chloroform:1.0 M phosphate buffer (pH 7.5) solvent system (30:70:4). The ITLC-Type SA media requires 45 minutes for development.

Serum samples could not be chromatographed without prior protein precipitation; therefore, 0.2-ml aliquots were added to a 12-ml centrifuge tube containing 2 ml of a tungstic acid deproteinization reagent (TDR) (Caraway, 1958). The reagent contains: 320 ml H_2O, 20 ml 10-percent $Na_2WO_4 \cdot 2H_2O$, 0.02 ml phosphoric acid, and 20 ml 0.67N H_2SO_4.

The TDR-serum mixture was placed on a Vortex Jr. Mixer for rapid equilibration, allowed to sit at room temperature for ten minutes, and then centrifuged. The supernatant was removed by suction and frozen until assayed.

With this chromatography system, if TDR is used for deproteinization, it is necessary that the final pH of the mixture be 3.5 to 4.0. If the pH is lower than 3.5, the chromatography characteristics of the system are changed. If the pH is higher than 4.0, incomplete deproteinization

will result and protein will accumulate at the origin.

To obtain reproducible R_f values, the TLC sheets must be preheated at 110°C for 15 minutes and brought to room temperature in a desiccator. The spotted chromatography sheet must also be pre-equilibrated with the solvent system by suspending the sheet in the chromatography chamber for 30 minutes before development. Unlabeled solutions of ara-C and ara-U as well as solutions of the ^{3}H-labeled compounds, treated in the same fashion as the samples, were utilized as reference compounds for chromatographic controls.

After development, the sheets are dried at room temperature. For qualitative work the spots are visualized under UV light; for quantitative work the individual channels are marked crosswise every 0.6 cm and cut from the sheet. The marked strips are then cut with a razor blade and placed into 20-ml scintillation counting vials without further cutting. One channel usually contains from 24 to 30 strips.

The radioactivity on each strip is then assayed as in the paper chromatography work described earlier.

For qualitative identification of sample components, Mallinckrodt ChromAR sheets may be substituted for the Gelman Chromatography Media. The sheets, containing a silicic acid sorbent, have the same solvent characteristics

as the Gelman sheets, with the added advantage of an inorganic phosphor for UV visualization of low concentration samples. These sheets require only 20 minutes for development with the above solvent system.

Cytidine Aminohydrolase Activity in Serum

Blood was collected either by venipuncture, as in the case of dogs, monkeys, and humans, or by heart puncture, as in the case of the smaller animals. The blood was allowed to clot, centrifuged, and the serum removed and immediately frozen.

For incubation, one milliliter of serum was transferred to a 20-ml Erlenmeyer flask and preincubated in a Dubnoff metabolic shaker at 37°C for 20 minutes. After preincubation, 0.1 ml of a 1.0-mg/ml solution of the ^{3}H-ara-C substrate (final concentration: 3.3×10^{-4}M, ca. 1×10^{7} cpm/ml) was added to each flask.

In incubations in which an inhibitor was used, 0.1 ml of a 1-mg/ml solution (final concentration: 1×10^{-4}M) was added 30 minutes prior to the addition of ara-C substrate.

At timed intervals, 0.2-ml aliquots of the incubation solution were removed and added to a 12-ml centrifuge tube containing 2 ml of TDR. The solution was quickly placed on a Vortex Jr. Mixer for mixing and equilibration. This procedure precipitates the protein and effectively stops

the enzymatic reaction. The mixture was allowed to sit at room temperature for 10 minutes and then centrifuged. The supernatant fluid was quickly frozen and later assayed for ara-C and ara-U content by the thin-layer chromatography system previously described. Robinson's medium (Robinson, 1949) was substituted for serum in control incubation studies.

Serum was obtained from monkeys and reserved as a stock enzyme source. This serum served as a standard, positive cytidine aminohydrolase control for all *in vitro* studies.

In the experiments in which monkey red blood cells were incubated with ara-C, the cells were separated from whole blood by centrifugation, washed twice with Robinson's medium, and resuspended in the medium for incubation. The cell suspension was handled the same as serum.

Aminohydrolase and Kinase Activity in Tissue Homogenates

Tissues were removed from each animal and immediately frozen. A weighed quantity (ca. 1 gm) was homogenized with 0.154 M KCl (1:5 dilution) in a Sorvall Omni-Mixer. Homogenation was performed on ice for three 30-second intervals with a 2-minute pause between each interval. The crude homogenate was then centrifuged at 14,000 x G for 30 minutes in an International refrigerated centrifuge equipped with a high-speed head. After centrifugation

the supernate was removed and frozen.

The reagents for the basic incubation mixture were prepared as follows:

(1) Tris-HCl Buffer (0.8 M)

9.7 g Tris diluted to 100 ml with distilled H_2O; pH was adjusted to 8.0 with 1N HCl

(2) $MgCl_2$-NaF Solution

40.6 mg $MgCl_2 \cdot 6H_2O$

50.0 mg NaF

diluted to 10 ml with Tris-HCl buffer (solution 1)

(3) Adenosine 5'-Triphosphate (ATP)

50 mg ATP disodium $3H_2O$ (Sigma)

diluted to 2 ml with $MgCl_2$-NaF (solution 2)

The solution of ATP is made just prior to the incubation with the tissue homogenate.

Other reagents were prepared as follows:

(4) Tetrahydrouridine (H_4U)

10 mg H_4U diluted to 10 ml with Tris-HCl buffer (solution 1)

(5) ^3H-Arabinosyl Cytosine (ara-C)

10 mg ^3H-ara-C (ca. 42 µc/ml)

diluted to 10 ml with Tris-HCl buffer (solution 1)

Each tissue supernate was incubated in three different incubation solutions as outlined below:

GROUP A. To Determine Kinase and Aminohydrolase Activity

One milliliter of supernate + 0.25 ml ATP solution + 0.10 ml Tris-HCl buffer

GROUP B. To Determine Kinase Activity While Inhibiting Aminohydrolase Activity

One milliliter of supernate + 0.25 ml ATP solution + 0.10 ml H_4U solution

GROUP C. To Determine Aminohydrolase Activity While Inhibiting Kinase Activity

One milliliter of supernate + 0.25 ml $MgCl_2$-NaF solution + 0.10 ml Tris-HCl buffer

The mixtures were preincubated for 15 minutes at 37°C before adding 0.25 ml of the ^{3}H-ara-C substrate.

The final concentration in 1.6 ml of incubation solution was $MgCl_2$, 5 µmoles; NaF, 30 µmoles; ATP, 11.5 µmoles; and ara-C, 900 nanomoles. The H_4U concentration in Group B was 360 nanomoles per 1.6 ml of solution.

At various time periods (0, 15, and 30 minutes) 0.2-ml aliquots of the incubation solution were removed and immediately added to 1.0 ml of TDR. The TDR-tissue homogenate mixture is then handled and assayed as outlined earlier for the TDR-serum mixtures.

Paper Chromatography Separation of Ara-C Nucleotides in Tissue Homogenates

In order to separate the various ara-C nucleotides it was necessary to employ two paper chromatography systems. Both systems utilized Whatman #1 paper as descending chromatography and the following solvent systems:

I. Isobutyric acid:NH_4OH:H_2O (240:23:97, running time 20 hours)

II. Absolute ethanol:1M NH_4Ac, pH 3.8: 1M NaEDTA, pH 8.2 (75:29:1, running time 24 hours)

The latter system overruns in 24 hours, requiring that 5'-phosphate ara-C be used as an R_m marker. To determine the R_m values for compound X in this system, the following equation is used:

$$R_m = \frac{\text{distance of compound X from origin}}{\text{distance of 5'-P-ara-C from origin}}$$

Therefore, some compounds have an R_m value greater than 1.00.

Determination of Protein Binding

The amount of binding of ara-C and Ara-U to the various serum proteins was determined by a modified, ultra-filtration procedure. The procedure consists of

tying a portion of moistened dialysis tubing at one end and placing the tied-off end into a 15-ml Buchner funnel with fritted glass disc of medium porosity. The open end of the tubing is then draped over the top of the funnel. The stem of the funnel is placed through a rubber stopper that is inserted into a small cup for collecting the filtrate. The tubing is air-dried before use. The aqueous-drug solution or serum-drug solution is placed in the dialysis sack and the whole apparatus is centrifuged at 100 x G for four hours at 4°C. Approximately 0.5 to 1.0 ml of filtrate is collected within this time period. Possible bag leakage is determined by performing a protein assay (Weichselbaum, 1946) on each sample filtrate. The difference in drug concentration per ml of filtrate and per ml of original drug-serum solution is estimated as the degree of protein binding. The aqueous drug solution is used similarly to determine nonbinding conditions and to estimate the amount of drug adsorption to the cellophane membrane.

VI. RESULTS

IN VIVO STUDIES

Use of H_4U as a Collection-Tube Additive

As will be shown later, the serum of monkeys and humans contains significant levels of cytidine aminohydrolase activity (see Table 24). Therefore, in collecting serum samples for *in vivo* studies, it is necessary that the enzyme be inhibited to avoid any *in vitro* deamination after the samples are withdrawn from these species. In all *in vivo* serum studies with monkeys and humans, tetrahydrouridine (H_4U) was used as a collection-tube additive to prohibit post-sampling deamination of ara-C.

To determine effectiveness of H_4U as a collection-tube additive, monkey serum from animals previously administered ara-C (50 mg/kg, i.v.) was collected at various time periods and divided into tubes with and without H_4U. The H_4U-containing tubes were prepared as described in METHODS. Ara-C serum levels were then determined for each sample as previously outlined. The data obtained for the two groups are shown in Table 1 and Figure 3. Total radioactivity (ara-C plus ara-U) was the same for both groups; however, ara-C was detected at the earlier time periods in serum collected in H_4U tubes. Samples without H_4U showed no detectable ara-C. The results clearly indicated the effectiveness and necessity of using collection tubes with H_4U

Table 1

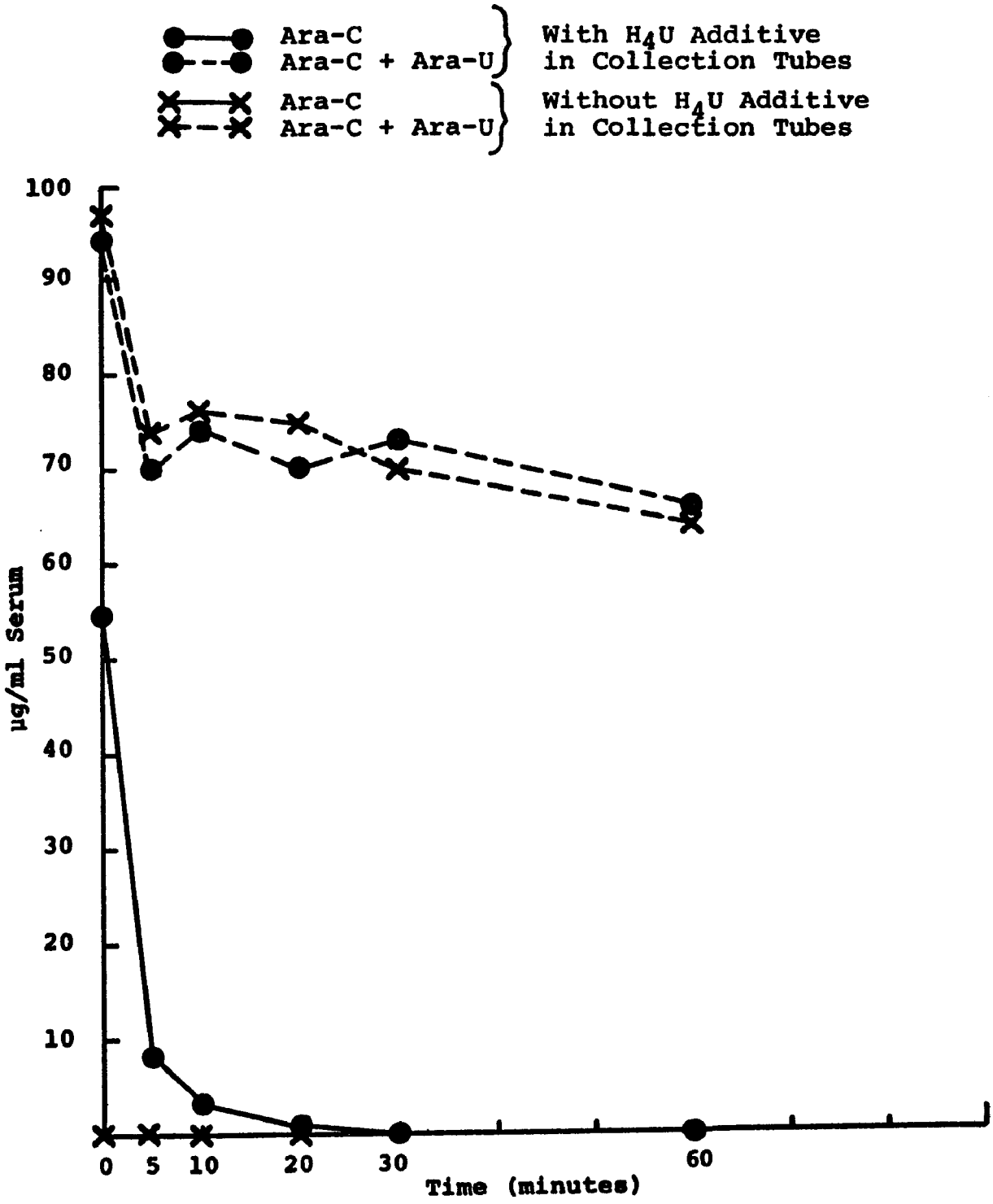
Ara-C Serum Levels in the Monkey as Determined With and Without H_4U Additive in the Sample Collection Tubes[1]

Group	Post-treatment Time Period (minutes)	Ara-C[2]		Ara-U[2]		Ara-C + Ara-U[3]
		Percent	µg/ml	Percent	µg/ml	µg/ml
A	0	0	0	100	97	97
	5	0	0	100	74	74
	10	-	-	-	-	76
	20	-	-	-	-	75
	30	-	-	-	-	70
	60	-	-	-	-	64
B[4]	0	85	80	15	14	94
	5	12	8	88	62	70
	10	4	3	96	71	74
	20	1	0.8	99	69	70
	30	-	-	-	-	73
	60	-	-	-	-	66

[1]Ara-C dose was 50 mg/kg, i.v.
[2]Data from thin-layer chromatography assay.
[3]Data represented by total radioactivity.
[4]Blood collected in tubes containing tetrahydrouridine (H_4U).

Figure 3

Serum Levels of Total Radioactivity (Ara-C + Ara-U) and Ara-C in the Monkey after Intravenous Administration of 50 mg/kg



as an additive in serum level determinations of ara-C in animals with significant serum cytidine aminohydrolase activity. Collection of blood on ice and rapid separation of serum at low temperature are not sufficient procedures to inhibit postsampling drug destruction.

Comparative Serum Levels

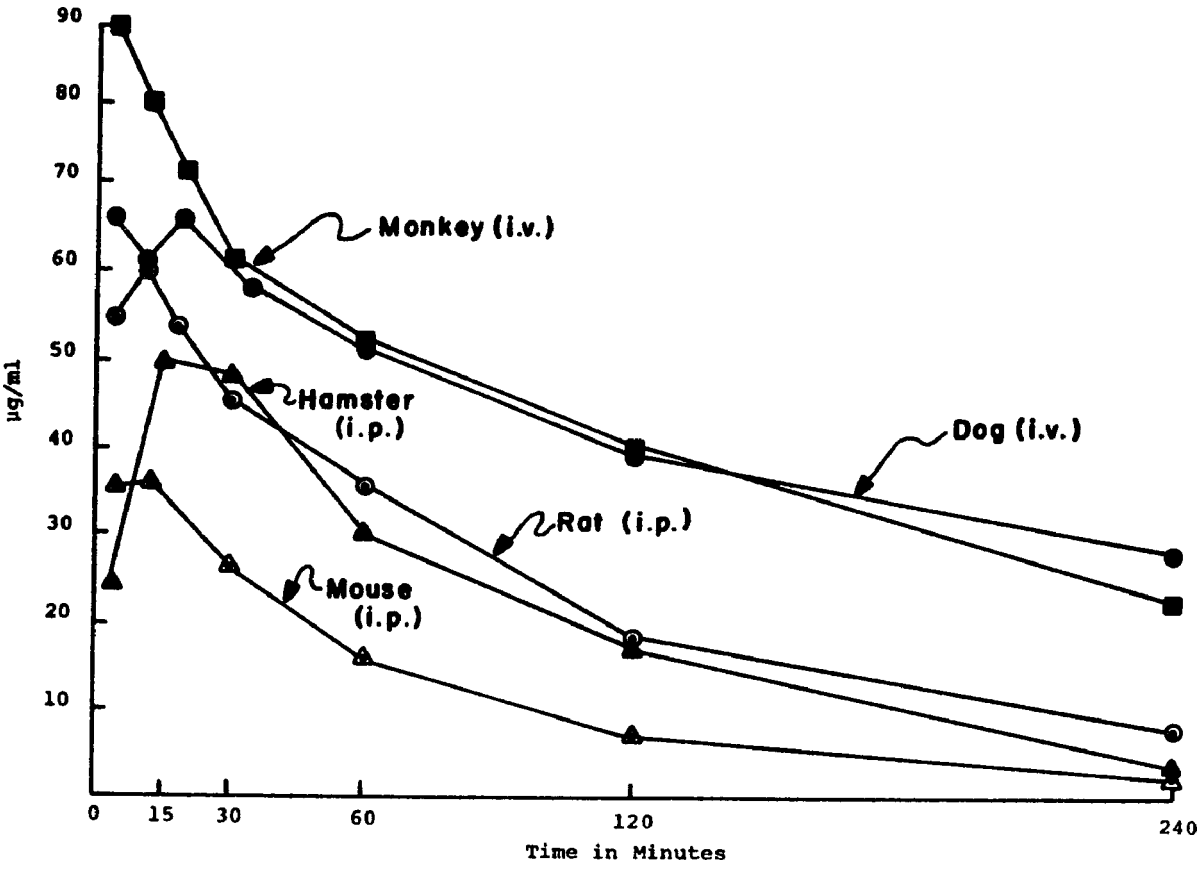
Figure 4 shows the serum levels of total radioactivity (ara-C plus ara-U) in various animal species after parenteral administration of ^{3}H-ara-C. The injected dose was 50 mg/kg. The ordinate is expressed in µg/ml since (1) ara-U is the sole metabolite of ara-C in serum, and (2) only one molecular weight unit difference exists between the two compounds. Therefore, when ^{3}H-ara-C is metabolized to ^{3}H-ara-U, a negligible change in total specific activity occurs.

The hamster, rat, and mouse data in Figure 4 were obtained from pooled samples, while those for the monkey and dog were from single animals. The curves for the latter two species were chosen because they represent typical serum levels from data of twelve monkeys and four dogs.

Serum drug levels of water-soluble compounds determined after parenteral administration may be affected by a number of factors. Some of the major factors to be considered in serum level studies are: protein binding,

Figure 4

Serum Levels of Total Radioactivity (Ara-C + Ara-U)
in Various Animal Species after
Parenteral Administration of 50 mg/kg



drug stability, metabolism, distribution and redistribution, and elimination. Exchange of labeled tritium is also a factor for consideration when compounds labeled with this isotope are used.

The degree of binding of ara-C to proteins of various animal sera was insignificant. Values found for dog, human, monkey, rat, and mouse sera were 4.0, 3.4, 1.6, 3.2, and 1.6 percent, respectively. Similar results were found for ara-U binding to these same sera proteins.

Both ara-C and ara-U are stable compounds at pH 7.0 and at 37°C. In 0.1 M buffered solutions at pH 5, 7, and 9, no breakdown of either compound was evident after incubation at 40°C for twenty-four hours.

In whole blood from a monkey previously dosed with ^{3}H-ara-C, 99.4 percent of the total radioactivity was found associated with the plasma portion and only 0.5 percent with the intracellular red blood cell fluid. Prior to assay, the red blood cells were washed and then lysed with a hypotonic salt solution.

The degree of tritium exchange involved in the use of ^{3}H-ara-C was studied by microdistillation of dog urine samples. The dog was injected with 35.7 mg/kg of ^{3}H-ara-C, intravenously, and a single 48-hour posttreatment urine sample collected. Twenty-five ml of urine or an aqueous standard drug solution containing 5 µg/ml of ^{3}H-ara-C

was distilled under vacuum until approximately 7 ml of distillate was collected. The distillate was then assayed for total radioactivity.

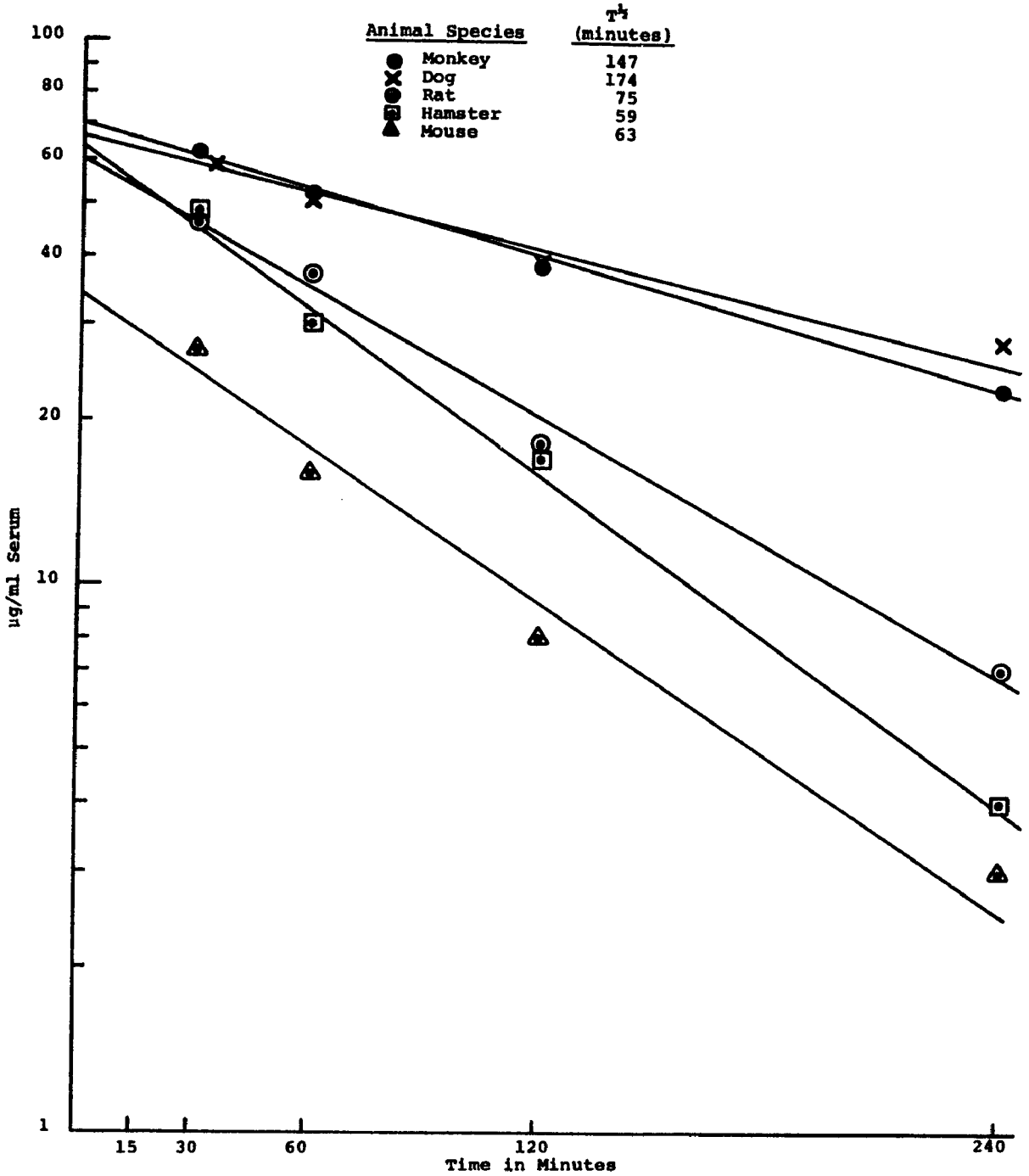
The amount of tritium exchange by this method was less than one percent for the aqueous drug standard and less than four percent for the urine sample. The urine distillate and residue represented ninety-nine percent of the total urine radioactivity, which in turn represented seventy-four percent of the total radioactive dose administered.

In summary, the serum levels of total radioactivity after parenteral administration of ^{3}H-ara-C are not affected by binding of the parent compound and its metabolite to various animal serum proteins. Nor is it apparently due to chemical instability, localization in erythrocytes, or exchange of the tritium label. Therefore, the major factors affecting the total radioactivity in serum are distribution and elimination processes.

The data presented in Figure 4 are plotted in semilogarithmic form in Figure 5. The $t_{\frac{1}{2}}$ for total radioactivity levels were purposely calculated after the 30-minute posttreatment time period so that any differences among the various species represent a difference in elimination process. The volume of distribution (V_D) (the fluid volume in which a drug appears to be dissolved)

Figure 5

Serum Levels of Total Radioactivity (Ara-C + Ara-U)
in Various Animal Species
after Parenteral Administration of 50 mg/kg



can be estimated from these plots. The V_D value is obtained by dividing the dose of the drug (mg/kg) by the serum concentration of the drug (μg/ml) at zero time period. The serum concentration at zero time period can be determined by extrapolation of the time versus drug concentration curve back to this point on the ordinate. Note that the concentration axis is a logarithmic scale (Goldstein et al., 1969). In these studies the volume of distribution of total radioactivity for the various species ranged between 70 and 150 percent of body weight.

Ara-C serum levels in the various animal species, as determined by bioassay, are shown in Figure 6. The bioassay (described in METHODS) utilized an ara-C-sensitive strain of Streptococcus faecalis. In the determination of ara-C, the bioassay was shown to be equivalent to the thin-layer method. The data in Figure 6 are presented in semilogarithmic form in Figure 7.

Human serum levels were not included in the above comparative graphs. To date, in this laboratory only one human has been studied as thoroughly as the animal species reported. Therefore, it is impossible to state with any confidence that the metabolism of ara-C by this human is typical of the general population. However, as will be seen in the DISCUSSION, other workers have reported human data that are in good agreement with data shown in Table 2 and Figures 8-10.

Figure 6

Serum Levels of Arabinosyl Cytosine in Various Animal Species after Parenteral Administration of 50 mg/kg

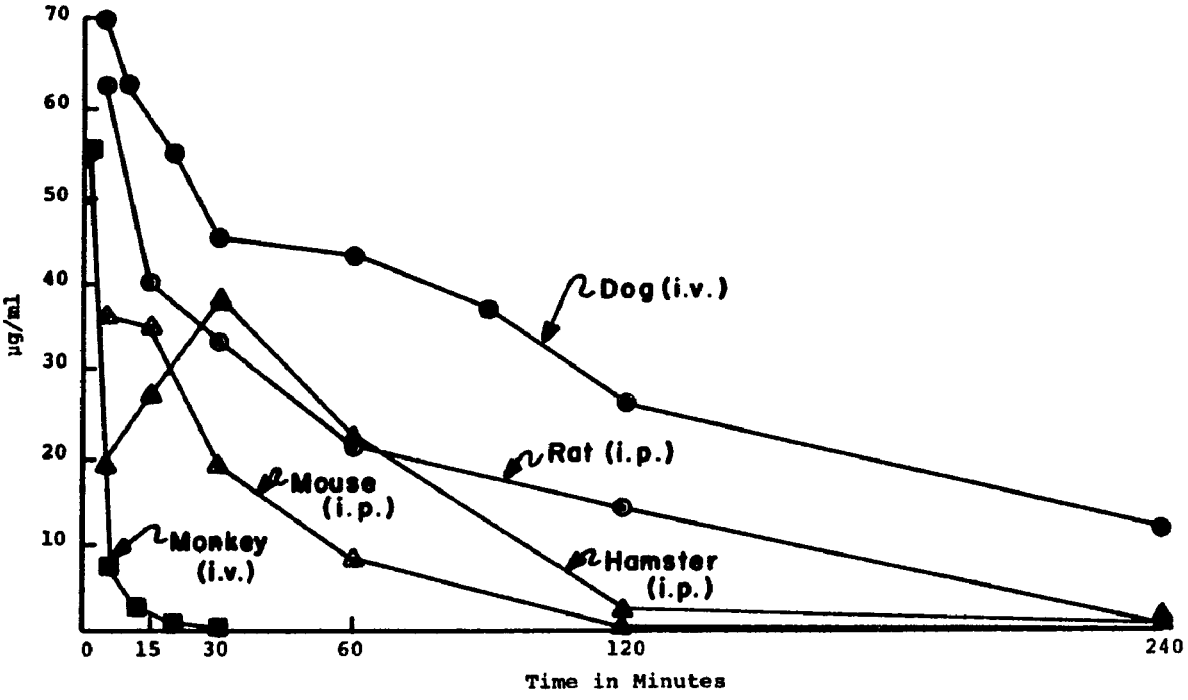


Figure 7

Serum Levels of Ara-C in Various Animal Species after Parenteral Administration of 50 mg/kg

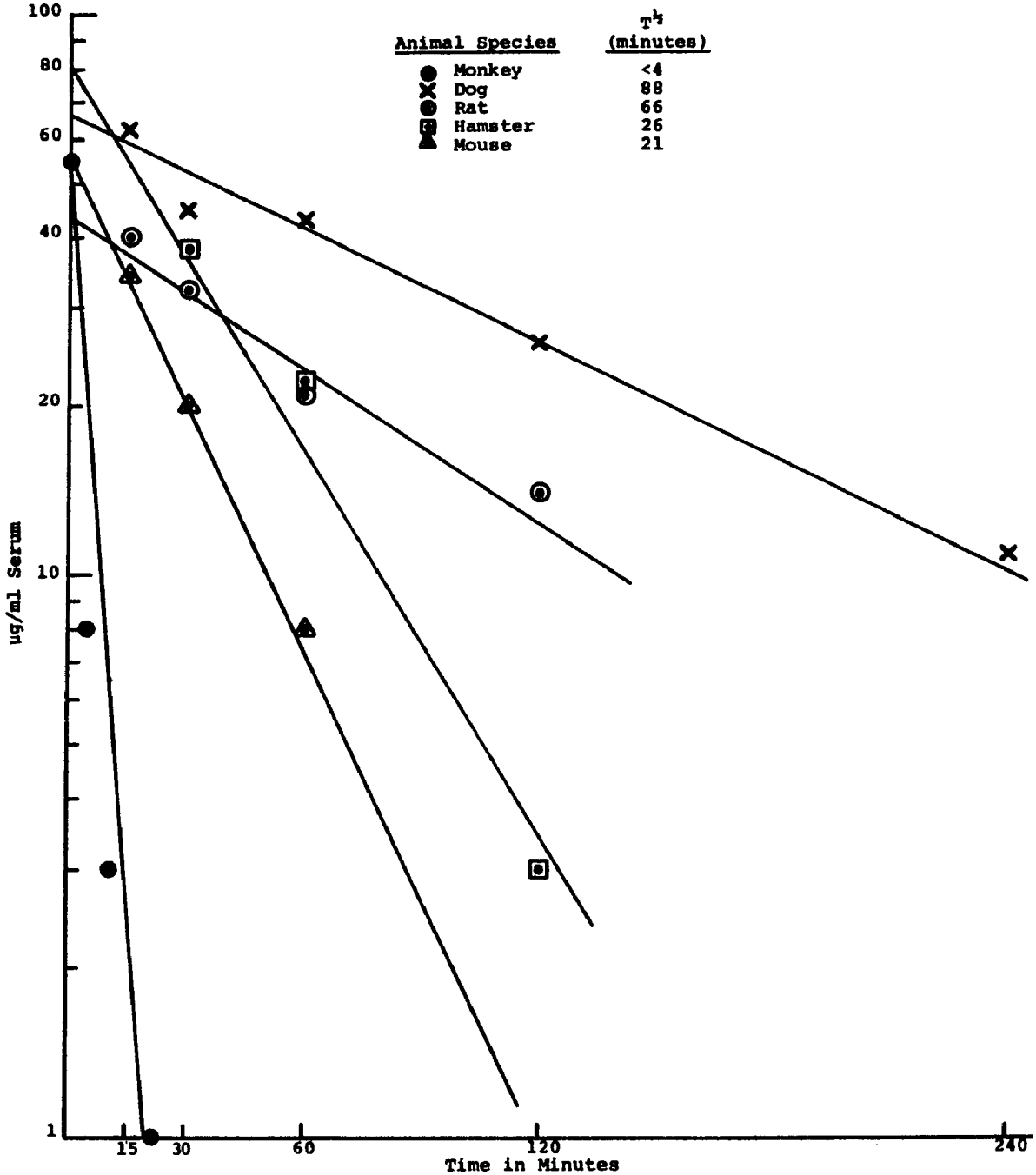


Table 2

Serum Levels of Total Radioactivity (Ara-C + Ara-U) and of Ara-C in the Human after 3.0 g/M^2 (86 mg/kg) of ^{3}H-Ara-C, Intravenously

Time Period	Ara-C + Ara-U (µg/ml)	Ara-C (µg/ml)
5 min	281	184
10 "	228	114
15 "	197	75
30 "	163	36
1 hr	145	14
2 "	119	5.5
4 "	79	-
8 "	41	-
24 "	8	-

Figure 8

Serum Levels of Total Radioactivity (Ara-C + Ara-U) and Ara-C in Human Serum after 3.0 g/M² (86 mg/kg) of Ara-C, I.V.

◎ Total Radioactivity (Ara-C + Ara-U)
✕ Ara-C

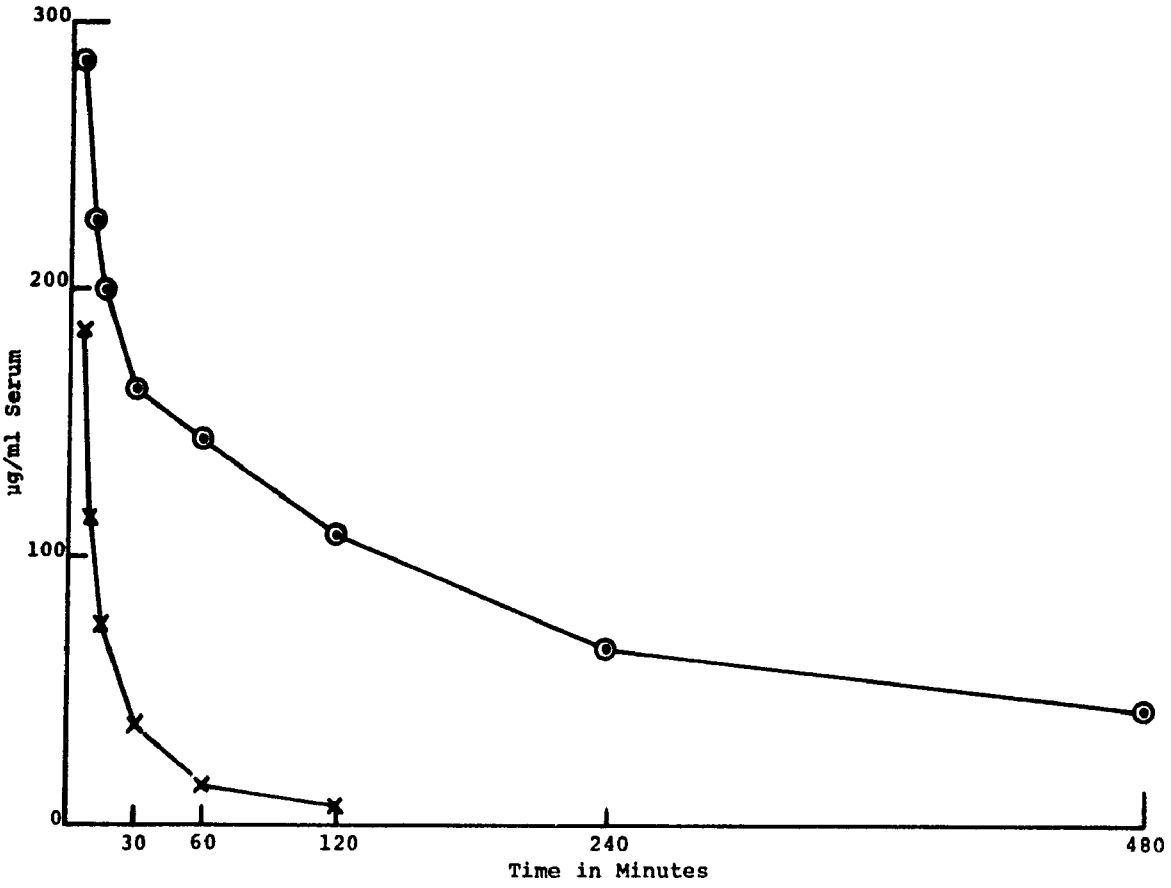


Figure 9

Serum Levels of Total Radioactivity (Ara-C + Ara-U) in Human Serum after 86 mg/kg of Ara-C, I.V.

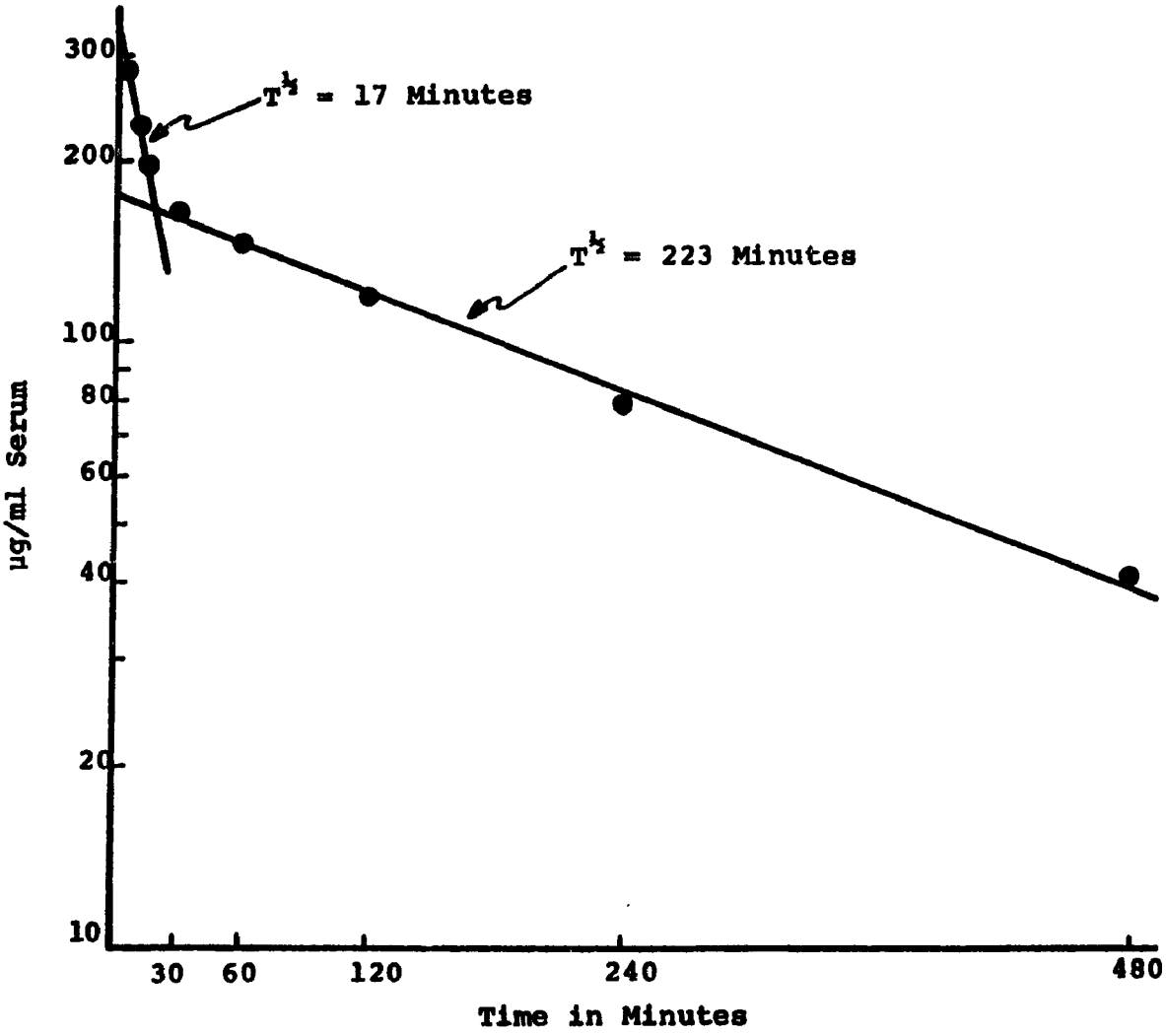
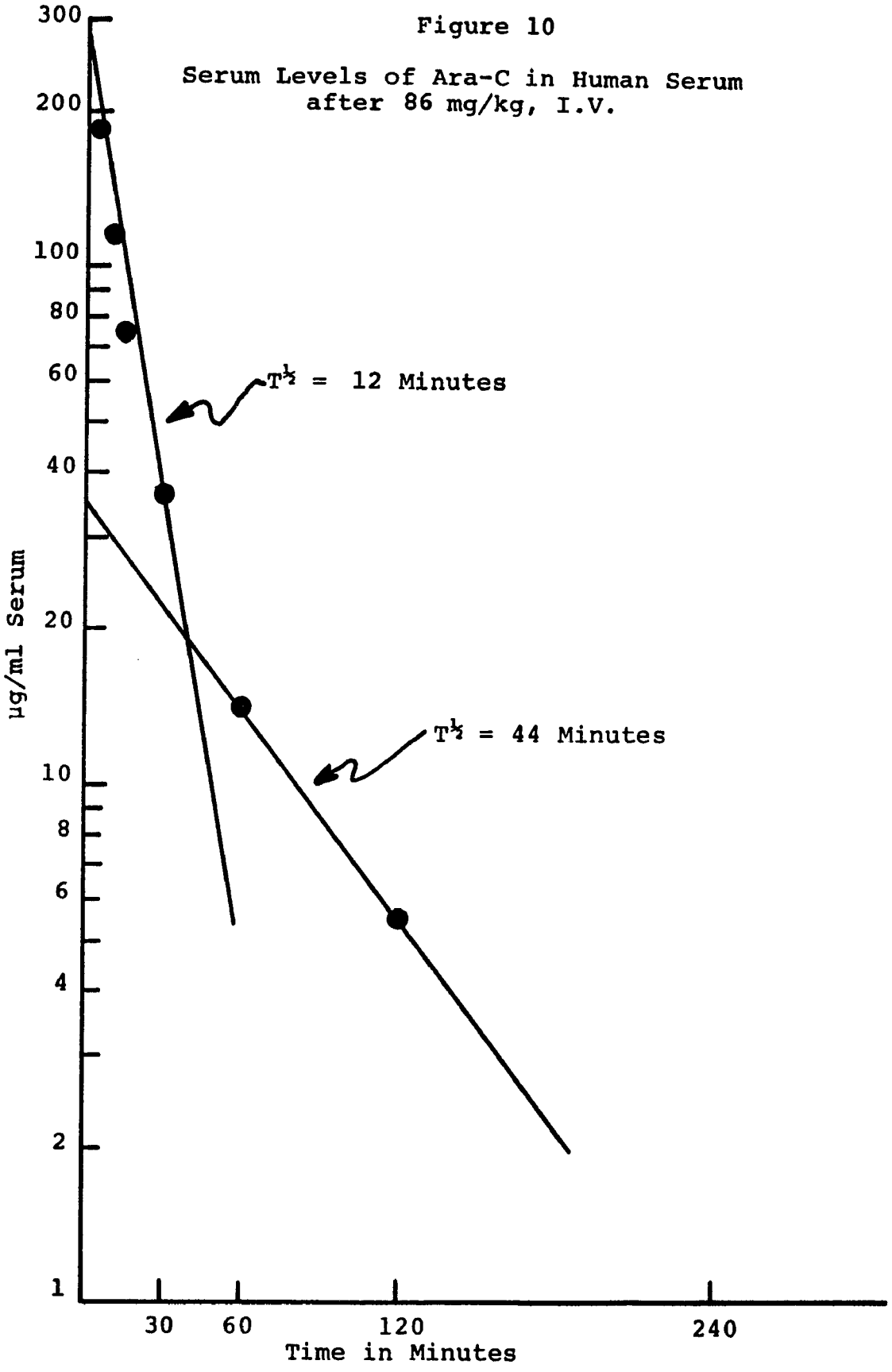


Figure 10

Serum Levels of Ara-C in Human Serum
after 86 mg/kg, I.V.



The serum levels of total radioactivity when plotted on semilogarithmic paper were divided into two components. The first component (between 5 and 30 minutes posttreatment) has a calculated value of 17 minutes and the second (between 30 and 240 minutes posttreatment), a value of 223 minutes. It is probable that the first component contains a distribution as well as an elimination factor.

Figure 10 shows the $t^{\frac{1}{2}}$ values for ara-C levels in the same patient. The first component has a value of 12 minutes, while the second component $t^{\frac{1}{2}}$ value is approximately 44 minutes. Both values are lower than those found for total radioactivity and reflect the role deamination plays as a determining factor in the half-life value of ara-C.

Comparative Urinary Excretion Patterns

The 24-hour urinary ara-C and ara-U excretion pattern in various animal species after ^{3}H-ara-C administration is shown in Table 3. The separation and assay of the parent compound and its metabolite were accomplished by either paper or thin-layer chromatography. Total 24-hour urinary recovery of the injected radioactivity and the percentage of this radioactivity associated with ara-C and ara-U are shown in Table 4 and Figure 11. The 24-hour urinary ratio of ara-C and ara-U after administration of ara-C correlated well with the tissue levels of cytidine aminohydrolase activity; i.e., in species with high enzyme activity,

Table 3

24-Hour Urinary Excretion Ratio of Ara-C and Ara-U from Various Animal Species after Parenteral Administration of ^{3}H-Ara-C

Animal Species	Number or Group	Sex	Dose Route	Dose mg/kg	Time Period (hours)	Percent Radioactivity Excreted[1] Ara-C	Ara-U
Monkey	6586	M	I.V.	50	0-4	7	93
					4-10	0	100
	6540	F	I.V.	50	0-6	5	95
					6-10	0	100
	6521	F	I.V.	50	0-6	5	95
					6-10	1	99
	6609	M	I.V.	50	0-4	1	99
					4-10	0	100
	6584	M	I.V.	50	0-2	13	87
					2-6	1	99
	6583	F	I.V.	50	0-6	4	96
					6-10	0	100
Dog	19	F	I.V.	50	0-0.5	77	23
					0.5-1	74	26
					1-2	74	26
					2-4	65	35
					4-6	61	39
					6-24	67	33
Dog	4	F	I.V.	50	0-0.5	92	8
					0.5-1	87	13
					1-2	80	20
					2-3	76	24
					3-4	71	29
					4-5	73	27
					5-6	67	33
					6-24	63	37
Rat	A	M	I.P.	100	0-24	76	24
	B	M	I.P.	100	0-24	74	26
Hamster	A	M	I.P.	300	0-6	45	55
					6-24	34	66
	B	M	I.P.	300	0-6	43	57
					6-24	29	71
Mouse	A	M	I.P.	300	0-24	53	47
	B	M	I.P.	300	0-24	40	60

[1]Calculated from total OCPM per chromatograph peak located by ultraviolet light and corresponding standards.

Table 4

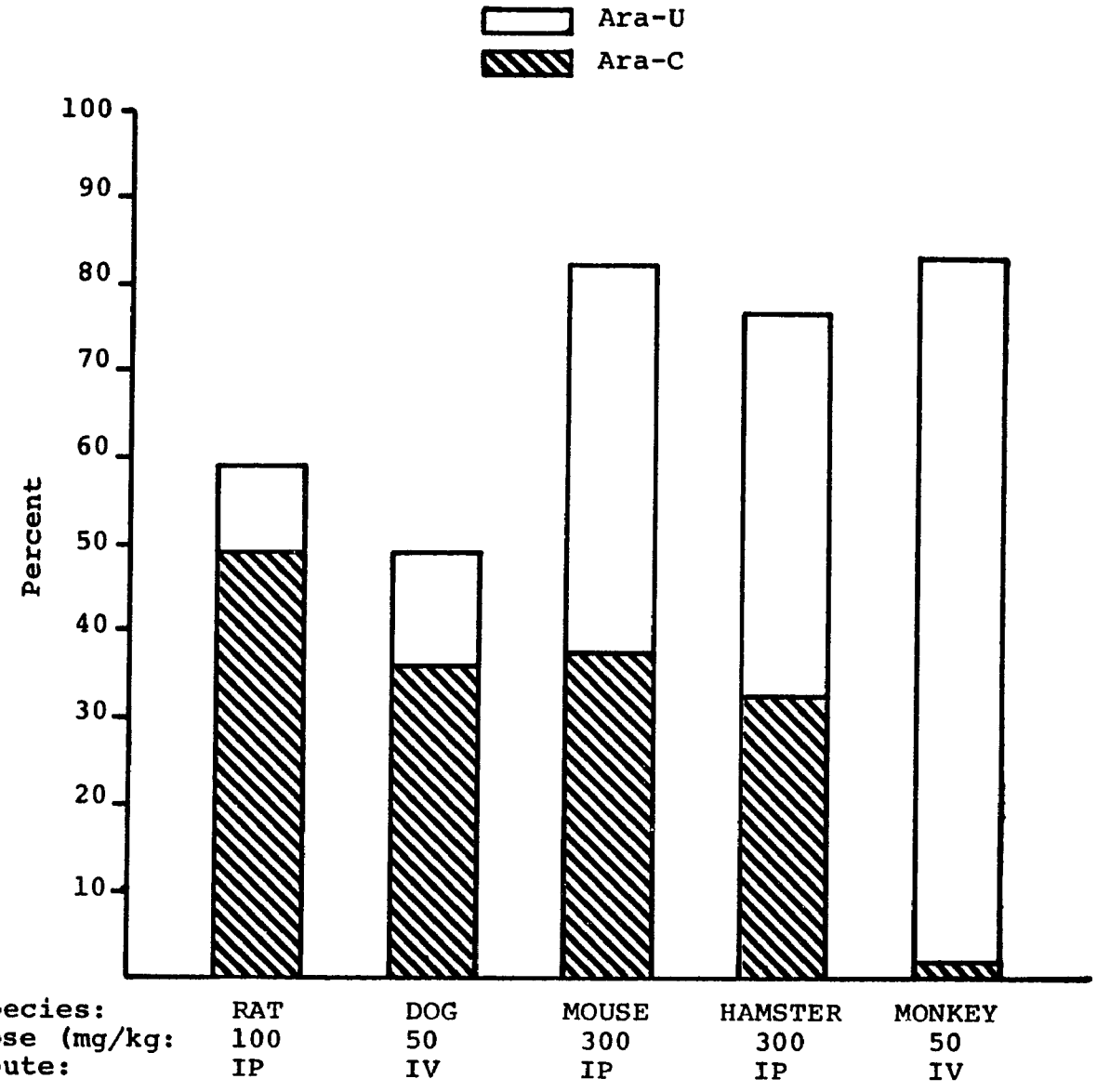
Total 24-Hour Urinary Recovery of Ara-C and Its Metabolite, Ara-U, from Various Animal Species after Parenteral Administration

Animal Species	Number or Group	Sex	Dose		Percent Dose Recovered	
			Route	mg/kg	Ara-C + Ara-U[1]	Ara-C[2]
Monkey	6586	M	I.V.	50	69	4
	6540	F	I.V.	50	62	3
	6521	F	I.V.	50	92	1
	6609	M	I.V.	50	87	<1
	6584	M	I.V.	50	98	4
	6583	F	I.V.	50	91	3
Dog	19	F	I.V.	50	55	43
	4	F	I.V.	50	42	28
Rat	A	M	I.P.	100	62	40
	B	M	I.P.	100	58	51
	C	M	I.P.	100	58	56
Hamster	A	M	I.P.	300	75	33
	B	M	I.P.	300	78	32
Mouse	A	M	I.P.	300	74	39
	B	M	I.P.	300	90	36

[1]Determined by total radioactivity assay.
[2]Determined by microbiological assay.

Figure 11

Total 24-Hour Urinary Recovery of Ara-C and Its Metabolite, Ara-U, from Various Animal Species after Parenteral Administration



the excretion of ara-C was low and excretion of ara-U was high. (Enzyme activity levels are reported in the section on IN VITRO STUDIES.)

Tables 5 and 6 show the urinary excretion data from the human included in the study. Over 90 percent of the excreted ara-C was recovered in the first sample (0-2 hours posttreatment). In 24 hours, 72.7 percent of the total radioactive dose was recovered in the urine and 7.5 percent of this total was the parent compound, ara-C.

Oral Versus Parenteral Route of Ara-C Administration

The oral absorption of ara-C and ara-U is reflected in the serum levels of total radioactivity after oral administration of ^{3}H-ara-C (Figure 12). The degree and rate of absorption of these compounds by the various animals extend from total and rapid in the rat to slight and slow in the hamster. The mouse showed a slight but rapid absorption, while the monkey showed a slight but slow absorption. The dog absorbed the compounds rapidly and maintained a relative high level of radioactivity in the serum.

An unexpected finding was the identical serum levels of total radioactivity in the rat after oral and intraperitoneal administration of ara-C. Although ara-C and ara-U are poorly absorbed in the gut of the mouse and hamster, it is not the case in the rat.

Table 5

Urinary Recovery of Total Radioactivity (Ara-C + Ara-U) in the Human after 3.0 g/M^2 of Ara-C, Intravenously

Time Period (hours)	TTL ml Voided	Ara-C + Ara-U mg/ml	Ara-C + Ara-U mg/TTL	Percent Recovery per Time Period	Percent Recovery Cumulative
0-2	254	3.14	797.6	17.7	17.7
2-4	178	3.81	662.2	14.7	32.4
4-8	126	6.76	851.8	18.9	51.3
8-16	685	1.00	687.1	15.2	66.5
16-24	445	0.63	280.8	6.2	72.7
24-32	61.5	1.52	93.7	2.0	74.7
32-48	Sample lost				
48-56	315	0.03	8.8	0.1	74.8

Table 6

Urinary Recovery of Ara-C in the Human after 3.0 g/M^2 of ^{3}H-Ara-C, Intravenously

Time Period (hours)	Ara-C	
	µg/TTL	Percent Recovery Cumulative
0-2	209	7.0
2-4	7.9	7.2
4-8	3.4	7.3
8-16	3.4	7.5
16-24	1.9	7.5
24-32	0	7.5
32-48	Sample lost	
48-56	0	7.5

Figure 12

Serum Levels of Total Radioactivity (Ara-C + Ara-U)
in Various Animal Species

After I.P. or I.V. Administration
After Oral Administration

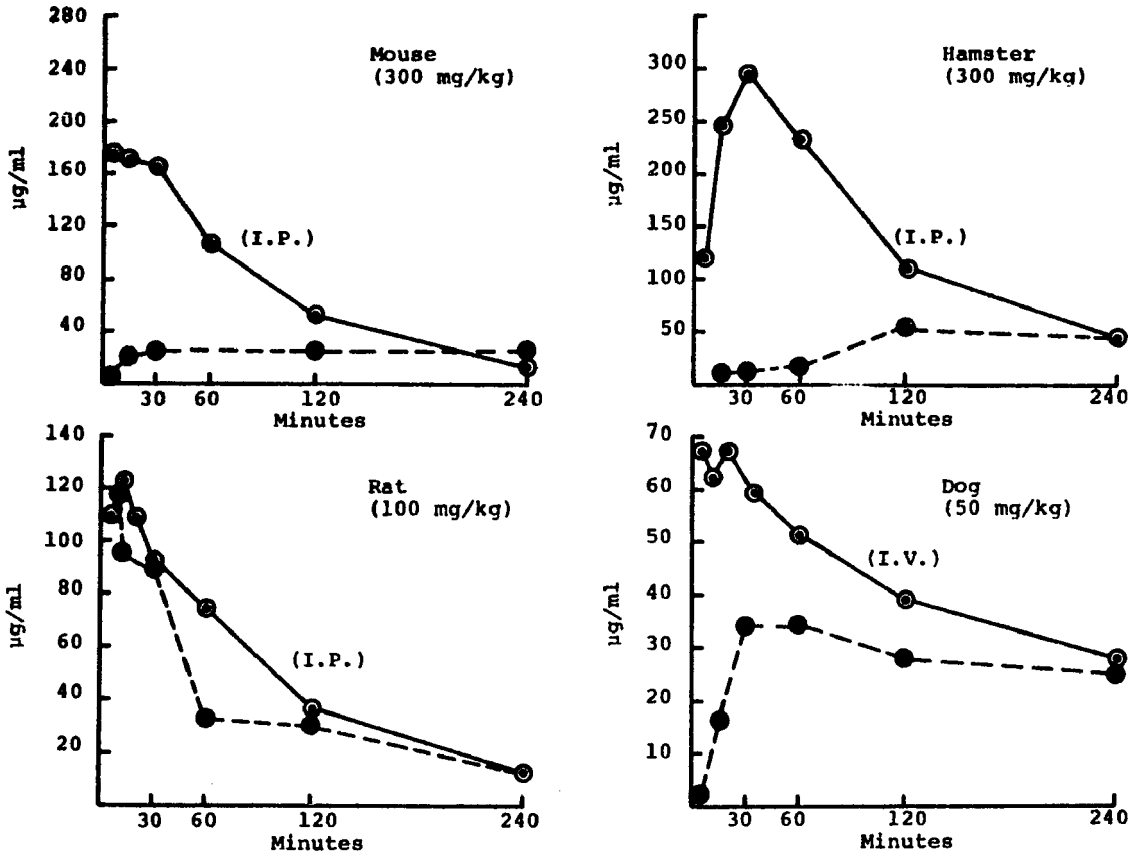


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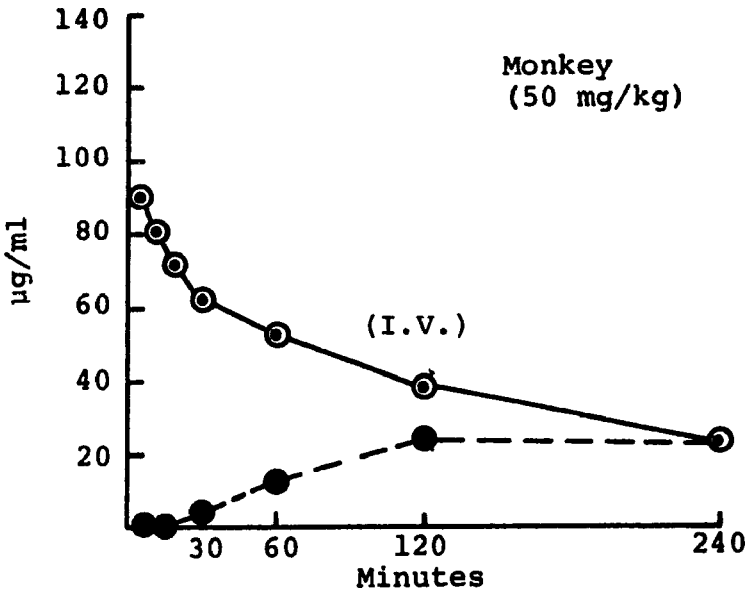
Figure 12 (continued)



The serum levels of ara-C after oral administration in various animal species are shown in Figure 13. The results can be explained by considering cytidine aminohydrolase activity in the small intestine (see Table 34). The dog, rat, and mouse — all contain very low aminohydrolase activity in this tissue — ara-C serum levels after oral administration of ara-C are essentially the same as the total radioactivity levels for the first 120 minutes after dosing. However, after 120 minutes the levels begin to drop below the total radioactivity levels and at 240 minutes are slightly greater than zero. On the other hand, no ara-C was detected in any monkey or hamster serum samples, which correlates well with the high cytidine aminohydrolase activity in the small intestine of these animals.

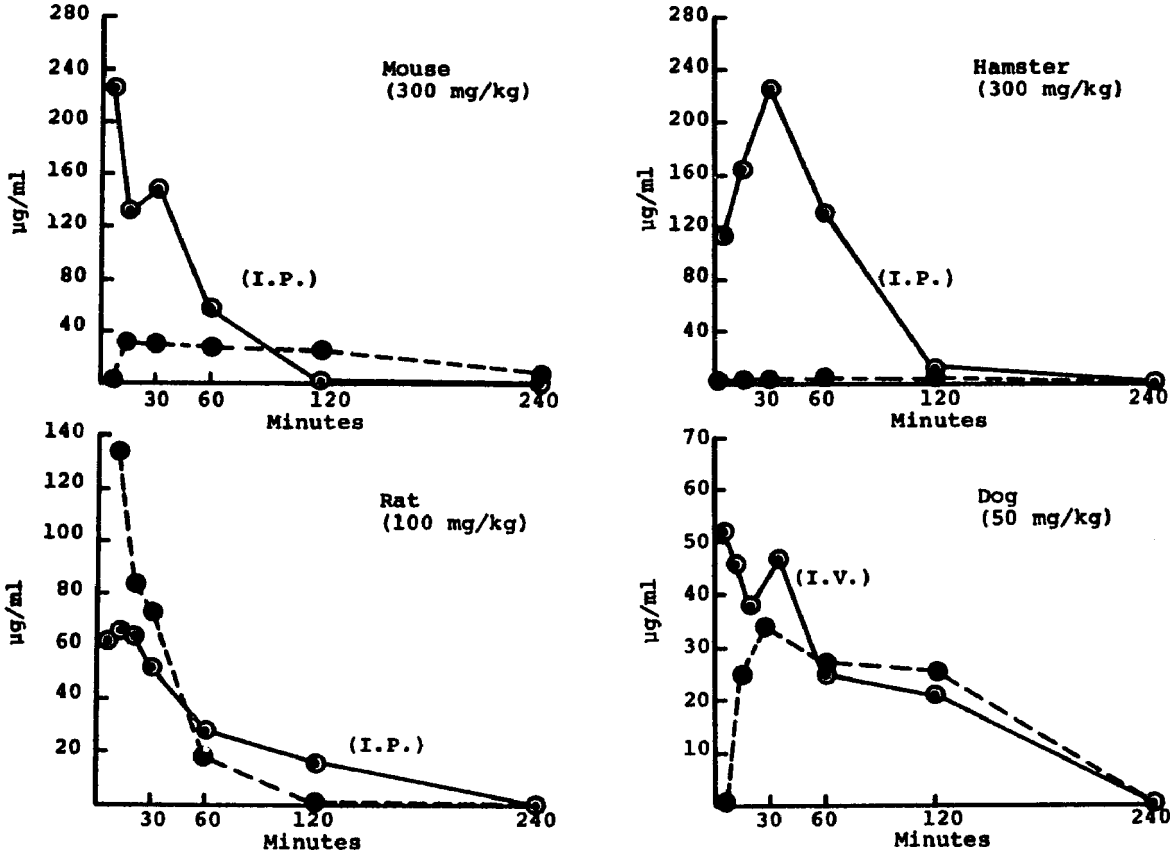
As with the total radioactivity, the rat showed an absorption rate of ara-C when administered orally which was equal to that found on parenteral administration of the drug. In the dog, the same serum levels of ara-C were reached after both routes of administration, although approximately 30 minutes are required for the orally-administered drug to reach the same serum level as the parenterally administered dose.

In 240 minutes all total radioactivity and ara-C serum levels were the same, whether ara-C was given orally or parenterally.

Figure 13

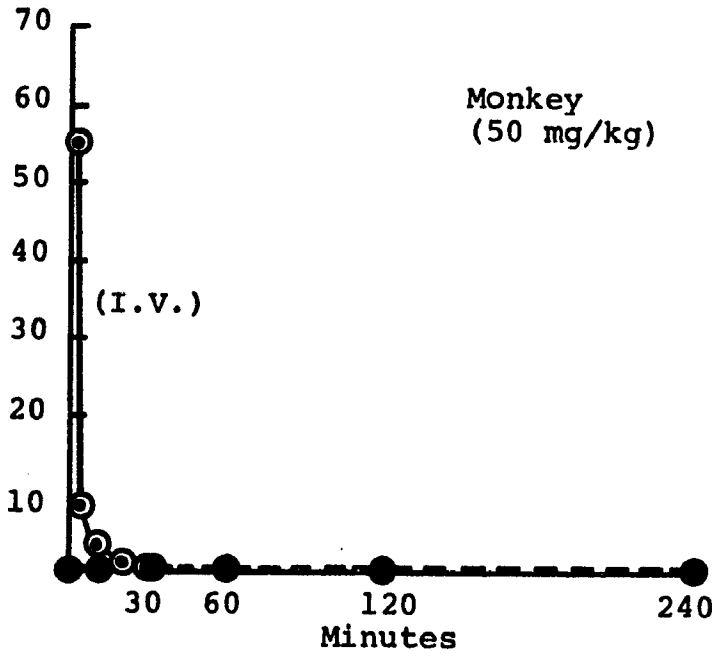
Serum Levels of Arabinosyl Cytosine in Various Animal Species

After I.P. or I.V. Administration
After Oral Administration



(continued)

Figure 13 (continued)



Effect of H_4U on the In Vivo Metabolism of Ara-C

A series of experiments were initiated to determine the effects of varying doses of H_4U on the *in vivo* deamination of ara-C. The rhesus monkey was selected as a model species for the investigation since this animal, in terms of distribution and activity of the aminohydrolase enzyme, most resembles man.

Male and female monkeys weighing between 3.0 and 4.1 kg were employed in the studies. The animals served as their own controls and received 50 mg/kg of ara-C, intravenously. Approximately one month after control data were obtained, two animals each received 100, 50, 25, and 12.5 mg/kg of H_4U, intravenously, 30 minutes prior to again receiving 50 mg/kg of ara-C. Serum levels and urinary excretion of ara-C and ara-U were determined for each animal.

The animals employed in the studies were identified as follows:

Group	Monkey Number	Sex	Dose of Ara-C mg/kg	Dose of H_4U mg/kg	Weight kg
A	6586	M	50	100	3.0
	6540	F	50	100	3.0
B	6542	M	50	50	3.8
	6585	M	50	50	3.6
C	6521	F	50	25	3.9
	6609	M	50	25	4.1
D	6584	M	50	12.5	3.1
	6583	F	50	12.5	3.6

Blood sampling times were: pre-R_x; and 15, 30, 120, 240, 360, and 600 minutes post-R_x. Samples were collected in tubes containing H_4U sufficient to give a concentration of 10^{-4}M, allowed to clot, centrifuged, and serum removed and frozen until assayed.

Urine sampling times were 0-10, 10-24, and 24-48 hours. Urine volumes were measured and aliquots removed and frozen until assayed.

Total radioactivity was determined for each serum and urine sample. The previously outlined thin-layer chromatography assay method was utilized for the determination of ara-C and ara-U in each serum and urine sample. This method was also utilized to determine the cytidine aminohydrolase activity of each pre-R_x serum sample.

Hematologic data, blood urea nitrogens (BUN) (Gentzkow, 1942), serum glutamic-oxalacetic transaminases (SGOT) (Reitman and Frankel, 1957), and alkaline phosphatase activity (Bodansky, 1933) were determined on all animals for three separate control periods prior to ara-C, then on days 2, 4, 6 or 7, and 9 following ara-C and H_4U-plus-ara-C.

Table 7 and Figure 14 summarize the recovery of total radioactivity in monkey serum at various time periods after ^{3}H-ara-C administration.

Table 7

Total Radioactivity, Expressed as Arabinosyl Uracil, in Monkey Sera after I.V. Administration of ^{3}H-Arabinosyl Cytosine (50 mg/kg)[1,2]

Group	Monkey Number	Post-Treatment Time Period in Minutes					
		15	30	120	240	360	600
A	6586	58.7	56.8	44.0	29.6	20.5	10.9
	6540	63.1	58.8	41.8	28.3	19.6	10.0
B	6585	60.1	53.5	37.8	25.5	15.7	6.9
	6542	51.2	46.3	27.6	16.8	9.9	3.3
C	6521	65.7	53.5	34.8	22.4	13.0	6.1
	6609	71.8	69.4	52.7	35.0	25.3	14.2
D	6584	75.4	73.5	44.6	29.4	19.6	9.7
	6583	68.7	64.4	38.6	22.0	13.4	5.8

[1]Data expressed as microgram of ara-U per milliliter of serum.

[2]All but trace amounts of radioactivity were found by thin-layer chromatography to be associated with a single metabolic product, ara-U. A microbiological assay failed to detect any unmetabolized ara-C.

Figure 14

Serum Levels of Total Radioactivity (Ara-C + Ara-U) in the Monkey after Intravenous Administration of ^{3}H-Arabinosyl Cytosine (50 mg/kg)

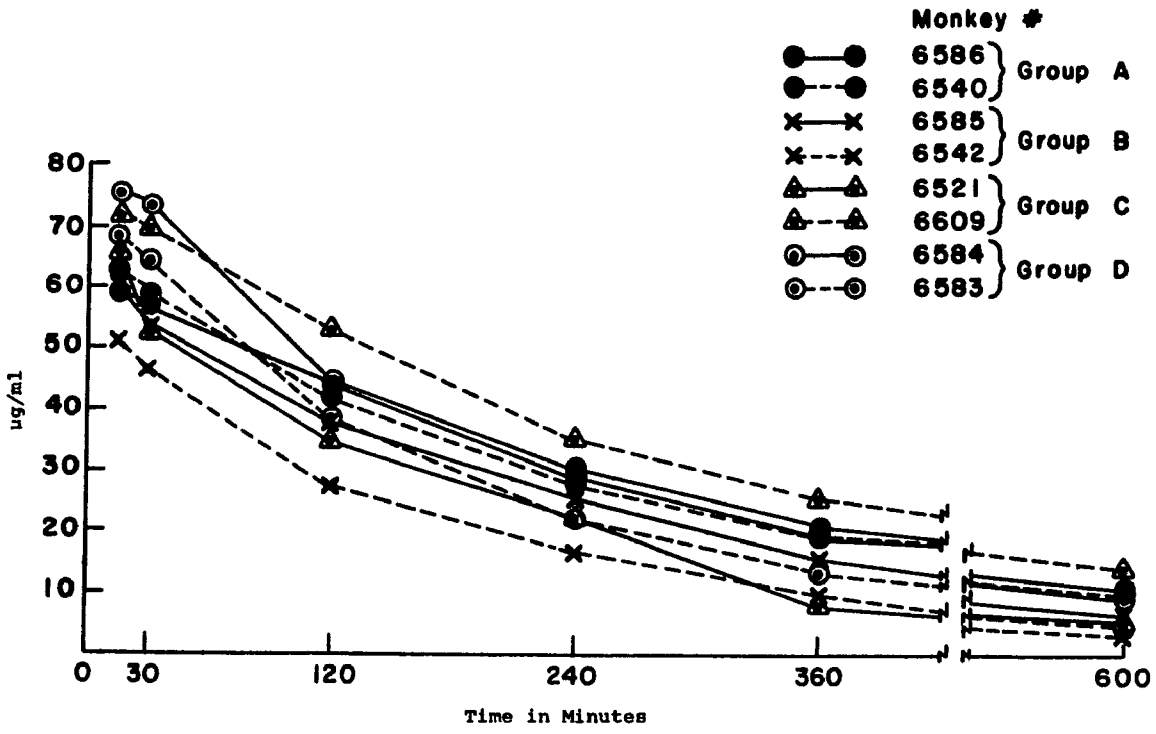


Table 8 shows the serum levels of ara-C and ara-U as assayed by TLC. Samples from the various animals assayed at 15 and 30 minutes displayed levels of 0.6 to 4.1 μg/ml, which is at the maximum limit of sensitivity of the assay as the experiment was designed.

Total radioactivity recovered in urine for the various posttreatment time periods is shown in Table 9. Table 10 summarizes the recovery of total radioactivity for 24 hours. These values range from 62 to 98 percent of the dose. Less than 4 percent of this radioactivity was associated with ara-C, and the remaining activity was associated with ara-U. These data are shown in bar-graph form in Figure 15.

The second phase of the studies involves the treatment of the same animals with varying doses of H_4U (100, 50, 25, and 12.5 mg/kg) 30 minutes prior to the standard dose of ara-C (50 mg/kg). Serum levels and urinary excretion patterns were followed as in the studies without H_4U. Hematological effects, BUNs, SGOTs, and alkaline phosphatases were determined as in the control study. Serum aminohydrolase activity was assayed prior to treatment.

Total radioactivity values (ara-C plus ara-U) in the serum of animals receiving H_4U-plus-ara-C are shown in Table 11. The calculated biological half-life for

Table 8

Serum Levels of Ara-C and Ara-U in Monkeys after I.V. Administration of ^{3}H-Arabinosyl Cytosine (50 mg/kg)[1]

Group	Monkey Number	Time Period (minutes)	Percent Total Radioactivity		Ara-C (µg/ml)
			Ara-C	Ara-U	
A	6586	15	4.8	86.2	2.8
		30	3.3	87.2	1.9
	6540	15	5.7	85.3	3.6
		30	6.6	79.2	3.9
B	6585	15	4.6	88.8	2.8
		30	3.5	90.1	1.9
	6542	15	2.9	94.7	1.5
		30	1.3	95.7	0.6
C	6521	15	4.3	88.7	2.8
		30	2.9	87.1	1.6
	6609	15	3.3	89.2	2.4
		30	2.9	90.6	2.0
D	6584	15	5.2	85.5	3.9
		30	3.9	91.1	2.9
	6583	15	6.0	85.8	4.1
		30	3.9	87.0	2.5

[1]Determined by thin-layer chromatography assay.

Table 9

Total Radioactivity in Monkey Urine after I.V. Administration of ^{3}H-Arabinosylcytosine (50 mg/kg)[1]

Group	Monkey Number	Dose (mg)	Post-Treatment Time Period (hours)	Ara-C + Ara-U[2] (mg Excreted per Time Period)	Total Recovery (% Dose)
A	6586	150	0-4	52.8	35.2
			4-10	49.5	68.2
			10-24	1.1	68.9
			24-48	0.3	69.1
A	6540	150	0-6	58.9	39.2
			6-10	27.5	57.6
			10-24	6.4	61.8
			24-48	2.0	63.1
B	6585	180	0-4	85.6	47.5
			4-10	- [3]	>47.5
			10-24	14.5	>55.6
			24-48	2.1	>56.7
B	6542	190	0-4	92.4	48.6
			4-10	32.1	65.5
			10-24	- [3]	>65.5
			24-48	0.2	>65.6
C	6521	195	0-6	135.9	69.7
			6-10	26.5	83.3
			10-24	16.6	91.8
			24-48	2.4	93.0
C	6609	205	0-4	78.7	38.4
			4-10	69.2	72.2
			10-24	30.1	86.8
			24-48	7.0	90.3
D	6584	155	0-2	41.9	27.0
			2-6	48.3	58.2
			6-10	28.5	76.5
			10-24	33.6	98.2
			24-48	2.7	100.0
D	6583	180	0-6	107.8	59.9
			6-10	41.9	83.1
			10-24	13.6	90.7
			24-48	1.1	91.3

[1]Expressed as ara-C + ara-U.

[2]All radioactivity was found by thin-layer chromatography to be associated with either ara-C or ara-U.

[3]Samples lost.

Table 10

Total 24-Hour Urinary Recovery of Ara-C and Its Metabolite, Ara-U, from Monkeys after I.V. Administration of ^{3}H-Ara-C (50 mg/kg)

Group	Monkey Number	Percent Dose Recovered	
		Ara-C + Ara-U[1]	Ara-C[2]
A	6586	69	4
	6540	62	3
B	6585	>56	-[3]
	6542	>66	-[3]
C	6521	92	1
	6609	87	<1
D	6584	98	4
	6583	91	3

[1]Determined by total radioactivity assay. A 3% ara-U impurity subtracted from each value.

[2]Determined by thin-layer chromatography assay.

[3]Samples lost.

Figure 15

Total 24-Hour Urinary Recovery of Ara-C and Ara-U in the Monkey after I.V. Administration of ^{3}H-Ara-C (50 mg/kg)

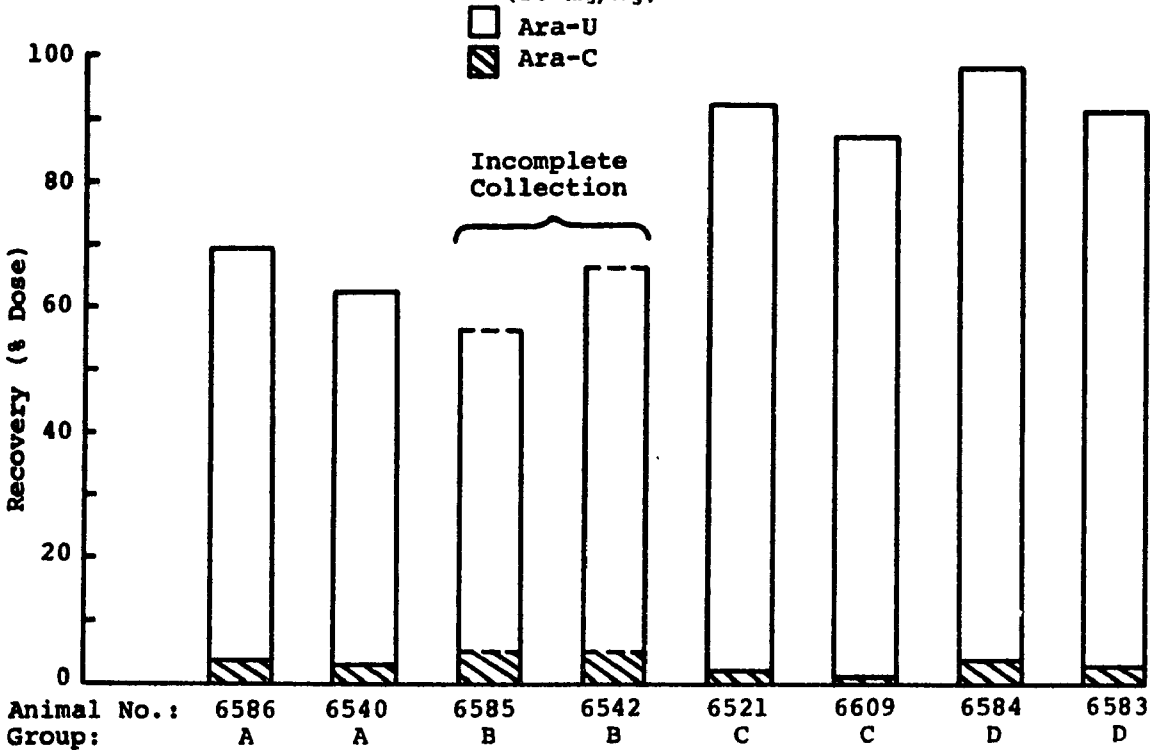


Table 11

Total Radioactivity in Monkey Sera after I.V. Administration of ^{3}H-Arabinosyl Cytosine (50 mg/kg) Animals Had Been Pretreated with Tetrahydrouridine[1]

Group	Monkey Number	H_4U Dose (mg/kg)	Post-treatment Time Period in Minutes 15	30	120	240	360	600
A	6586	100	64.8	51.9*	42.0	32.3	23.7	14.1
	6540	100	63.5	55.8	37.7	23.9	17.2	8.6
B	6585	50	55.0	50.4	30.4	18.8	11.8	5.5
	6542	50	59.9	53.0	34.4	22.0	12.9	6.2
C	6521	25	65.8	58.9	38.7	22.6	15.0	8.3
	6609	25	62.7	54.2	34.9	24.0	17.0	8.5
D	6584	12.5	84.7	76.4	48.1	25.9	17.9	8.3
	6583	12.5	79.5	71.0	46.8	26.5	17.8	11.0

[1]Data expressed as microgram of ara-C + ara-U per milliliter of serum.

*Forty-five minute sample.

serum total radioactivity for these animals and the ara-C control animals ranged from 156 to 282 and 135 to 231, respectively. Serum levels of ara-C and ara-U determined by the TLC assay are shown in Table 12.

The data on serum levels of ara-C and ara-U in the individual animals are shown in Figures 16-19. H_4U effectively blocked deamination of ara-C at all dose levels tested.

The biological half-life for ara-C serum levels in two monkeys each for the 100, 50, 25, and 12.5 mg/kg H_4U dose is 160-216, 84-96, 82-102, and 60-78 minutes, respectively (Figures 20-23).

The initial aminohydrolase activity ranged from 440 to 898 nanomoles of ara-U produced per ml of pre-R_x serum per 60 minutes incubation (Table 13). These values were within the 288 to 900 range found for normal monkeys in this laboratory. Although seven out of eight animals displayed an increased enzyme activity before the H_4U pretreatment, this increase was within the day-to-day fluctuation found for this enzyme activity in monkeys.

Total radioactivity recovered in urine for the various posttreatment time periods is shown in Table 14. Table 15 summarizes the recovery of total radioactivity for 24 hours. These values correlated with the ara-C/ara-U ratio found in the serum of these animals. These data are shown in bar-graph form in Figure 24.

Table 12

Serum Levels of Ara-C and Ara-U in Monkeys after I.V. Administration of ^{3}H-Arabinosyl Cytosine (50 mg/kg). Animals Had Been Pretreated with Tetrahydrouridine.

Group	Monkey Number	Dose H_4U (mg/kg)	Time Period (minutes)	Percent Total Radioactivity [1] Ara-C	Ara-U [2]	Ara-C (µg/ml)
A	6586	100	15	89	1	58
			45	89	2	46
			120	88	3	37
			240	83	9	27
			360	78	14	19
			600	67	27	9
A	6540	100	15	85	2	54
			30	92	2	51
			120	84	8	32
			240	75	17	18
			360	66	27	11
			600	43	53	4
B	6542	50	15	83	5	50
			30	75	12	40
			120	54	36	19
			240	33	59	7
			360	21	68	3
			600	11	76	1
B	6585	50	15	82	6	45
			30	81	10	41
			120	58	23	18
			240	48	44	9
			360	34	60	4
			600	11	88	1
C	6521	25	15	88	2	58
			30	77	11	45
			120	58	35	22
			240	31	61	7
			360	20	71	3
			600	7	79	1
C	6609	25	15	84	2	53
			30	79	8	43
			120	74	19	26
			240	52	41	13
			360	32	61	5
			600	12	78	1
D	6583	12.5	15	70	21	56
			30	60	32	43
			120	26	64	12
			240	10	82	3
			360	7	88	1
			600	4	91	<1
D	6584	12.5	15	85	10	72
			30	79	10	60
			120	60	30	29
			240	36	60	9
			360	19	74	3
			600	7	83	1

[1] Determined by thin-layer chromatography assay.
[2] Three percent ara-U impurity subtracted from each value.

Figure 16

Serum Levels of Total Radioactivity (Ara-C + Ara-U) and Ara-C after 100 mg/kg H_4U and 50 mg/kg Ara-C

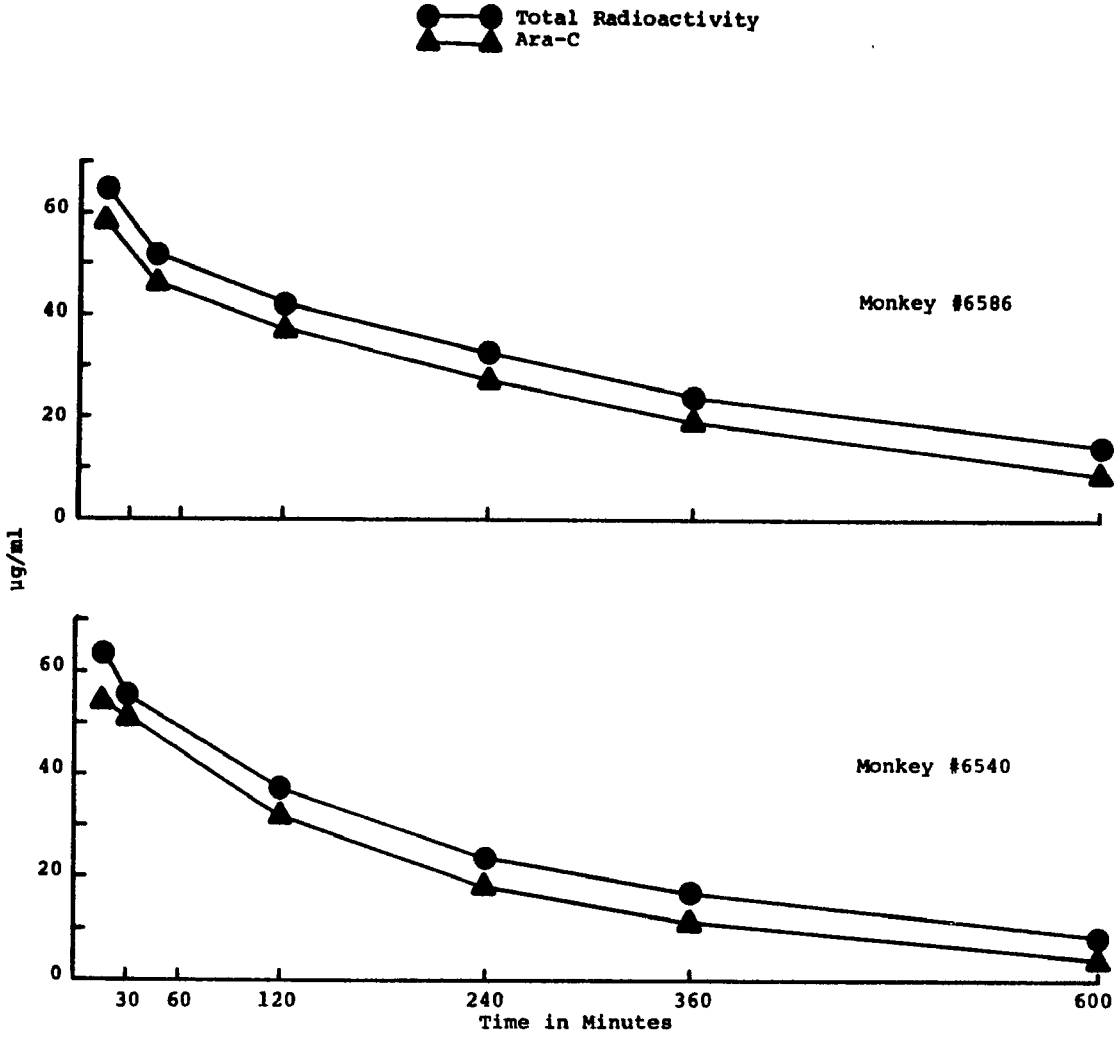


Figure 17

Serum Levels of Total Radioactivity (Ara-C + Ara-U) and Ara-C after 50 mg/kg H_4U and 50 mg/kg Ara-C

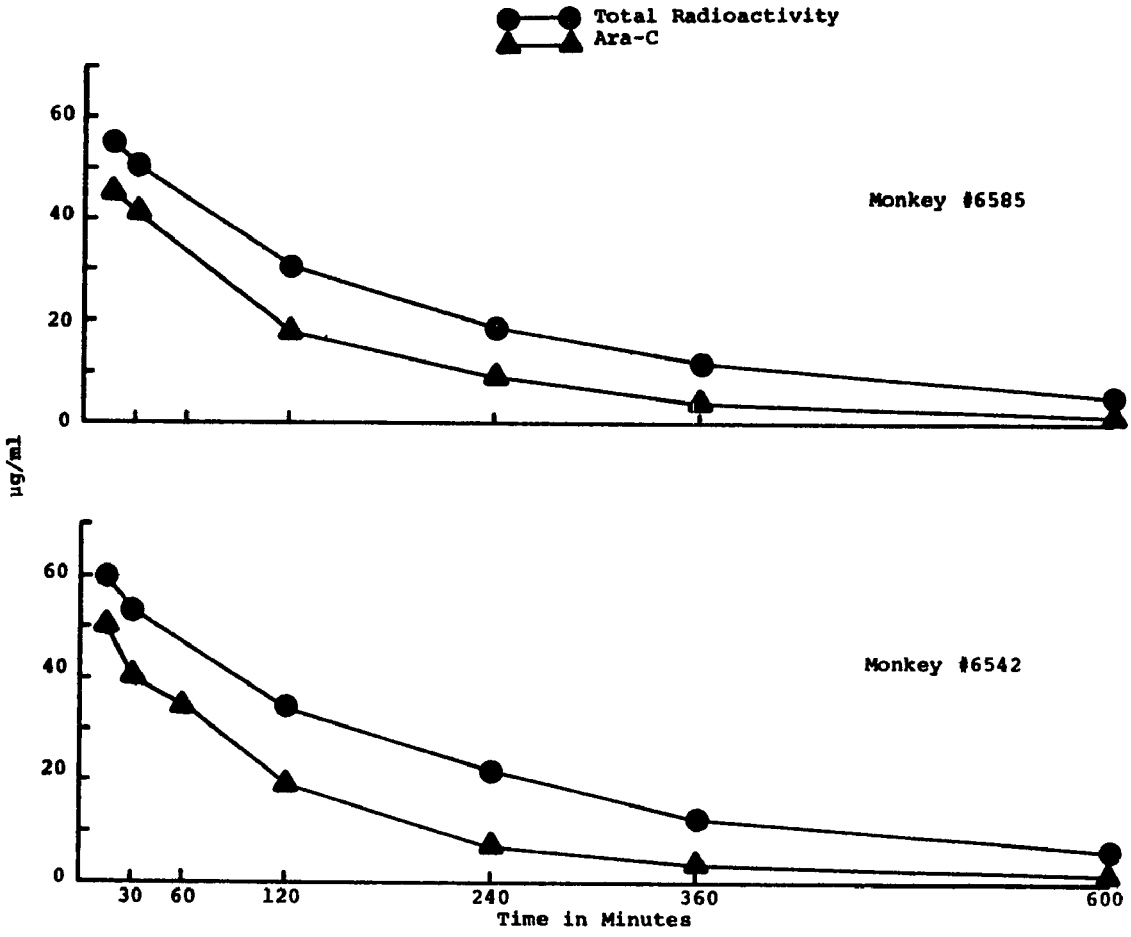


Figure 18

Serum Levels of Total Radioactivity (Ara-C + Ara-U) and Ara-C after 25 mg/kg H_4U and 50 mg/kg Ara-C

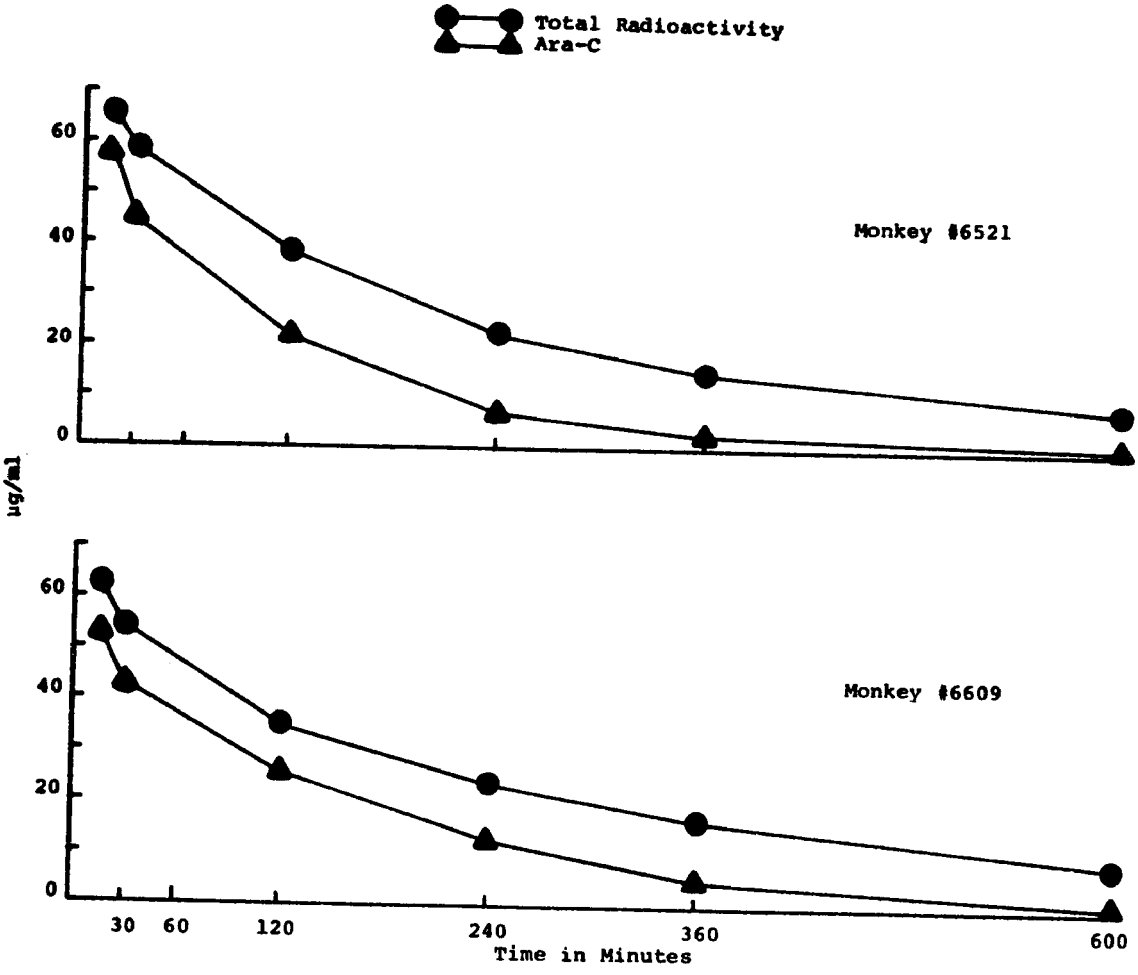


Figure 19

Serum Levels of Total Radioactivity (Ara-C + Ara-U) and Ara-C after 12.5 mg/kg H_4U and 50 mg/kg Ara-C

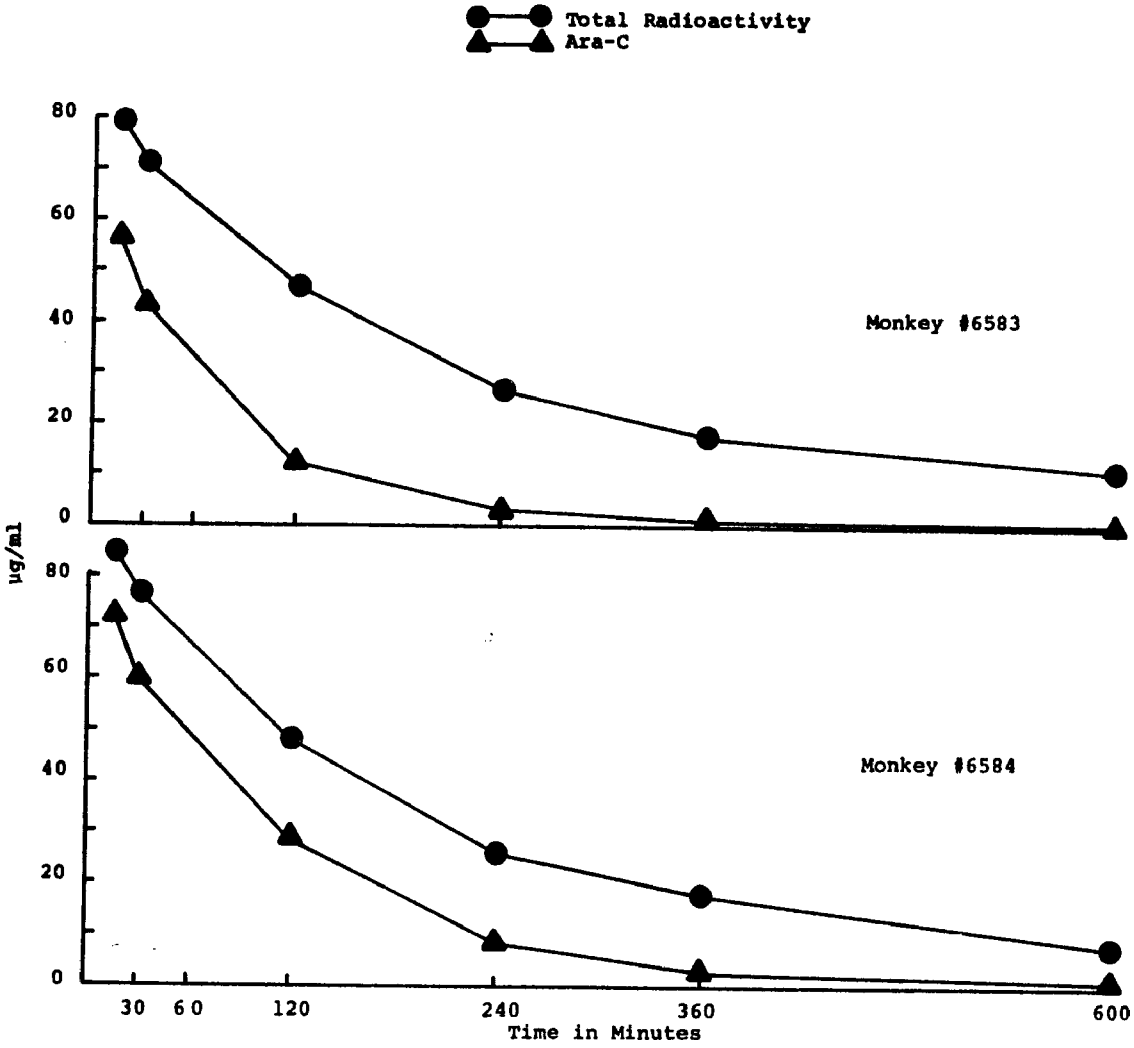


Figure 20

Serum Levels of Ara-C after 100 mg/kg H_4U and 50 mg/kg Ara-C

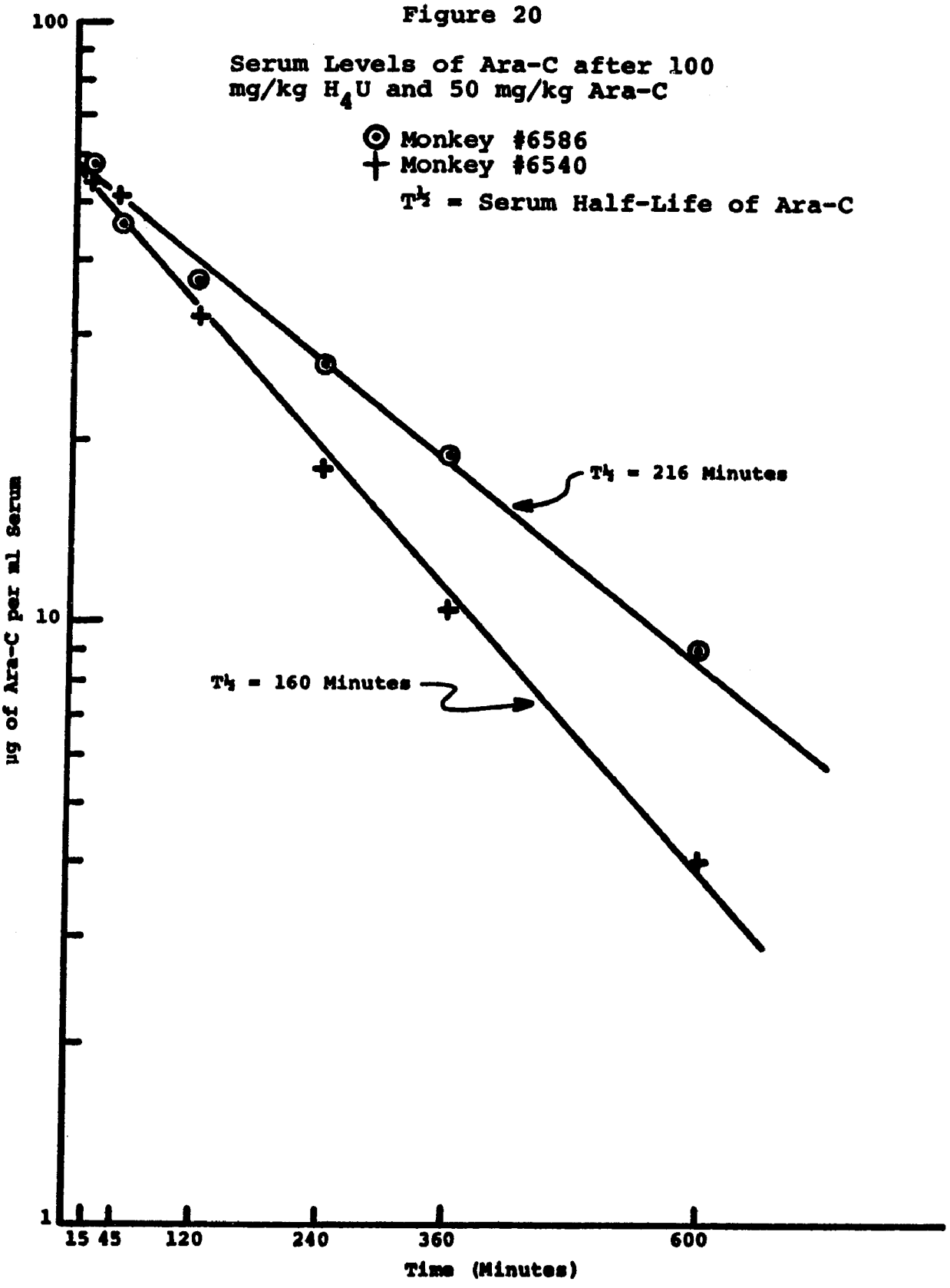


Figure 21

Serum Levels of Ara-C after 50 mg/kg H_4U and 50 mg/kg Ara-C

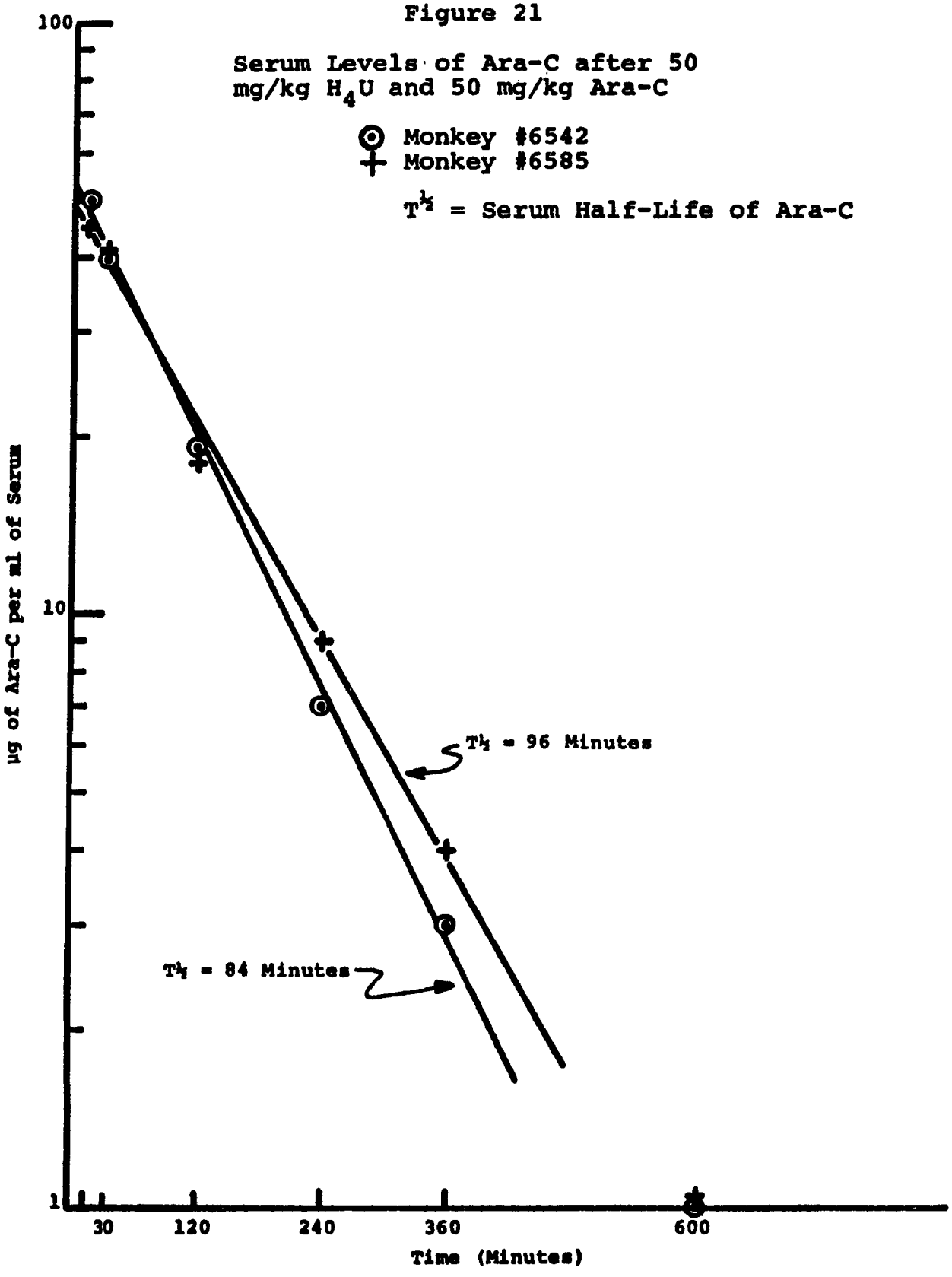


Figure 22

Serum Levels of Ara-C after 25 mg/kg H_4U and 50 mg/kg Ara-C

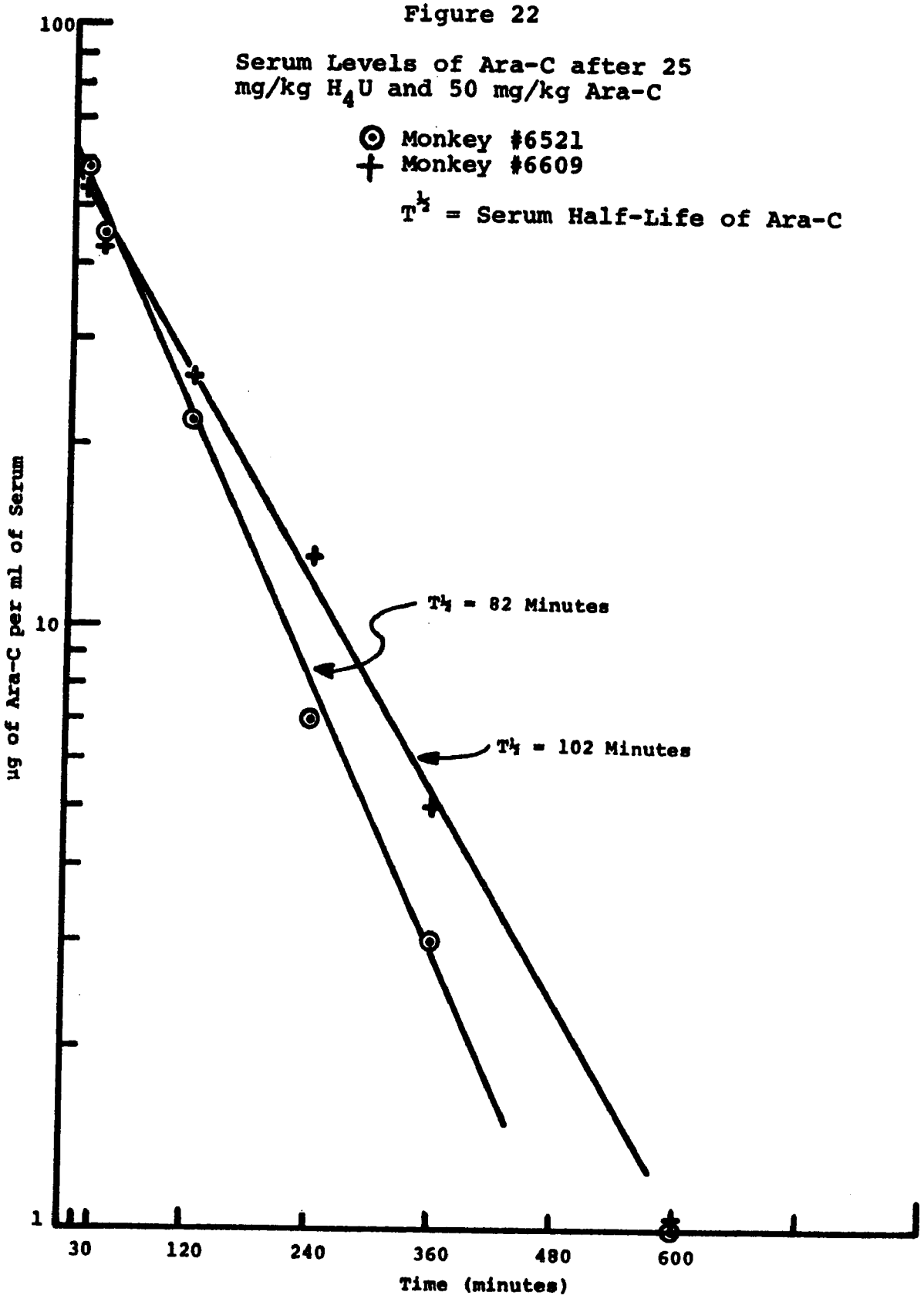


Figure 23

Serum Levels of Ara-C after 12.5 mg/kg H_4U and 50 mg/kg Ara-C

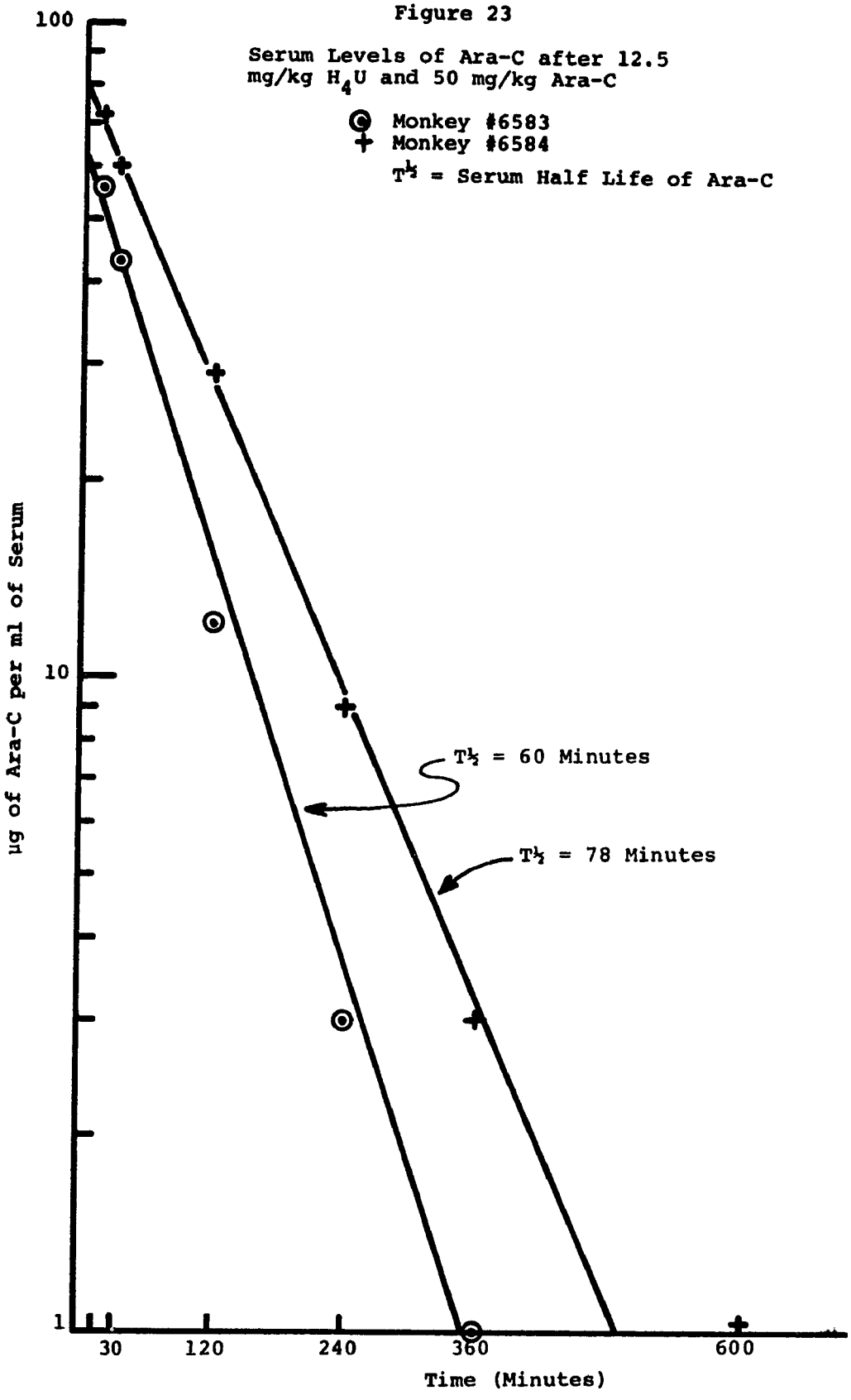


Table 13

Aminohydrolase Activity in Monkey Sera Prior to Treatment with Ara-C or H_4U + Ara-C[1]

Group	Monkey Number	Aminohydrolase Activity[2]	
		Controls	Pre-H_4U Treated
A	6586	440	712
	6540	528	627
B	6585	502	739
	6542	502	396
C	6521	898	1056
	6609	581	739
D	6584	440	581
	6583	581	686

[1]Determined after incubation with ^3H-ara-C. Substrate concentration: 3.3×10^{-4}M.

[2]Nanomoles of ara-U produced per ml of serum per 60-minute incubation.

Table 14

Total Radioactivity in Urine of Monkey after Administration of ^{3}H-Ara-C,[1] I.V., 50 mg/kg. Animals Were Pretreated with Tetrahydrouridine.

Group	Monkey Number	Total Dose Ara-C (mg)	Post-treatment Time Period (hours)	Ara-C + Ara-U[2] (mg excreted/ time period)	Total Recovery (% dose)
A	6586	150	0-4	50	36
			4-10	33	58
			10-24	34	81
			24-48	7	86
A	6540	150	0-4	67	44
			4-10	40	70
			10-24	21	84
			24-48	4	87
B	6585	170	0-4	91	54
			4-10	57	87
			10-24	16	97
			24-48	5	100
B	6542	190	0-4	106	55
			4-10	56	85
			10-24	22	96
			24-48	6	99
C	6521	198	0-4	107	54
			4-10	49	78
			10-24	22	89
			24-48	6	92
C	6609	198	0-4	27	13
			4-10	125	76
			10-24	22	87
			24-48	11	93
D	6583	180	0-4	91	50
			4-10	56	81
			10-24	24	95
			24-48	5	98
D	6584	160	0-4	48	30
			4-10	69	74
			10-24	31	93
			24-48	5	96

[1]Expressed as ara-C + Ara-U.

[2]All radioactivity was found by thin-layer chromatography to be associated with either ara-C or ara-U.

Table 15

Total 24-Hour Urinary Recovery of Ara-C and Its Metabolite, Ara-U, from Monkeys after Administration of ^{3}H-Ara-C, I.V., 50 mg/kg. Animals Had Been Pretreated with Tetrahydrouridine.

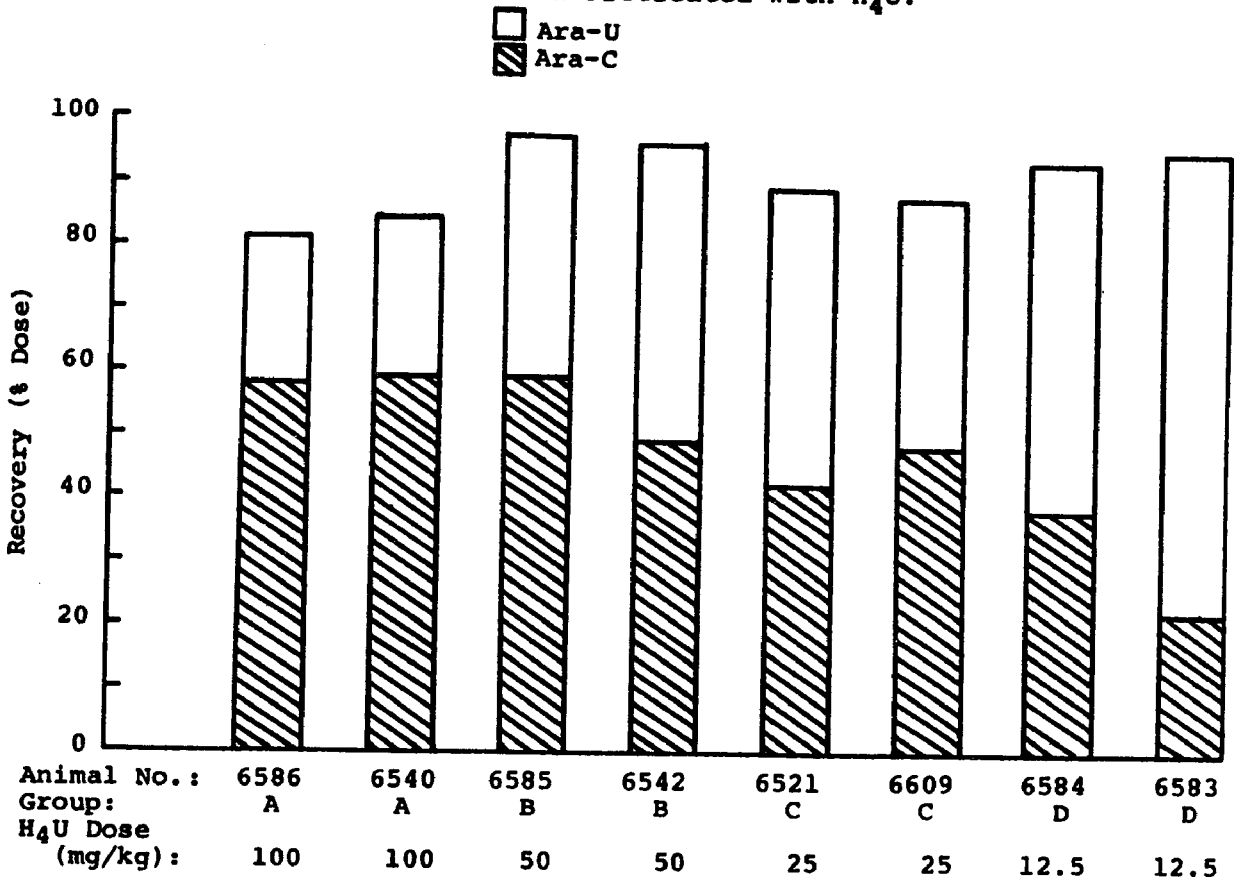
Group	Monkey Number	H_4U Dose (mg/kg)	Percent of Dose Recovered	
			Ara-C + Ara-U[1]	Ara-C[2]
A	6586	100	81	58
	6540	100	84	59
B	6585	50	97	59
	6542	50	99	49
C	6521	25	89	42
	6609	25	87	48
D	6584	12.5	93	38
	6583	12.5	95	22

[1]Determined by total radioactivity assay.

[2]Determined by thin-layer chromatography assay.

Figure 24

Total 24-Hour Urinary Recovery of Ara-C and Ara-U in the Monkey after I.V. Administration of ^{3}H-Ara-C (50 mg/kg). Animals Had Been Pretreated with H_4U.



If H_4U is to be used clinically to potentiate the chemotherapeutic effects of ara-C, it is of major importance that the compound be essentially nontoxic in its own right and when used in conjunction with ara-C. It is essential also that H_4U increase the therapeutic index of ara-C.

For the control ara-C studies, all animals displayed a modest drop in RBC four to nine days after treatment (Table 16). Only two of eight animals displayed a drop in WBC, at two to seven days posttreatment. A mild drop in percent reticulocytes was evident from two to five days after treatment in five of the eight animals. Differential counts were within normal ranges. BUN, SGOT, and alkaline phosphatase values were unaffected by ara-C treatment.

After treatment with H_4U followed by ara-C, all animals displayed significantly greater depression of WBC than with ara-C alone, especially at the higher H_4U dose levels. A modest decrease in RBC was again noted in all animals. A severe drop in percent reticulocytes was evident from two to seven days after treatment in all animals. Differential counts were not significantly different than with ara-C alone. BUN, SGOT, and alkaline phosphatase values were within normal limits.

Table 16

Hematologic Studies on Recipient of Ara-C Alone and With Tetrahydrouridine Pretreatment

	Monkey Number	Dose Ara-C (mg/kg	Day Post-Rx 2	4	5	7	9	Dose (mg/kg) Ara-C	H4U	Day Post-Rx 2	4	5	7	9
A. Erythrocyte Count	6586	50	++	++		++	++	50	100	+	++		++	++
	6540	50	+	+		+	++	50	100	N	++		+	++
	6542	50	+		+	++	++	50	50	+		+	+	++
	6585	50	N			+	++	50	50	N		++	++	++
	6521	50	+	++		++	++	50	25	+	++		+	+
	6609	50	N	+		+	+	50	25	N	+		+	+
	6584	50	N		+	+	++	50	12.5	+		++	++	++
	6583	50	N		++	++	++	50	12.5	+		++	++	++
B. Leukocyte Count	6586	50	N	N		N	N	50	100	++	++		++	+++
	6540	50	N	N		N	N	50	100	N	N		++	++
	6542	50	N		N	N	N	50	50	N		++	+	+
	6585	50	+			+	N	50	50	+		++	+	++
	6521	50	+	N		N	N	50	25	+	++		++	+
	6609	50	N	N		N	N	50	25	N	N		N	N
	6584	50	N		N	N	N	50	12.5	N		N	N	N
	6583	50	N		N	N	N	50	12.5	N		++	+	+
C. Reticulocyte (%)	6586	50	N	N		N	N	50	100	N	++		+++	N
	6540	50	+	++		N	N	50	100	N	+++		++	N
	6542	50	+		N	N	N	50	50	+++		++++	N	N
	6585	50			N	N	N	50	50	+		+++	+++	N
	6521	50	+	+		N	N	50	25	N	++++		N	N
	6609	50	+	++		N	N	50	25	++	++++		N	N
	6584	50	+++		++	N	N	50	12.5	N		+++	N	N
	6583	50	N		N	N		50	12.5	++		++++	N	N

Criteria for grading changes in formed elements of peripheral blood:

Erythrocyte counts: Reduction 500,000 - 1,000,000 re control = +
Reduction 1,100,000 - 2,000,000 re control = ++
Reduction 2,100,000 - 3,000,000 re control = +++
Reduction >3,000,000 re control = ++++

Leukocyte counts: 5,000 - 7,500/cmm = +
2,500 - 4,990/cmm = ++
1,000 - 2,490/cmm = +++
<1,000/cmm = ++++

Reticulocyte (%): Reduction 1/2 to 1/4 control = +
Reduction <1/4 to 1/20 = ++
Reduction <1/20 but countable = +++
Reduction to TFC = ++++

A single animal receiving ara-C alone vomited once between the third and fourth hour after administration. All animals receiving 100, 50, and 25 mg/kg of H_4U followed by ara-C vomited between three and six hours. One of two animals receiving 12.5 mg/kg of ara-C vomited at three hours.

To determine the effect of H_4U alone on these parameters, an experiment was initiated in which two monkeys each received 100 and 12.5 mg/kg of H_4U. The hematologic and biochemical data for samples from a similar sampling schedule as above showed no significant difference between the pre- and posttreatment samples.

To determine the <u>in vivo</u> duration of H_4U inhibitory effects, serum was collected at various time periods from the above H_4U-dosed monkeys and incubated with ^3H-ara-C. The conditions of the incubation were identical to those outlined later in studies determining the levels of aminohydrolase activity in the sera of various animal species. Whether the animals had received 12.5 or 100 mg/kg of H_4U, the same inhibitory effects were obtained. The aminohydrolase activity in the pretreatment sera ranged from 494 to 714 units (see Table 23 for definition of an aminohydrolase unit). All animal posttreatment sera up to 120 minutes showed the aminohydrolase activity to be completely inhibited. Only 4 to 5 percent of the original enzymatic

activity was present after 240 minutes, and 7 to 10 percent after 360 minutes. In the monkeys receiving 100 mg/kg of H_4U, the aminohydrolase activity was the same for the 10-hour posttreatment sera as for the 360-minute sera.

IN VITRO STUDIES

Thin-Layer Chromatography Assay Method

Arabinosyl cytosine and arabinosyl uracil have such similar solubility properties that a liquid-liquid extraction method which is adequate for complete separation of the two compounds has not been found. Until initiation of the work presented here, paper chromatography has been the major means of separating the ara-C parent compound from its metabolic products. Paper chromatography, however, is time-consuming and requires that at least 50 strips be counted for complete analysis of a single chromatogram. This separation procedure also requires that samples be protein-free, since the protein will build up at the origin during the spotting process. The most frequently utilized deproteinization reagent in this work has been trichloroacetic acid (TCA) (Camiener and Smith, 1965).

The use of TCA in this laboratory, however, caused significant loss of ara-C and ara-U. The compounds are apparently trapped within the precipitated proteins, since no direct protein binding has been demonstrated.

To alleviate these problems, a separation and assay method was developed which utilized thin-layer chromatography in conjunction with a tungstic acid deproteinization reagent (TDR). Coupled with the use of tritium-labeled

ara-C and computerized handling of data, the method (described in detail in METHODS) is quicker and more accurate than any method previously reported.

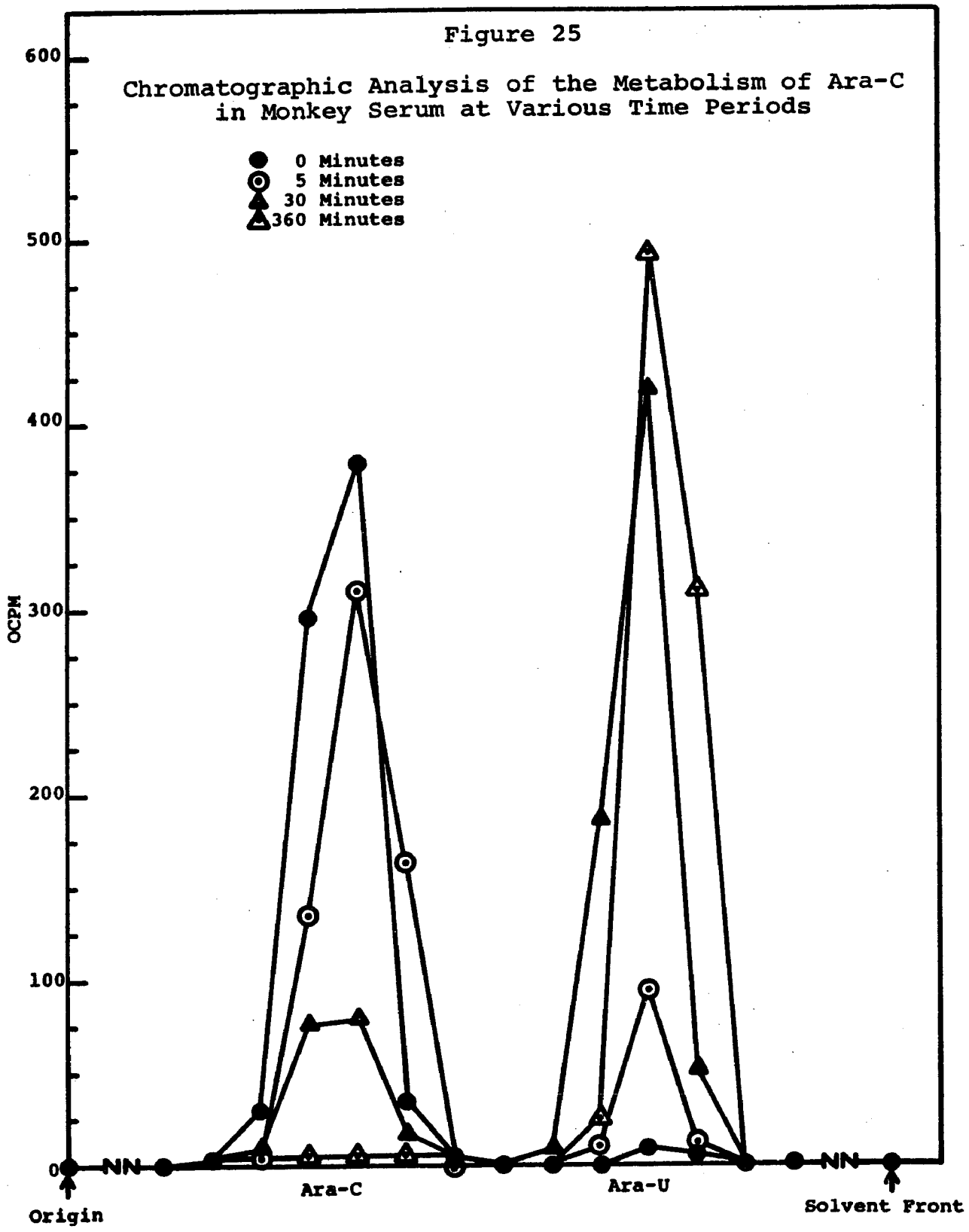
The Rf values found for ara-C and ara-U standards in the thin-layer system are 0.38 and 0.60, respectively. Values for other related compounds are: Cytidine, 0.28; cytosine, 0.40; uridine, 0.55; uracil, 0.72; and 1-(5-phosphate-β-D-arabinofuranosyl)cytosine, 0.00. The spots, visualized under ultraviolet light of 254 nm wavelength, were well localized, with no apparent tailing.

The efficiency of the system in separating ara-C from ara-U in serum is demonstrated in Figure 25. The plot represents data obtained on incubating ^{3}H-ara-C (8.3×10^{-3}M) with one milliliter of freshly collected monkey serum. After incubating at 37°C, samples were removed at various time periods and quickly added to the TDR. Following separation from the precipitated proteins, the TDR supernatant was spotted on the thin-layer media and developed as previously described. No metabolite other than ara-U was found to be present in any of the serum samples chromatographed.

Figure 26 shows a plot of data obtained with the same system but with the substitution of 0.1 M phosphate buffer (pH 7.4) for the monkey serum. No degradation of the parent compound, ara-C, occurred under the latter

Figure 25

Chromatographic Analysis of the Metabolism of Ara-C in Monkey Serum at Various Time Periods



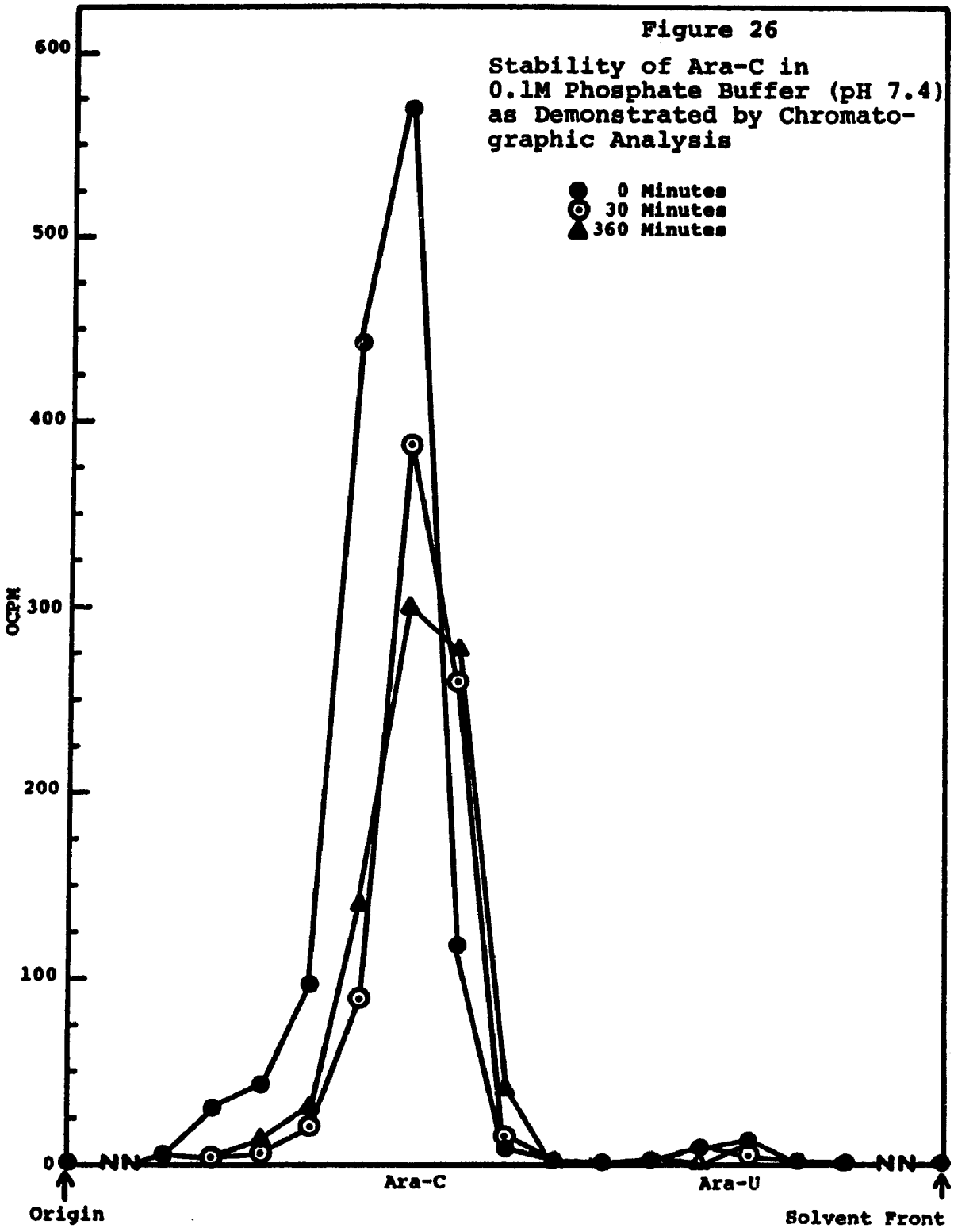


Figure 26
Stability of Ara-C in 0.1M Phosphate Buffer (pH 7.4) as Demonstrated by Chromatographic Analysis
0 Minutes
30 Minutes
360 Minutes
600
500
400
300
200
100
0
OCPM
NN
NN
Ara-C
Ara-U
Origin
Solvent Front

conditions. More detailed data from this work are shown in Table 17.

Before utilizing the above-mentioned TLC system as an assay method for cytidine aminohydrolase activity, the following information had to be known:

(1) the effectiveness of TDR in stopping the enzymatic reaction;

(2) the stability of ara-C and ara-U in the TDR supernate;

(3) recovery of ara-C and ara-U from the serum samples after precipitation of proteins with TDR;

(4) degree of elution of ara-C, ara-U, and any of their degradation products from the thin-layer media;

(5) the effect of ara-U concentration on the metabolism of ara-C; and

(6) the relationship between the concentration of ara-U produced and levels of cytidine aminohydrolase activity.

The results of experiments designed to answer these questions are summarized in the following paragraphs.

On incubating ara-C with monkey serum, the compound is rapidly metabolized to ara-U. However, a zero-time-period sample removed from the incubation solution and

Table 17

Chromatographic Analysis of *in Vitro* Metabolism of Ara-C in Monkey Serum and Phosphate Buffer Mixtures

Incubation Mixture	Time Period (minutes)	Percent Total Radioactivity		
		Ara-C	Ara-U	Ara-C + Ara-U
Serum + ara-C	0	94	1	98
	5	84	13	98
	10	68	27	97
	20	46	50	98
	30	29	67	98
	60	2	94	99
	120	<2	92	97
	240	<2	93	97
	360	<2	92	97
Buffer + ara-C	0	96	0	98
	30	94	0	97
	360	96	0	98

immediately transferred into a centrifuge tube containing TDR showed that less than one percent of the ara-C substrate had been metabolized to ara-U. TDR was far more effective in stopping this enzymatic reaction than TCA, freezing, or boiling at 100°C.

Tritium-labeled ara-C and ara-U are quite stable in TDR. Solutions which were frozen for over four months and then reanalyzed still showed the same ara-C/ara-U ratio, as determined by thin-layer and paper chromatography. No other radiolabeled products appeared.

The stability of phosphorylated ara-C metabolites was established by the following experiment. Two TDR supernates of calf thymus homogenates, which had been previously incubated with ^{3}H-ara-C, were reassayed by TLC for ara-C and its phosphorylated products. The samples had been frozen for 30 days. The initial assay found the phosphorylated products to be 22 percent and 36 percent in the two supernates. Ara-C represented 72 and 67 percent of the total radioactivity. Reassay of the samples showed 23 and 38 percent phosphorylated products and 72 and 66 percent ara-C.

The TLC system does not separate the individual phosphorylated products, since all are left at the origin. It can only be concluded that the nucleotides did not break down to nucleosides. No conclusion regarding

interconversion of nucleotides can be drawn from this study.

The recovery of ara-C from dog serum, rat tissue homogenates, and saline, after treatment with TDR, is shown in Table 18. The homogenates were prepared by adding four milliliters of saline to one gram of tissue and homogenizing in an Omni-Mix homogenizer for 3 minutes. To assay for ara-C recovery, 0.1 ml of ^{3}H-ara-C solution (1 mg/ml) was added to one milliliter of the various preparations (serum, homogenate, or saline). The solutions were mixed well and 0.2 milliliters were removed and added to 2 ml of TDR. After standing at room temperature for 15 minutes, the solutions were centrifuged for an additional 15 minutes. Since each preparation was devoid of aminohydrolase activity, ara-C content was determined by assaying for total radioactivity in each TDR supernate. The assay was accomplished by adding 0.1 ml of the supernate to a vial containing 15 ml of special aqueous scintillation solution and counting each vial in a liquid scintillation spectrometer. The ara-C associated radioactivity found in the serum and homogenate TDR solutions ranged from 86 to 91 percent of that recovered in the nonprotein saline standard after the addition of TDR. The assays were done in triplicate for each preparation.

Table 18

Recovery of Ara-C from Biological Materials after Tungstic Acid Deproteinization

Sample	Run	DPM[1]	Average DPM	Average Percent Recovery
Dog Serum	1	34,044	31,827	87
" "	2	31,132		
" "	3	30,305		
Rat Liver Homogenate	1	29,142	30,866	86
" " "	2	32,875		
" " "	3	30,582		
Rat Kidney Homogenate	1	33,497	32,523	91
" " "	2	28,694		
" " "	3	34,377		
Saline	1	36,310	35,925	100[2]
"	2	36,630		
"	3	34,836		

[1]Corrected for supernatant volume.
[2]Value set at 100 percent.

The thin-layer assay method includes a water elution of ara-C, ara-U, and the phosphorylated metabolites from the thin-layer media prior to adding the scintillation cocktail. A comparison of the DPMs in vials containing TLC strips on which standards were spotted to the counts after the strips were removed from the vials demonstrates the effectiveness of this procedure. The results indicated that over 98 percent of the radioactivity was eluted from the TLC strip.

In an experiment designed to determine the effect of various concentrations of ara-U on the in vitro metabolism of ara-C, the ara-U was added to monkey serum before adding the substrate ^{3}H-ara-C (350 nanomoles). After incubation for 15 minutes, the amount of ^{3}H-ara-C produced in each sample was determined by the TLC procedure. The results (Table 19) show that ara-U, within the concentration range studied (0-350 nanomoles), had no significant effect on the metabolism of ara-C in monkey serum.

Monkey serum was utilized as a source of aminohydrolase enzyme to determine the effect of enzyme and substrate concentration on the rate of deamination of ara-C. The effect of various enzyme concentrations (in reality, dilutions of monkey serum containing high enzymatic activity) is shown in Table 20. The data are plotted in Figure 27 and demonstrate good linearity.

Table 19

Effect of Varying Concentrations of Ara-U on the In Vitro Metabolism of Ara-C by Monkey Serum[1]

Ara-U Added (nanomoles)	Aminohydrolase Activity[2]
350	368
213	368
175	344
88	368
0	344

[1]Incubation solution:
0.5 ml serum +
0.4 ml Robinson's solution +
0.1 ml ara-U standard solution +
0.1 ml ^{3}H-ara-C (1 mg/ml; 350 nanomoles)
Incubation at 37°C for 15 minutes.

[2]Nanomoles ^{3}H-ara-U produced per milliliter of serum per 60 minutes incubation.

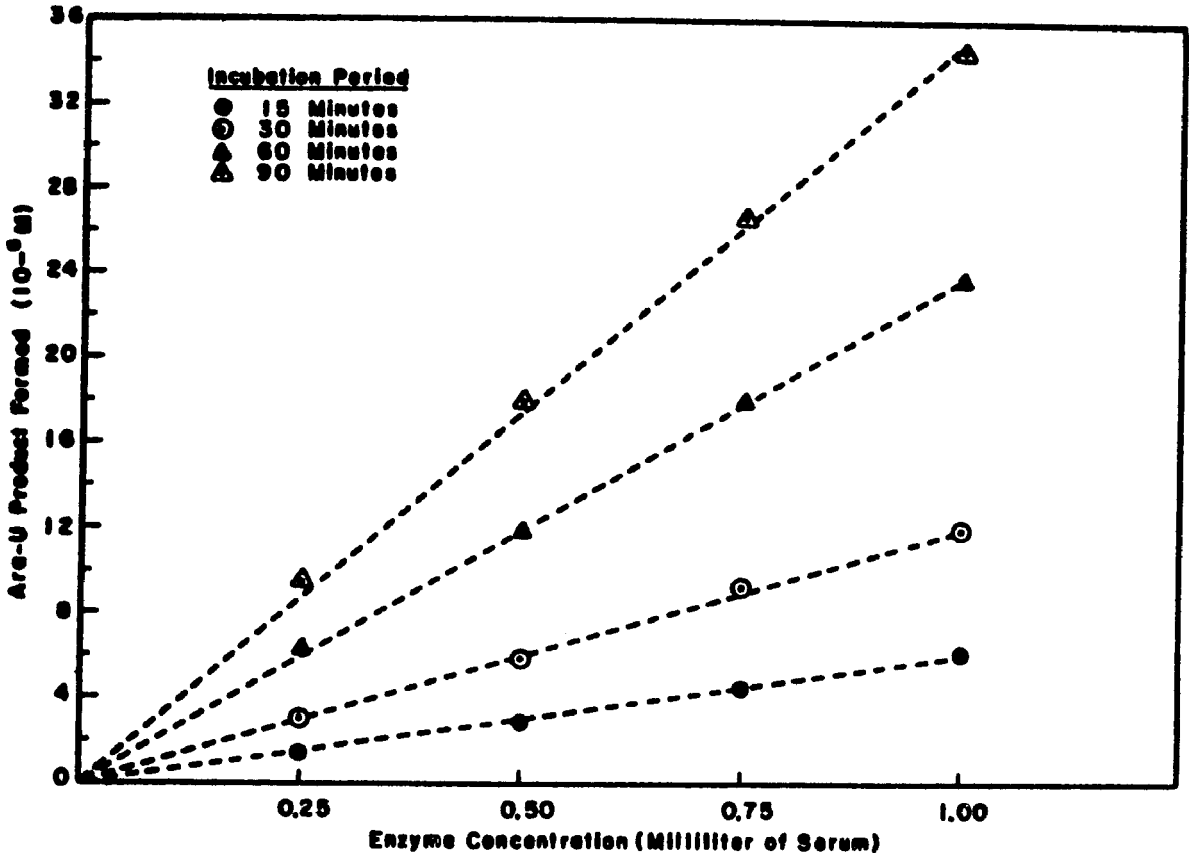
Table 20

Effect of Enzyme Concentration on Rate of Deamination of Ara-C in Monkey Serum

Enzyme Concentration (ml of Serum)	Time Period (minutes)	Percent Radioactivity		
		Ara-C	Ara-U	Ara-C + Ara-U
1.00	15	84.5	9.7	97.4
0.75	15	87.2	7.1	97.5
0.50	15	90.5	4.5	98.2
0.25	15	92.9	2.2	98.3
1.00	30	75.3	19.0	97.5
0.75	30	79.9	14.8	97.9
0.50	30	85.4	9.4	98.0
0.25	30	89.8	4.8	97.7
1.00	60	56.5	37.8	97.5
0.75	60	65.9	28.5	97.6
0.50	60	76.0	18.9	98.1
0.25	60	85.3	9.9	98.4
1.00	90	39.8	54.8	97.8
0.75	90	52.1	42.1	97.4
0.50	90	66.2	28.4	97.8
0.25	90	80.5	15.0	98.7

Figure 27

Effect of Enzyme Concentration on the Rate of Deamination of Ara-C in Monkey Serum



The effect of sbustrate concentration on the rate of deamination of ara-C by monkey serum animohydrolase activity is shown in Tables 21 and 22. The data are plotted as $\frac{1}{V}$ versus $\frac{1}{S}$ in Figure 28. For the reactions involving small changes in substrate concentration, the K_m values for this crude enzyme system were 1.3 and 1.5×10^{-4}M. These values agree with the K_m range of 1.2 to 1.6×10^{-4}M found by Camiener (1967) in a crude enzyme system from human liver homogenates.

In summary, it was concluded that the thin-layer system used in conjunction with TDR was satisfactory as a specific and accurate assay method for the determination of cytidine aminohydrolase activity in biological materials.

Serum Cytidine Aminohydrolase Activity

The aminohydrolase activities in the sera of mice, rats, hamsters, dogs, monkeys, and humans are shown in Table 23. One milliliter of serum was incubated at 37°C with 350 nanomoles of ^{3}H-ara-C for a time period sufficient to allow greater than 10 percent but less than 30 percent conversion of the substrate to the ara-U product. A zero-time-period sample, a non-serum control, and a stock monkey serum sample — containing a predetermined amount of enzyme activity — was assayed with each serum sample. The percent of ara-U formed was determined by the thin-layer assay procedure as outlined earlier.

Table 21

Effect of Substrate Concentration on Rate of Deamination of Ara-C in Monkey Serum

Substrate Concentration (10^{-4}M)	Time Period (minutes)	Percent Radioactivity		
		Ara-C	Ara-U	Ara-C + Ara-U
6.33	5	88.8	5.3	94.1
3.17	5	84.3	8.6	92.9
1.58	5	82.6	13.2	95.8
0.32	5	68.7	22.3	91.0
6.33	10	83.0	10.2	93.2
3.17	10	73.8	17.1	90.9
1.58	10	61.7	27.1	88.8
0.32	10	47.4	42.7	90.1
6.33	20	67.4	27.1	94.5
3.17	20	51.4	42.6	94.0
1.58	20	31.8	58.1	89.9
0.32	20	19.8	64.6	84.4
6.33	30	57.6	36.6	94.2
3.17	30	38.8	54.1	92.9
1.58	30	21.0	72.4	93.4
0.32	30	10.0	74.3	84.3
6.33	60	31.8	63.5	95.3
3.17	60	11.7	82.2	93.9
1.58	60	4.3	91.7	96.0
0.32	60	11.4	72.5	83.9

Table 22

Kinetic Analysis of the Effect of Substrate Concentration on Rate of Deamination of Ara-C in Monkey Serum

Time Period (minutes)	S (10^{-4}M)	$\frac{1}{S}$ (10^{3}M)	V (10^{-5}M)	$\frac{1}{V}$ (10^{3}M)	Km (10^{-4}M)
5	6.33	1.58	3.35	29.9	1.5
5	3.17	3.15	2.73	36.6	
5	1.58	6.33	2.09	47.8	
5	0.32	31.25	0.71	140.8	
10	6.33	1.58	6.46	15.5	1.3
10	3.17	3.15	5.42	18.5	
10	1.58	6.33	4.28	23.4	
10	0.32	31.25	1.37	73.0	
20	6.33	1.58	17.15	5.8	2.5
20	3.17	3.15	13.50	7.4	
20	1.58	6.33	9.18	10.9	
20	0.32	31.25	2.07	48.3	
30	6.33	1.58	23.17	4.3	3.3
30	3.17	3.15	17.15	5.8	
30	1.58	6.33	11.44	8.7	
30	0.32	31.25	2.38	42.0	
90	6.33	1.58	40.20	2.5	9.1
90	3.17	3.15	26.06	3.8	
90	1.58	6.33	14.49	6.9	
90	0.32	31.25	2.32	43.1	

Figure 28

Effect of Substrate Concentration on the Rate of Deamination of Ara-C in Monkey Serum

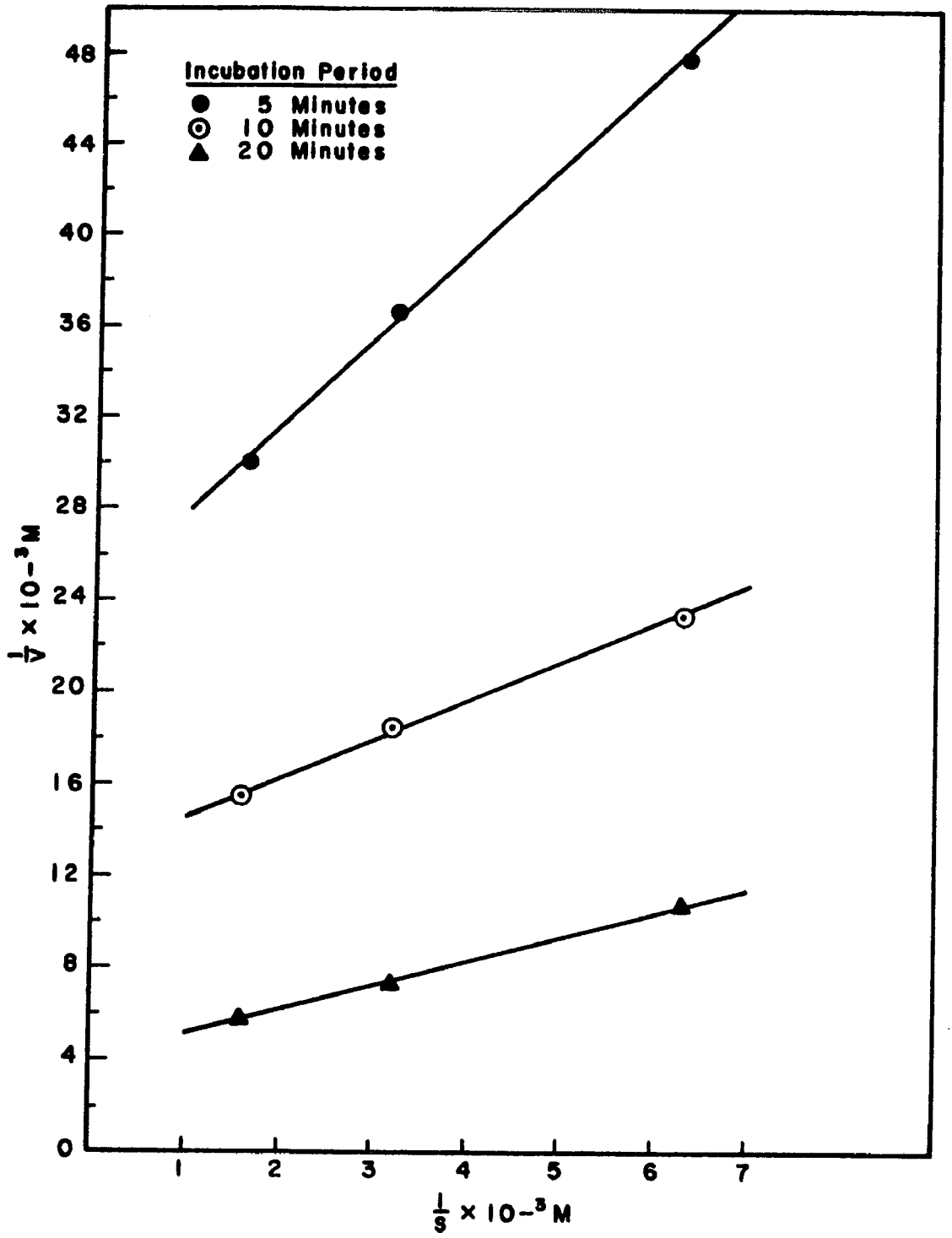


Table 23

Aminohydrolase Activity in Sera of Mice, Rats, Hamsters, Dogs, Monkeys, and Humans as Determined by Incubation with Arabinosyl Cytosine

Species		Sex	Incubation Period (minutes)	Aminohydrolase Activity*
MOUSE:	Group 1	M	60	10
	Group 2	M	60	10
	Group 3	M	60	13
RAT:	Group A	M	60	7
	Group B	F	60	7
	Group C	M	60	7
HAMSTER:	Group 1	M	60	7
	Group 2	M	60	<5
DOG #:	8	F	60	20
	65	F	60	13
	S-297	F	60	13
MONKEY #:	301	F	15	784
	303	F	15	900
	380	F	15	444
	20	M	15	392
	24	M	15	288
	697	M	15	432
	6586	M	15	440
	6540	F	15	528
	6585	M	15	502
	6521	F	15	898
	6609	M	15	581
HUMAN:	MEH	M	60	26
	MEK	M	60	30
	JDS	F	60	36
	MGW	F	60	33
	MGW	F	60	23
	LBM	M	60	17
	RLW	M	60	23
	ST	M	60	13
	REW	M	60	23
CONTROL: Phosphate buffer (0.2 M) pH 7.4			60	<5

*Nanomoles ara-U produced per ml serum per 60 minutes incubation.

One aminohydrolase activity unit is equal to one nanomole of ara-U produced per one milliliter of serum incubated at 37°C for 60 minutes.

The results show the mouse, rat, and hamster sera had a very low level of aminohydrolase activity (<5 to 20 units). The level in human and dog sera was slightly higher (13 to 36 units), whereas in monkey sera levels were extremely high (288 to 900 units) — 10 to 25 times the maximum level in normal human sera. The magnitude of individual variation in monkey sera was approximately threefold. Due to its extremely high serum aminohydrolase activity, the monkey, or monkey serum, was used as an ultimate challenge for both in vivo and in vitro inhibitors of this enzyme.

The aminohydrolase activity in sera of various cancer patients as determined by incubation with ^{3}H-ara-C is shown in Table 24. Sera from Group-A patients were frozen for up to seven months before assay. Sera from Group-B patients were refrigerated for several days before freezing; these samples were frozen for about one week before assay. Group-C samples were frozen for approximately one month.

The aminohydrolase activity units in sera from cancer patients had a much wider range (0 to 192) than normal humans (13 to 36). There is no apparent correlation between level of aminohydrolase activity and sex,

Table 24

Aminohydrolase Activity in Sera of Various Cancer Patients as Determined by Incubation with Arabinosyl Cytosine

Patient[1]	Sex	Diagnosis	Aminohydrolase Activity[2]
Group A			
70217	M	C.M.L.	10
64941	M	A.M.L.	7
69779	F	Melanoma	13
66874	M	Hodgkin's	26
68650	M	Hodgkin's	10
70099	M	A.L.L.	7
69160	M	Sarcoma	30
60558	F	Fibrosarcoma	7
62110	F	Melanoma	10
65053	M	Metastatic cancer	10
64821	M	C.L.L.	52
70218	M	C.M.L.	7
52897	M	A.M.L.	7
68-2265	F	Melanoma	17
Group B			
HW	F	C.L.L.	30
GMA	F	Hodgkin's	0
RL	F	C.L.L.	33
NC	F	C.L.L.	23
CW	M	Lymphosarcoma	17
EB	M	C.L.L.	20
VS	F	C.L.L.	13
CAW	M	C.G.L.	23
EY	F	C.M.L.	46
EY	F	C.G.L.	7
ACS	M	Lymphoma	43
Group C			
KF	M	A.M.L.	3
ED	F	A.M.L.	192
GO	F	A.M.L.	3
KF	M	A.M.L.	26

[1]Serum provided by:
Group A: Dr. James K. Luce, M. D. Anderson Hospital, Houston, Texas
Group B: Dr. Charles Butterworth, U. of Ala., Birmingham, Alabama
Group C: Dr. Thomas C. Hall, U. of Rochester, Rochester, N. Y.

[2]Nanomoles ara-U produced per ml serum per 60 min. incubation.

source of samples, or diagnosis of disease.

The degree of variation of serum aminohydrolase activity in the various patients may explain some of the variable clinical response to ara-C therapy when the drug is administered by low-level, continuous infusion.

Effects of Tetrahydrouridine on the In Vitro Deamination of Ara-C

Effects of various concentrations of tetrahydrouridine on the rate of deamination of ara-C by monkey serum are shown in Table 25. The serum utilized was from Monkey #24 and contained 288 units of aminohydrolase activity (see Table 23). Under the incubation conditions described, the minimum H_4U concentration completely inhibiting deamination of ara-C substrate (6.2×10^{-4}M) was 6.2×10^{-5}M. On this basis, 1×10^{-4}M was assumed a sufficient concentration of H_4U as a collection-tube additive for the *in vivo* work.

Tissue Cytidine Aminohydrolase and Dexoycytidine Kinase Activity

To determine tissue distribution of both aminohydrolase and kinase activities involved in the metabolism of ara-C, a series of experiments was designed in which various tissue homogenates (liver, kidney, spleen, and small intestine) were incubated with ara-C and assayed for the formation of ara-U and nucleotide metabolites. The tissues assayed were from the same animal species

Table 25

Effects of Various Concentrations of Tetrahydrouridine (H_4U) on the Rate of Deamination of Ara-C by Monkey Serum[1]

H_4U Concentration (M)	Total OCPM	Percent Radioactivity[2]		
		Ara-C	Ara-U	Ara-C + Ara-U
6.24×10^{-4}	1092	92.9	0.0	96.1
3.12×10^{-4}	1062	94.6	0.0	96.8
1.56×10^{-4}	1044	93.1	0.8	97.2
7.80×10^{-5}	1084	94.1	0.0	97.3
6.24×10^{-5}	1188	91.0	0.0	94.3
3.12×10^{-5}	1198	92.5	0.5	96.3
1.56×10^{-5}	1213	92.4	1.4	97.1
7.80×10^{-6}	1146	92.4	1.9	97.6
3.12×10^{-6}	1185	87.9	5.8	96.9
1.56×10^{-6}	1180	84.0	9.6	96.9
Controls[3]: A	1234	29.0	64.7	97.0
B	1164	25.3	71.4	98.7
C	1232	26.1	66.1	95.5

[1]Substrate concentration = 6.24×10^{-4}M.
[2]Determined by thin-layer chromatography.
[3]Serum without H_4U.

as those used in the in vivo studies. One unit of aminohydrolase activity is equal to one nanomole of ara-U produced per gram of wet tissue per 30 minutes incubation at 37°C. One unit of kinase activity is equal to one nanomole of nucleotides (ara-C equivalents) formed per gram of wet tissue per 30 minutes incubation. In defining the unit of kinase activity, neither the type nor molecular weight of nucleotide is taken into consideration.

The design of the experiment (see METHODS) allowed for duplicate assays and controls, while limiting the number of samples. Duplication was accomplished by inhibiting one enzyme while assaying for the other. H_4U was used to inhibit aminohydrolase activity, while the absence of ATP inhibited kinase activity. Monkey serum and calf thymus homogenates were used as positive aminohydrolase and kinase controls, respectively.

Since two enzyme activities were being assayed simultaneously, it was important to ascertain the effect of incubation conditions on each enzyme system.

Incubation temperature and pH were adjusted for maximum kinase activity since the aminohydrolase activity was insensitive, within a wide range, to both effects. On the other hand, it was necessary to determine the effect of NaF, ATP, and H_4U by further investigation.

Effect of F^- on phosphatase activity

Kinase activity was assayed by determining the amount of ara-C nucleotides formed. This method is valid only if the nucleotides formed are not metabolized back to the original nucleoside. Therefore, F^- is used in the incubation mixture in order to inhibit any phosphatase enzyme which might be present. The effectiveness of the inhibitor in this system was determined by the following experiment:

To two flasks (A and B) containing rat liver homogenate (phosphatase enzyme source) was added the basic incubation mixture as outlined earlier. Two additional flasks (C and D) were treated similarly, except no NaF was added. The substrate used for the reaction was the 5'-monophosphate derivative of ara-C. After incubation, protein precipitation, and thin-layer chromatography of the TDR supernates, the formed ara-C was located under UV light, cut from the ITLC sheets, and placed in counting vials. To the vials was added 5 ml of 0.1N HCl and the vials were shaken for several minutes. The solution was then poured into tubes and centrifuged. The clear supernate was assayed for ara-C content by determination of optical density readings on a Beckman DU spectrophotometer at 280 nm. Blanks were established by cutting out the area located in a blank channel adjacent to the ara-C spots.

The comparison of the phosphatase activity, as determined by the degradation of 5'P-ara-C to ara-C in the two groups of flasks, is shown in Table 26. The flasks containing NaF exhibited less than three percent of the enzymatic activity found in the flasks with no NaF. This inhibition would be sufficient to eliminate any dephosphorylation of formed nucleotides in the above assay.

Although F^- was an effective inhibitor of the phosphatase enzyme, it was necessary to demonstrate that it does not also inhibit the aminohydrolase and kinase enzymes.

The effect of NaF on kinase activity in the above procedure was determined by the following experiment:

To two flasks (A and B) containing calf thymus homogenate (kinase enzyme source) was added the basic incubation mixture as outlined earlier. Two other flasks (C and D) were treated in the same manner as A and B, except no NaF was added. The incubation and assay procedures were the same as in the earlier sections.

Kinase activity assayed in the incubation solution containing NaF was 109-119 percent of the activity in the solution without NaF (Table 27). Apparently NaF slightly enhanced the kinase activity.

To determine the effect of NaF on the aminohydrolase assay, the following experiment was performed:

Table 26

Inhibition of Phosphatase Activity
in Rat Liver Homogenates as Determined by Incubation
with 5'-Monophosphate Derivative of Arabinosyl Cytosine

Sample	Incubation Solution[1]	Time Period	Optical Density Reading	Phosphatase Activity
A	Basic without NaF	0	0.0055	
		60	0.0465	100[2]
B	Basic without NaF	0	0.0087	
		60	0.0464	90
C	Basic	0	0.0065	
		60	0.0077	2.6
D	Basic	0	0.0066	
		60	0.0077	2.6

[1]The basic incubation mixture contained NaF, $MgCl_2$, ATP, and Tris-HCl buffer (pH 8.0).

[2]Maximum phosphatase activity set at a value of 100.

Table 27

Effect of F^- on Aminohydrolase and Kinase Activity as Determined by Incubation with Arabinosyl Cytosine

Tube	Enzyme Source	Incubation Solution[1]	Percent Radioactivity Total Ara-U	Percent Radioactivity Nucleotide[2]	Enzyme Activity[3]
A	Calf thymus	Basic with NaF	-	9.8	109
B	"	Basic with NaF	-	10.7	119
C	"	Basic without NaF	-	8.4	93
D	"	Basic without NaF	-	9.0	100
E	Mouse kidney	Basic with NaF	73.0	-	101
F	"	Basic with NaF	72.5	-	100
G	"	Basic without NaF	72.6	-	100
H	"	Basic without NaF	72.3	-	100

[1]The basic incubation mixture contained NaF, $MgCl_2$, ATP, and Tris-HCl buffer, pH 8.0. Substrate concentration was 1.13×10^{-5}M.

[2]Radioactivity remaining at origin.

[3]Maximum enzymatic activity set at a value of 100.

To two flasks (E and F) containing mouse kidney homogenate was added the basic incubation mixture. An additional two flasks (G and H) were treated similarly, except no NaF was added. Incubation and assay were as outlined earlier.

Aminohydrolase activity in incubation solutions containing NaF was 100-101 percent of that determined in the solution without NaF. Therefore, NaF had no effect on aminohydrolase activity in mouse kidney homogenates.

Effect of ATP and H_4U on aminohydrolase and kinase activity

Data on enzyme activity in mouse, hamster, and monkey tissue homogenates (Tables 28, 30, and 32) indicate that ATP had no effect on aminohydrolase activity (good duplication in Groups A and C) and was a necessary co-factor for the kinase activity (no activity in Group C). These data also demonstrate the inhibitory effect of H_4U on the aminohydrolase activity (no activity in Group B) and lack of effect on the kinase actiivty (good duplication in Groups A and B).

Tissue Cytidine Aminohydrolase Activity

One aspect of the in vitro metabolism studies involved the determination of cytidine aminohydrolase activity in the tissues of various animals. Fresh tissue was removed,

Table 28

Kinase and Aminohydrolase Activity in Mouse Tissue Homogenates as Determined by Incubation with Arabinosyl Cytosine

Group	Tissue	Incubation Solution[1]	Kinase Activity[2]	Amino-hydrolase Activity[3]
A	Liver	Basic	<5	65
	Kidney		5	1314
	Spleen		65	<5
	Small Intestine		<5	125
B	Liver	Basic	<5	5
	Kidney	with H_4U	5	15
	Spleen		65	<5
	Small Intestine		<5	20
C	Liver	Basic	<5	60
	Kidney	without ATP	<5	1301
	Spleen		<5	<5
	Small Intestine		<5	105

[1]Basic incubation mixture contained NaF, $MgCl_2$, ATP, and Tris-HCl buffer (pH 8.0).

[2]Nanomoles nucleotides (ara-C equivalents) formed per gram wet tissue per 30 minutes incubation.

[3]Nanomoles arabinosyl uracil formed per gram wet tissue per 30 minutes incubation.

Table 29

Kinase and Aminohydrolase Activity in Rat Tissue Homogenates as Determined by Incubation with Arabinosyl Cytosine

Group	Tissue	Incubation Solution[1]	Kinase Activity[2]	Amino-hydrolase Activity[3]
A	Liver	Basic	5	<5
	Kidney		5	<5
	Spleen		15	<5
	Small Intestine		<5	15
	Thymus		95	<5
	Bone Marrow		36	9
B	Liver	Basic	5	<5
	Kidney	with H_4U	5	<5
	Spleen		10	<5
	Small Intestine		<5	<5
C	Liver	Basic	5	<5
	Kidney	without ATP	5	<5
	Spleen		<5	<5
	Small Intestine		<5	10

[1]Basic incubation mixture contained NaF, $MgCl_2$, ATP, and Tris-HCl buffer (pH 8.0).

[2]Nanomoles nucleotides (ara-C equivalents) formed per gram wet tissue per 30 minutes incubation.

[3]Nanomoles arabinosyl uracil formed per gram wet tissue per 30 minutes incubation.

Table 88

Kinase and Aminohydrolase Activity in Hamster Tissue Homogenates as Determined by Incubation with Arabinosyl Cytosine

Group	Tissue	Incubation Solution[1]	Kinase Activity[2]	Amino-hydrolase Activity[3]
A	Liver	Basic	<5	702
	Kidney		<5	864
	Spleen		36	200
	Small Intestine		<5	630
B	Liver	Basic with H_4U	<5	27
	Kidney		<5	18
	Spleen		36	18
	Small Intestine		<5	36
C	Liver	Basic without ATP	9	774
	Kidney		<5	774
	Spleen		9	206
	Small Intestine		<5	630

[1]Basic incubation mixture contained NaF, $MgCl_2$, ATP, and Tris-HCl buffer (pH 8.0).

[2]Nanomoles nucleotides (ara-C equivalents) formed per gram wet tissue per 30 minutes incubation.

[3]Nanomoles arabinosyl uracil formed per gram wet tissue per 30 minutes incubation.

Table 31

Kinase and Aminohydrolase Activity in Dog Tissue Homogenates as Determined by Incubation with Arabinosyl Cytosine

Group	Tissue	Incubating Solution[1]	Kinase Activity[2]	Amino-hydrolase Activity[3]
A	Liver	Basic	<5	315
	Kidney		<5	<5
	Spleen		<5	<5
	Small Intestine		<5	<5
B	Liver	Basic	<5	<5
	Kidney	with H_4U	<5	<5
	Spleen		<5	<5
	Small Intestine		<5	<5
C	Liver	Basic	<5	315
	Kidney	without ATP	<5	<5
	Spleen		<5	<5
	Small Intestine		<5	<5

[1]Basic incubation mixture contained NaF, $MgCl_2$, ATP, and Tris-HCl buffer (pH 8.0).

[2]Nanomoles nucleotides (ara-C equivalents) formed per gram wet tissue per 30 minutes incubation.

[3]Nanomoles arabinosyl uracil formed per gram wet tissue per 30 minutes incubation.

Table 32

Kinase and Aminohydrolase Activity in Monkey Tissue Homogenates as Determined by Incubation with Arabinosyl Cytosine

Group	Tissue	Incubation Solution[1]	Kinase Activity[2]	Amino-hydrolase Activity[3]
A	Liver	Basic	<5	1,890
	Kidney		<5	1,440
	Spleen		45	6,390
	Small Intestine		<5	4,680
B	Liver	Basic	<5	<5
	Kidney	with H_4U	<5	<5
	Spleen		45	<5
	Small Intestine		<5	<5
C	Liver	Basic	<5	2,070
	Kidney	without ATP	<5	1,440
	Spleen		<5	6,300
	Small Intestine		<5	4,680

[1]Basic incubation mixture contained NaF, $MgCl_2$, ATP, and Tris-HCl buffer (pH 8.0).

[2]Nanomoles nucleotides (ara-C equivalents) formed per gram wet tissue per 30 minutes incubation.

[3]Nanomoles arabinosyl uracil formed per gram wet tissue per 30 minutes incubation.

homogenized, and incubated as described under METHODS.

Tables 28 through 32 show the results obtained for each animal. Aminohydrolase activity was present in all monkey and hamster tissues assayed. Significant activity was found only in mouse kidney and small intestine and dog liver. Essentially no activity was present in any rat tissues. Monkey spleen and small intestine contained extremely high activity. All monkey tissues exhibited higher activity than any other animal tissues studied. In the mouse, the only tissue which showed a high level of activity was the kidney. All hamster tissue assayed displayed an unexpected high level of aminohydrolase activity; levels in kidney, liver, and small intestine were equal to and approximately three times that found in the spleen. The only enzymatic activity in the dog was found in liver tissue.

The minimum activity for the enzymatic assay was set at a value of <5 units for both aminohydrolase and kinase activity determinations. This value represents <0.5 percent of substrate conversion to produce — either as ara-U or as a phosphorylated derivative of ara-C.

When product formation exceeded 50 percent of substrate concentration, the samples were reassayed using a shorter incubation period.

Results from Group A (basic incubation mixture) and Group C (incubation mixture without ATP) demonstrate good duplication for aminohydrolase activity determination. The results of Group B (incubation mixture plus H_4U) indicate essentially no ara-U is produced if the aminohydrolase enzyme is inhibited.

Table 33 is a comparative grading system for the aminohydrolase activity in the five animals studied.

Tissue Deoxycytidine Kinase Activity

Deoxycytidine kinase activities in the liver, kidney, spleen, and small intestine of the various animals are shown in Tables 28-32. Thymus and bone marrow of the rat and spleens of all animals except the dog displayed kinase activity. No activity was found in any of the other animal tissues.

The results from Group A (basic incubation mixture) and Group B (incubation mixture plus H_4U) demonstrate good duplication for the kinase activity determinations. The results of Group C (incubation mixture without ATP) indicate no nucleotides were produced unless ATP was present in the incubation mixture.

Table 34 is a comparative grading system for the kinase activity in the three animals studied.

Table 33

Cytidine Aminohydrolase Activity
in Various Animal Tissue Homogenates

Tissue	Aminohydrolase Activity*				
	Mouse	Rat	Hamster	Dog	Monkey
Liver	2+	0	5+	4+	>5+
Kidney	5+	0	5+	0	>5+
Spleen	0	0	4+	0	>5+
Small Intestine	3+	1+	5+	0	>5+
Thymus	-	0	-	-	-
Bone Marrow	-	1+	-	-	-
Serum	0	0	0	0	>5+

*Nanomoles arabinosyl uracil formed per gram wet tissue per 30 minutes incubation:

0 = 5 or <5
1+ = 6-49
2+ = 50-99
3+ = 100-199
4+ = 200-399
5+ = 400-1000

Table 34

Kinase Activity in Various Animal Tissue Homogenates

Tissue	Kinase Activity*				
	Mouse	Rat	Hamster	Dog	Monkey
Liver	0	0	0	0	0
Kidney	0	0	0	0	0
Spleen	3+	1+	2+	0	3+
Small Intestine	0	0	0	0	0
Thymus	-	3+	-	-	-
Bone Marrow	-	2+	-	-	-

*Nanomoles of nucleotides (ara-C equivalents) formed per gram wet tissue per 30 minutes incubation:

0 = 5 or <5
1+ = 6-24
2+ = 25-49
3+ = 50-99
4+ = 100-149
5+ = 150->150

Comparison of Assay of Ara-C Nucleotides by Paper and Thin-Layer Chromatography

In the thin-layer procedure utilized in most of the ara-C *in vitro* metabolism studies, aqueous standards of 5'-monophosphate ara-C (ara-CMP), cytidine 5'-monophosphate (CMP), cytidine 5'-diphosphate (CDP), and cytidine 5'-triphosphate (CTP) remained at the origin after development of the chromatogram. The 5'-di- and triphosphate derivatives of ara-C were not available for R_f determination. However, it was assumed that the values would be essentially the same as those found for CDP and CTP.

Other than ara-U, ara-C nucleotides have been the only other metabolites of ara-C found in biological systems. It was logical, therefore, to assume that the radioactivity remaining at the origin after TLC assay of tissue homogenates — previously incubated with ara-C and the necessary co-factors for kinase activity — was associated with ara-C nucleotides.

To further strengthen this assumption and to separate the various ara-C nucleotides, selected samples were assayed by both paper and thin-layer chromatography. The paper chromatography systems were those described earlier under METHODS. The R_f values for various compounds chromatographed in these systems are shown in Table 35.

Non-labeled compounds were located by visualization

Table 35

R_f Values for Various Compounds after Development on Thin-Layer and Paper Chromatograms

Compound	Chromatography Systems		
	Thin-Layer[1]	Paper System-I[2]	Paper System-II[3]
Ara-C	0.38	0.82	2.30
Ara-U	0.60	0.60	2.50
Ara-CMP	0.00	0.56	1.00
CMP	0.00	0.56	0.89
CDP	0.00	0.42	0.36
CTP	0.00	0.29	0.13

[1]Gelman's ITLC-Type SA chromatography media; methanol-chloroform-1.0M phosphate buffer, pH 7.5 (30:70:4).

[2]Whatman #1 paper; isobutyric acid-NH_4OH-H_2O (240:23:97).

[3]Whatman #1 paper; absolute ethanol-1M NH_4Ac, pH 3.8-1M NaEDTA, pH 8.2 (75:29:1). See METHODS for details on the determination of R_f values for this system.

under UV light at 250 nm and/or by spraying the chromatogram with a molybdic acid spray for detecting phosphorus-containing compounds (Hanes and Isherwood, 1949). The spray solution contained 60 percent w/w $HClO_4$ (5 ml), 1N HCl (10 ml), and 40 percent w/v $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25 ml). The mixture is diluted with water to 100 ml. After spraying and air-drying, the chromatograms are placed under UV light for 10-20 minutes. Compounds containing phosphorus begin to appear as blue spots against a white background.

Labeled compounds are located as described for the paper chromatography assay procedure used in the in vivo studies.

To compare the TLC assay of ara-C nucleotides with the paper chromatography systems, the mouse spleen homogenate assayed by TLC in Table 28 was reassayed by paper chromatography. The results are shown in Table 36. The thin-layer system showed 14 percent of the total radioactivity remained at the origin after development. The paper systems showed 8 and 9 percent of the radioactivity was associated with ara-C nucleotides. Of the total nucleotides, 75-77 percent was in the form of 5'-triphosphate and the remaining 23-25 percent was equally divided between the mono- and diphosphate derivatives. The lower nucleotide recovery in the paper systems as compared to the thin-layer system was due to incomplete

Table 36

Comparative Assay of Ara-C Nucleotides by Thin-Layer and Paper Chromatography[1]

Compound[2]	Percent Total Radioactivity		
	Thin-Layer System	Paper System-I	Paper System-II
Ara-CTP	14	7	6
Ara-CDP		1	1
Ara-CMP		1	1
Ara-C	82	82	86
Ara-U	2	4	3

[1]Ara-C nucleotides were the products of a crude enzyme system from mouse spleen homogenate incubated with ara-C and the necessary co-factor for kinase activity.

[2]Identified from R_f values of standards and/or related compounds.

elution of the ara-C nucleotides from the Whatman paper media. On recalculating the recoveries after correcting for this source of error, the paper and thin-layer assays gave the same results. The ara-C and ara-U associated radioactivity was completely eluted from both the thin-layer and paper chromatography media.

Chromatography of rat thymus homogenates after similar incubation conditions showed 14 percent of the total radioactivity to be associated with nucleotides when assayed by TLC and 9-10 percent by the two paper systems. The only nucleotide present was the monophosphate derivative. A calf thymus homogenate showed 17 percent nucleotides as assayed by TLC and 10 percent by paper system II. The triphosphate derivative represented 70 percent of the formed nucleotides.

Again it must be emphasized that standards of the di- and triphosphate derivatives of ara-C were not available, and therefore, any identification of these products depends on the validity of the assumption that they have essentially the same R_f values as CDP and CTP in these chromatography systems.

VII. DISCUSSION

Clinical investigations with ara-C have clearly indicated the necessity of obtaining and maintaining a significant ara-C serum level for good therapeutic response to the drug. Although, obviously, the serum level concentrations are a function of the amount of drug administered, the level also depends upon the extent and rate of its absorption, distribution, and binding or localization in tissues. Degradation, activation, and excretion will also significantly influence serum levels. The work presented here is a comparison of rate and extent of the absorption of ara-C in various animal species after oral and parenteral administration. Activation, degradation, and excretion of the drug by these animal species were also studied. Distribution and binding by serum protein were not found to be significant factors in the control of ara-C serum levels.

It is hoped that the data obtained will provide the clinician and/or basic researcher with the necessary knowledge to design future protocols that will derive maximum therapeutic response from ara-C. The results, therefore, will be discussed in this context.

In this study, the ara-C serum levels after parenteral administration for the various animal species were

in good agreement with those obtained by other investigators. An apparent disagreement was with the results obtained by Dixon and Adamson (1965). These authors reported serum levels of total radioactivity (ara-C + ara-U) in the dog after 100 mg/kg, i.v., which were higher initially than the values obtained in our laboratory. The rate of decline of total radioactivity was also greater than that observed in the experiments reported here. It should be pointed out, however, that Dixon and Adamson employed an intravenous saline infusion during the course of their experiments which may have influenced their results. These authors did not separate ara-C from ara-U.

In the BDF_1 mouse, our results showed a serum $t^{\frac{1}{2}}$ of 21 minutes after administration (i.p.) of 300 mg/kg. Borsa et al. (1969) reported a serum $t^{\frac{1}{2}}$ of 20 minutes for ara-C. The ara-C serum level was determined by an L-cell tissue culture bioassay after an intraperitoneal injection of 250 mg/kg.

In the human, Talley et al. (1967) reported work with 89 patients who had received up to 50 mg/kg of ara-C by various dosage regimens. The results showed no detectable ara-C in the serum 15 minutes after drug administration. These authors employed a bioassay involving the use of KB cultured cells but did not have access to

an aminohydrolase inhibitor, such as H_4U, as a collection-tube additive. Therefore, it is not known how much in vitro deamination occurred in the samples after they were collected and prior to being assayed. Creasey et al. (1966) in a similar study with 5 patients receiving 5 or 10 mg/kg of ^{3}H-ara-C (i.v.) failed to find ara-C more than 5 minutes after injection in two subjects, and after 20 minutes in a third. These authors used a paper chromatography assay method for whole blood samples after acidifying with perchloric acid. The acidification step probably succeeded in stopping the aminohydrolase activity, and therefore some credence can be given to their results.

A comparative study of serum radioactivity levels after administration of ^{3}H-ara-C by various routes (i.v., s.c., and i.m.) was reported by Finklestein et al. (1970). Levels of radioactivity, studied in one of two patients who received the drug i.v., fell rapidly with a $t_{\frac{1}{2}}$ of less than 30 minutes. Two patients who received the drug i.m. and one of two patients who received the drug s.c. demonstrated peak blood levels of radioactivity between 25 minutes and one hour after injection of the drug. Plasma values were highest for i.v. and i.m. injections. The levels of radioactivity were reported to be too low to permit separation of the radioactivity into ara-C and ara-U.

Data for the serum level of ara-C in the human, as collected in our laboratory, must be compared to other reported data in the context of our utilization of H_4U as a collection-tube additive. In the human receiving 86 mg/kg of ^{3}H-ara-C (i.v.) we detected total radioactivity up to 24 hours after drug injection and ara-C up to 2 hours after administration. The two ara-C serum components (described in RESULTS) had a half-life of 12 minutes and 44 minutes. The usefulness of H_4U collection tubes on the precise determination of ara-C in serum can be illustrated by results from an on-going study of ara-C serum levels in cancer patients (T. C. Hall). Post-treatment serum samples (15 minutes after i.v. administration of ara-C) were collected in tubes with and without H_4U. The results for two patients were 0.54 and 0.90 µg/ml of ara-C without H_4U added, and 5.01 and 4.78 µg/ml with H_4U added. The erroneous estimates obtained for ara-C serum levels when the samples are not collected in tubes containing an aminohydrolase inhibitor are evident from this work.

In examining the data obtained for serum levels of radioactivity after oral administration of ^{3}H-ara-C (Figure 12), some conclusions can be made as to the absorption of ara-C and/or ara-U by this route. The oral absorption of radioactivity associated with either

or both of these compounds was nearly complete in the monkey and dog and poor in the mouse and hamster. Radioactivity in rat serum was the same whether the compound was administered orally or intraperitoneally.

The absorption of ara-C after oral administration of the compound cannot be accurately determined due to the effects of intestinal and serum aminohydrolase activity. However, a resulting level which combines absorption and deamination can be determined. The monkey and hamster showed no detectable serum level of ara-C after oral administration. The mouse, rat, and dog ara-C serum levels were essentially identical to serum levels of total radioactivity. These results indicate the lack of intestinal metabolism of ara-C by these animals. The serum levels are therefore directly related to the oral absorption of the compound. The effects of serum and intestinal aminohydrolase activity on orally administered ara-C will be discussed in more detail later in this section.

Urinary recovery of ara-C and total radioactivity after parenteral administration of ^{3}H-ara-C were in good agreement with published data. Again, the major disagreement was with the data of Dixon and Adamson (1965) for the dog. These authors found a 24-hour urinary recovery (after 100 mg/kg, i.v.) of total radioactivity of 90 percent of the dose. In our laboratory the recovery of

total radioactivity was 50 percent and the recovery of ara-C was 35 percent of the injected dose. The experimental conditions (continuous infusion of saline) could have influenced these results. The 24-hour urinary recovery of total radioactivity (85 percent) for the CD_2F_1 mouse as found by these authors is in good agreement with the 82 percent for the BDF_1 mouse as determined in our laboratory. The CD_2F_1 mice were injected with 20 mg/kg, s.c., and the BDF_1 mice received 300 mg/kg, i.p. Smith (1966) found a total radioactivity recovery of 70 percent for 24 hours in the rat. We found a recovery of 50 percent for this animal in our laboratory.

The reported values for the human for 24-hour urinary excretion of radioactivity after administration of ^3H-ara-C averages approximately 90 percent. This agrees with the results obtained in our laboratory. Creasey et al. (1966) reported 86-96 percent of the radioactivity administered to five patients in the form of ^3H-ara-C (5 or 10 mg/kg, i.v.) was excreted in the urine in 24 hours. Only 6-9 percent of this radioactivity was associated with ara-C. Talley et al. (1967) found 3.8 to 10 percent of the injected radioactivity excreted in human urine in the form of ara-C 24 hours after administration of 50 mg/kg of ^3H-ara-C. These authors did not report total radioactivity recoveries. The patient studied in our laboratory had a

24-hour urinary recovery of total radioactivity of 73 percent and an ara-C recovery of 7.5 percent after receiving 86 mg/kg of ^{3}H-ara-C, intravenously.

Finklestein et al.(1970) found only a small difference in the amount of ara-C + ara-U excreted in 24 hours by patients receiving ara-C by s.c., i.m., and i.v. administration. The values reported were: s.c, 68 and 71 percent; i.m., 79 and 81 percent; and i.v., 87 and 91 percent.

As mentioned earlier, degradation of ara-C by the cytidine aminohydrolase enzyme is an important factor in the control of the serum levels of this compound. This enzyme is found in the serum and tissues of various animal species. However, the distribution and quantity differs from one species to another and also within strains of the same species. It is essential, therefore, when attempts are made to control ara-C serum levels that the levels of aminohydrolase activity of the particular species be known. This factor becomes extremely important in such species as the monkey and human which contain very high activity in serum and tissues. Although the human normally does not contain a high serum aminohydrolase activity, an occasional cancer patient is found who has high serum enzymatic activity. Such a patient has been reported from this laboratory and from that of Dollinger and

co-workers (1967). The patient studied in our laboratory had a disease diagnosis of AML. In Dollinger's study the diagnosis was CGL. The latter patient was found to tolerate high doses of ara-C without hematologic effects.

The tissue levels of aminohydrolase activity varied from species to species. Since most metabolism of drugs is carried out either by the liver or kidney, the levels of aminohydrolase activity in these two tissues would exert a major effect on the serum levels of ara-C. In work presented here, the rat was the only species which did not contain a significant enzyme activity in one or both of these tissues. Except for the moderate aminohydrolase activity in mouse liver, the tissue activities in this study agreed with those reported by Camiener and Smith (1965) and Durham and Ives (1969). These authors reported finding no aminohydrolase activity in mouse liver. The disagreement could be the results of technical procedures or species differences. Camiener and Smith used Swiss mice; Durham and Ives, CF_1 mice; and in our study BDF_1 mice were employed.

As stated in the INTRODUCTION, ara-C is inactive as an anticancer agent unless it is intracellularly phosphorylated to form the nucleotide. Tissue kinase activity is, therefore, extremely important for therapeutic response to this compound. Part of the work presented here was designed to determine the level of this enzyme in various tissues of species studied in the <u>in vivo</u> experiments.

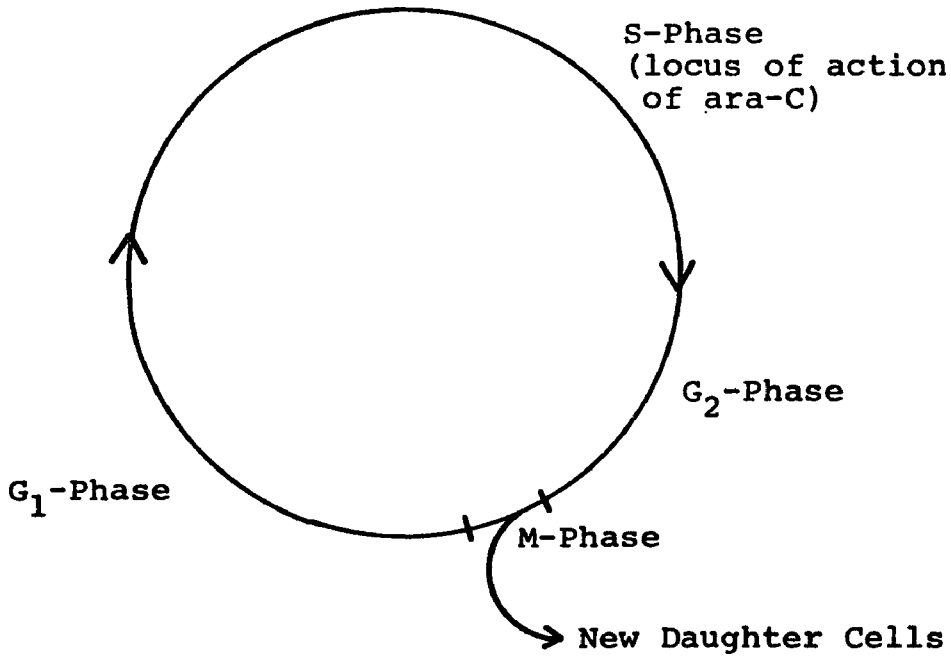
Deoxycytidine kinase activity was found in rat thymus and bone marrow and the spleens of all animals except the dog. The results were in agreement with those of Durham and Ives (1969). The only apparent disagreement was the slight kinase activity found in the small intestine of the rat in the present study which was not detected by these authors. Our study employed Sprague-Dawley rats, while Durham and Ives used weanling rats from Harlan Industries. Although some of the sensitivity of the kinase assay was sacrificed for the aminohydrolase assay, the kinase activity data was quite adequate for identifying those tissues containing significant activity.

"Unbalanced growth" is that mechanism, resulting in cell death, whereby DNA synthesis is blocked while active RNA and protein synthesis continues. This mechanism was first reported by Cohen and Barner (1955) in the death of E. coli cells produced by specific interference with DNA replication. The authors also showed that simultaneous inhibition of RNA and protein synthesis, as well as DNA synthesis, temporarily stopped bacterial cell division but did not kill or sterilize the cells. When RNA and protein synthesis — but not DNA synthesis — was restored, the E. coli cells again were permanently sterilized. The mechanisms of action whereby many known antileukemic drugs kill leukemic cells have been attributed to "unbalanced growth" (Skipper et al., 1967).

Ara-C is an antileukemic drug which produces unbalanced growth (Cohen, 1966). It inhibits DNA synthesis in various systems (Chu and Fischer, 1962; Kim and Eidinoff, 1965; Pizer and Cohen, 1960; Slechta, 1961) without effecting RNA and protein synthesis.

Karon and Shirakawa (1969) demonstrated with asynchronous Don-C cells in culture that the locus of action of ara-C was the S-phase of the mitotic cycle. These investigators found that cells in G_1, M, and G_2 at the time of drug exposure proceeded through the cycle until they entered the sensitive phase and were killed.

As pointed out in the diagram below, the cell division cycle is composed of four phases, G_1, S, G_2, and M.



S-phase is that part of the cell cycle in which new DNA synthesis takes place. The DNA is doubled in preparation for cell division. During the G_1-phase, RNA and protein synthesis takes place but no DNA is synthesized. The M-phase involves mitosis in which a new cell is formed.

The S-phase specificity of ara-C has prompted Skipper et al. (1967) to investigate the therapeutic advantages of matching the dose and schedule of ara-C to the cell cycle characteristics of L1210 leukemic cells grown in BDF_1 mice. The size of the inoculum was 10^4 to 10^6 cells. The authors found that effective blood concentrations of ara-C must be provided at intervals slightly shorter than median T_s (time of S-phase) or constantly until all viable cells have been exposed to effective concentrations of ara-C while in their S-phase. Although the median $T_{G_1} + T_M + T_{G_2}$ of rapidly growing L1210 leukemic cells is about 3.8 hours, about 24 hours were necessary for 99.999 percent of these cells to have passed into the vulnerable S-phase. The authors summarized the results of four <u>in vivo</u> experiments in which the individual dose of ara-C and the number of doses were kept constant. Only the interval between doses was varied. In every experiment the q3 hrs (x8) and q6 hrs (x8) schedules provided greater increase in hosts survival time than any other schedule, e.g., q10 min (x8), q30 min (x8), q1 hr (x8).

The less successful schedules were thought to be inferior because they did not maintain effective blood concentrations for a long enough time period. An every-12-hours (x8) schedule, also found inferior, was thought to produce too long an interval between effective blood concentrations (i.e., longer than median T_S, thus allowing some leukemic cells to traverse S-phase without being exposed to effective levels of ara-C). Therefore, cells which attempt DNA replication (S-phase) in the presence of effective concentrations of ara-C were sterilized and cells which were in the G_1, M, and G_2 phases were unharmed.

If an effective blood concentration of ara-C can be maintained long enough in an animal bearing a nonsynchronous leukemic cell population, theoretically all of the leukemic cells will pass into S-phase in the presence of ara-C and be killed. However, if an effective blood concentration cannot be maintained, because of host toxicity, until all G_1 + M + G_2 cells have passed into S-phase, there will be viable leukemic cells remaining at the end of that course of treatment.

Numerous investigators are presently searching for an appropriate therapeutic schedule which would take advantage of the cell cycle characteristics of human ALL and AML cell populations. The major problem in relating

the schedules found effective with the L1210 cells in mice to these human cells is the large difference in the time of the $G_1 + M + G_2$ phases. Whereas the median length of these phases for L1210 cells is 3.8 hours, the median length for human ALL and AML cells is ca. 35 hours. Since the extreme length of the $G_1 + M + G_2$ for the L1210 cells was found by Skipper et al. (1967) to be 24 hours or greater, then the extreme length for these phases for the ALL and AML cells must be much greater than 35 hours. However, it must be kept in mind that only a maximally effective ara-C serum concentration of about 3 μg/ml is necessary to obtain a maximum rate of leukemic cell kill. The rate of cell kill, therefore, is almost completely concentration-independent above 3.0 μg/ml (Skipper et al., 1967).

Other than alteration of treatment schedules as an approach to improve the clinical effectiveness of ara-C, choice of route of administration and dose level has also gained much attention. Early clinical work established that the response to ara-C by patients with a wide variety of malignant neoplasms was greater after administration in multiple small daily doses (ca. 3-10 mg/kg/day x 10) than a single large injection (ca. 50 mg/kg) (Talley and Vaitkevicius, 1963; Henderson and Burke, 1965; Loo et al., 1965).

The drug is now being administered predominantly either as a continuous slow infusion (ca. 0.3-1 mg/kg/day to toxicity) (Ellison et al., 1967, 1968; Freireich, 1969) or in multiple daily injections (Howard et al., 1968; Freireich, 1969).

A most recent approach to the enhancement of ara-C serum levels is through the use of cytidine aminohydrolase inhibitors. The most potent inhibitor among those reported is tetrahydrouridine. This compound, a reduced cytidine, was found to be effective in vivo in monkeys in our laboratory (see RESULTS) and in vitro by Camiener (1967c). Camiener (1968) studied the kinetics of this inhibition and found that its action was only partial (or of a regulatory type). The inhibited enzyme was still able to form products, but at a reduced rate. In the study by Camiener, enzyme inhibition was dependent on a preincubation reaction with H_4U, and the substrate appeared to interact preferentially with uninhibited enzyme.

Clinically, if H_4U is to be used in combination with ara-C, its effective inhibitory dose level and toxicity (in conjunction with ara-C) must be established. In this work, toxicity and ara-C serum levels were determined in monkeys receiving ara-C and H_4U (at varying dose levels). The toxicity, as determined by hemato-

logical and biochemical parameters, was greater after 100 mg/kg of H_4U + 50 mg/kg of ara-C than with ara-C alone. However, the serum $t^{\frac{1}{2}}$ for ara-C was increased in the animals receiving H_4U from the control value of <4 minutes to 160-216 minutes. At a lower H_4U dose level (12.5 mg/kg) toxicity was only slightly greater than the controls and the serum $t^{\frac{1}{2}}$ for ara-C was 60-78 minutes. In all instances, toxicity was reversible, returning to normal within two weeks.

In animals receiving H_4U only at dose levels of 12.5 and 100 mg/kg, blood samples were collected at various time periods and assayed for uninhibited aminohydrolase activity. The serum aminohydrolase activity was found to be completely inhibited up to 120 minutes after the H_4U injection. Only 2-3 percent of the pretreatment enzymatic activity was present in the 240 and 360 minutes posttreatment samples. The amount of serum enzyme inhibiiton was the same for 6 hours at both dose levels. In the context of these studies one might postulate that the greater $t^{\frac{1}{2}}$ for ara-C after 100 mg/kg H_4U than after 12.5 mg/kg is due to aminohydrolase activity which is not located in serum. To what extent this nonserum enzymatic activity was inhibited is not known at this time.

Since the presently employed ara-C schedules require multiple doses or continuous infusions, it would be advantageous to have an orally active form of this drug. Evans (1961) first reported the oral activity of ara-C in a paper concerning the antitumor activity of the compound in rats. The drug was found to be 1/3 to 1/5 as active orally as it was parenterally. Most of this decreased response is likely due to intestinal aminohydrolase activity. Neil (1969) utilized H_4U in conjunction with ara-C in hopes of increasing the oral response of mice carrying L1210 leukemic cells. His findings indicated that when administered orally H_4U does increase the effectiveness of oral ara-C. The author found a similarity between the therapeutic effects obtained with an oral combination of ara-C + H_4U and parenteral administration of ara-C alone. Although the parenterally administered ara-C gave a much higher maximum ara-C serum level, the H_4U + ara-C combination greatly increased the length of time during which effective levels of ara-C were present. These data suggest that oral combinations of H_4U and ara-C have promising clinical application. Further studies with oral combinations of H_4U and ara-C in the monkey are presently being pursued in our laboratory.

SUMMARY

Arabinosyl cytosine (ara-C) is a potent antitumor and antiviral agent. It inhibits growth of a variety of transplantable tumors in mice and rats, mammalian cells in culture, bacteria, and DNA-containing viruses in cell culture and whole organisms. In humans, the agent has been studied for the treatment of neoplasms and of virus infection of the eye. Its activity, however, is limited by its metabolic pathways. Two enzymes directly responsible for the catabolism and anabolism of ara-C are cytidine aminohydrolase and deoxycytidine kinase, respectively. The major pathway for the degradation of the compound involves a deamination resulting in the production of arabinosyl uracil (ara-U). Activation of ara-C is catalyzed by a kinase enzyme which converts the nucleoside to a biologically active nucleotide derivative.

In this study, serum levels of total radioactivity and of ara-C were determined in various animal species after parenteral administration of tritium-labeled ara-C (50 mg/kg). The ara-C was determined by a microbiological assay method. Serum biological half-life ($t_{\frac{1}{2}}$) for total radioactivity 30 minutes after administration of the compound for the monkey, dog, rat, hamster, and mouse were: 147, 174, 75, 59, and 63 minutes, respec-

tively. Serum $t^{\frac{1}{2}}$ of ara-C for these same animals was: <4, 88, 66, 26, and 21 minutes, respectively.

The serum levels of total radioactivity for a single cancer patient after receiving 86 mg/kg of ^{3}H-ara-C when plotted on semilogarithmic paper were divided into two components. The first component (between 5 and 30 minutes posttreatment) has a $t^{\frac{1}{2}}$ value of 17 minutes and the second (between 30 and 240 minutes posttreatment), a value of 223 minutes. The serum $t^{\frac{1}{2}}$ values for ara-C in the same patient were 12 minutes and 44 minutes. Due to the aminohydrolase activity in the serum of monkeys and humans, it was necessary to utilize tubes containing tetrahydrouridine (H_4U) for collection of samples from these species.

Serum levels of total radioactivity and/or ara-C after parenteral administration of ^{3}H-ara-C were not affected by binding of ara-C and ara-U to various animal serum proteins. Nor were they apparently affected by chemical instability, localization in erythrocytes, or exchange of the tritium label.

The 24-hour urinary ratio of ara-C and ara-U after parenteral administration of ara-C correlated well with the tissue levels of cytidine aminohydrolase activity; i.e., in species with high enzyme activity the excretion of ara-C was low and excretion of ara-U was high. The

percent of dose recovered as ara-C 24 hours after administration of the compound was: monkey, <1-4; dog, 28-43; rat, 40-56; hamster, 32-33; and mouse, 36-39. The percent of dose recovered as total radioactivity (ara-C + ara-U) for these same animals was: monkey, 62-98; dog, 42-55; rat, 58-62; hamster, 75-78; and mouse, 74-90.

In a human cancer patient, in 24 hours after ara-C administration 72.7 percent of the total administered radioactive dose was recovered in the urine and 7.5 percent of this total was the parent compound, ara-C.

The oral absorption of radioactivity associated with either ara-C or ara-U was nearly complete in the monkey and dog, and poor in the mouse and hamster. Radioactivity in rat serum was the same whether ^{3}H-ara-C was administered orally or intraperitoneally. The monkey and hamster showed no detectable serum level of ara-C after oral administration. These findings are probably the results of the high aminohydrolase activity in the intestinal tissue and/or serum of these animals. The mouse, rat, and dog ara-C serum levels were essentially identical to serum levels of total radioactivity. These results indicate the lack of intestinal and serum metabolism of ara-C by these animals. The serum levels are therefore directly related to the oral absorption of the compound.

Tetrahydrouridine was administered in doses of 100, 50, 25, and 12.5 mg/kg, i.v., to monkeys to test tetrahydrouridine effectiveness as an inhibitor of the cytidine aminohydrolase enzyme. In each of two animals the serum $t^{\frac{1}{2}}$ of ara-C (50 mg/kg, i.v.) was 160 and 216, 84 and 96, 82 and 102, and 60 and 78 minutes, respectively, for the decreasing doses of H_4U. Without H_4U pretreatment ara-C was not detected in serum after 15 minutes. Control 24-hour urinary ara-C recovery was <4 percent of the injected dose. After H_4U pretreatment recovery extended from 59 percent (100 mg/kg of H_4U) to 22 percent (12.5 mg/kg of H_4U).

Control (ara-C without H_4U pretreatment) and H_4U-treated animals displayed a modest posttreatment drop in RBC. The H_4U-treated animals had a significantly greater depression of WBC and reticulocytes than controls. BUN, SGOT, and alkaline phosphatase levels were within normal limits for all animals.

To determine the effect of H_4U alone on the hematological and biochemical parameters, an experiment was initiated in which two monkeys each received 100 and 12.5 mg/kg of H_4U. The data for these toxicity parameters for samples from a similar sampling schedule as above showed no significant difference between the pre- and posttreatment samples.

The development of a thin-layer chromatography assay method for the separation and assay of ara-C and its metabolic products made possible the determination of cytidine aminohydrolase and deoxycytidine kinase activities in biological fluids. Serum aminohydrolase activity in monkeys ranged from 288-900 units. One unit equals one nanomole of ara-U produced per ml serum per 60 minutes incubation. Human and dog serum aminohydrolase activity ranged from 10-36 units, with the human showing a slightly higher average. No activity was found in the serum of hamsters, rats, or mice. The aminohydrolase activity units in sera from cancer patients had a much wider range (0 to 192) than normal humans. The degree of variation of serum aminohydrolase activity in the various patients may explain some of the variable clinical response to ara-C therapy when the drug is administered by low-level, continuous infusion.

The aminohydrolase enzymatic activity has a unique tissue distribution in the animal species studied. The monkey and hamster showed high activity in every tissue assayed. The rat had only slight activity in small intestine and bone marrow, and no activity in any other tissues. The mouse had highest activity in the kidney, with less in the small intestine and liver. The only activity found in dog tissue was in the liver.

Deoxycytidine kinase activity was found in the thymus and bone marrow of the rat, and in the spleen of all animals studied except the dog. No kinase activity was detected in any other tissues. Tetrahydrouridine had no effect on tissue deoxycytidine kinase activity in these tissues.

Clinical investigations with ara-C have clearly indicated the necessity of obtaining and maintaining a significant ara-C serum level for good therapeutic response to the drug. A major factor controlling ara-C serum levels, as shown by work presented here, is the level of cytidine aminohydrolase activity in the tissue and serum of the animal species. Tetrahydrouridine (12.5 to 100 mg/kg, i.v.) was effective in the monkey in increasing the serum $t_{\frac{1}{2}}$ of intravenously administered ara-C (50 mg/kg). Whether the inhibitor, given parenterally or orally, would also enhance ara-C serum levels when the drug is administered orally or by continuous infusion remains to be investigated. It may be possible to increase the therapeutic response of ara-C by administering H_4U in conjunction with the drug. However, additional dose-response and metabolism studies with H_4U and ara-C combinations administered by various routes and at various dose levels are needed.

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