Nucleoside Antibiotics. Biosynthesis of Arabinofuranosyladenine by *Streptomyces antibioticus*†

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ABSTRACT: Data are presented showing the distribution of ^{14}C from radioactive tracer experiments with [U-^{14}C]adenosine, [U-^{14}C]adenine, [6-^{14}C]glucose, [1-^{14}C]ribose, and D- and L-[1-^{14}C]arabinose following their incorporation into the nucleoside antibiotic, arabinofuranosyladenine, elaborated by *Streptomyces antibioticus*. The data clearly show that the biosynthesis of arabinofuranosyladenine involves a direct epimerization of the C-2′ hydroxyl group of adenosine or an adenosine derivative. This conclusion is based on the observation that the relative distribution of ^{14}C in the adenine and ribose moieties of the AMP isolated from the RNA of *S. antibioticus* were the same as the relative distribution of ^{14}C in the adenine and arabinose moieties of the arabinofuranosyladenine isolated from experiments in which [U-^{14}C]adenosine was added to the cultures. These ^{14}C ratios did not change if unlabeled adenine or unlabeled D-ribose were added simultaneously with [U-^{14}C]adenosine. Very little of the [U-^{14}C]-adenosine was hydrolyzed by *S. antibioticus*. The biosynthesis of arabinofuranosyladenine does not occur in the culture filtrates. Additional proof that all five carbons of the ribose moiety of the adenosine were involved in the formation of the arabinose moiety of arabinofuranosyladenine was obtained by degradation of the ribose and arabinose. The per cent distribution of ^{14}C in C-1 to C-5 of the arabinose from arabinofuranosyladenine was the same as in the ribose of the [U-^{14}C]adenosine added to the culture medium.

Arabinofuranosyladenine (ara-A)[1] is one of the most recent nucleoside antibiotics that have been isolated from the Streptomyces. The chemical synthesis of ara-A was reported before its isolation as a naturally occurring nucleoside antibiotic from *S. antibioticus* (Lee *et al.*, 1960; Barker and Fletcher, 1961; Reist *et al.*, 1962; Glaudemans and Fletcher, 1963, 1964; Cohen, 1966; Lepine *et al.*, 1966). In 1967 ara-A was isolated from the culture filtrates of *S. antibioticus* (Parke, Davis and Co., 1967). Ara-A has broad spectrum activity against DNA viruses in cell culture and significant antiviral activity against herpes keratitis infections in hamsters, herpes simplex virus infections in Swiss mice, herpes encephalitis in mice, vaccinia virus, and DNA containing pseudorabies or rabbit myxoma virus *in vitro* (Schabel, 1968; Miller *et al.*, 1968; Sidwell *et al.*, 1968, 1969; Schardein and Sidwell, 1968; Sloan *et al.*, 1968; Dixon *et al.*, 1968; Kurtz *et al.*, 1968). The biochemical properties of ara-A can be found in the reviews by Cohen (1966) and Suhadolnik (1970).

The biochemistry of D-arabinosyl nucleosides, their distribution and metabolic fate of D-arabinose have been studied in several laboratories. Levin and Racker (1959) were the first to report on the enzymatic conversion of D-ribose 5-phosphate to D-arabinose 5-phosphate.

D-Arabinose has been identified as the pentose moiety of spongothymidine and spongouridine as isolated from *Cryptotethya crypta* (Bergmann and Feeney, 1951). Three pathways for D-arabinose catabolism in bacteria have been described (Palleroni and Doudoroff, 1957; Mortlock and Wood, 1964; LeBlanc and Mortlock, 1971). The phosphorylation and interconversion of arabinose have been reported (Cohen and McNair Scott, 1950; Cohen, 1953; Volk, 1959; Tono and Cohen, 1962).

It was of interest to study the biosynthesis of ara-A to determine the mechanism by which *S. antibioticus* synthesizes this nucleoside antibiotic. Based on known, established biochemical reactions in carbohydrate and nucleoside chemistry, several pathways were considered for the biosynthesis of ara-A. One involved the direct epimerization of the C-2′ hydroxyl of a purine nucleoside or nucleotide. Another involved the conversion of a nucleoside diphosphate hexose or pentose to the arabinose derivative. This nucleotide diphosphate arabinoside would then react with adenine to form ara-A. Precedence for this proposal is based on the isolation of uridine diphosphate arabinose (Sandermann *et al.*, 1968). Two proposals that have been submitted by Lim and Cohen (1966) for the biosynthesis of other ara nucleotides or nucleosides may be expanded to include the biosynthesis of ara-A. They suggested that either (1) D-arabinose 1-phosphate could condense with uracil or thymine to form D-ara-U or D-ara-T or (2) D-arabinose 5-phosphate is converted to 1-pyrophosphoryl-D-arabinose 5-phosphate which condenses with a purine or pyrimidine base to form the ara nucleotide. This communication presents data strongly suggesting that ara-A biosynthesis by *S. antibioticus* proceeds *via* an epimerization of the C-2′ hydroxyl of adenosine or an adenosine derivative. A preliminary report of this work has been published (Suhadolnik and Farmer, 1971).

Materials and Methods

Melting points were taken with a Thomas-Hoover silicone bath apparatus and are uncorrected. Ultraviolet spectra and measurements were recorded on the Beckman Model DB spectrophotometer and a Gilford Model 2400 spectrophotometer. Radioactive measurement was made in a Packard liquid scintillation spectrometer with Bray's scintillation

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[1] Abbreviation used is: ara-A, arabinofuranosyladenine.

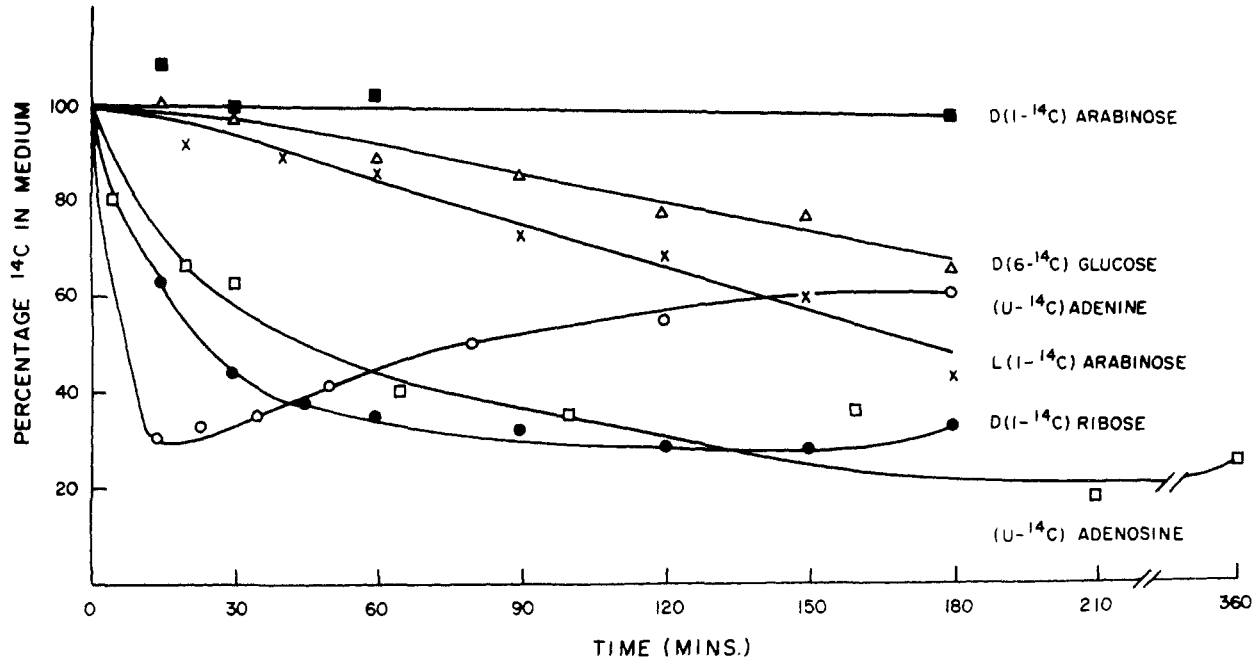

FIGURE 1: Per cent of ^{14}C remaining in the medium. [U-^{14}C]Adenine (○), [U-^{14}C]adenosine (□), D-[6-^{14}]glucose (Δ), D-[1-^{14}C]ribose (●), D-[1-^{14}C]arabinose (■), and L-[1-^{14}C]arabinose (×).

solution (Bray, 1960) and with a gas-flow counter (D-47), Nuclear-Chicago.

Culture flasks for ara-A production were shaken at 28° on a New Brunswick Gyrotort (Model G-25, speed setting 6). *S. antibioticus* (NRRL No. 3238) was maintained on nutrient agar slants. The culture medium for production of ara-A was prepared as described by Parke, Davis Laboratories (1967). Each 2-l. baffled flask contained 300 ml of medium. Inoculations were done by the addition of agar plugs (Uematsu and Suhadolnik, 1972). The uptake of the ^{14}C-labeled compounds added to the cultures is shown in Figure 1. ara-A was isolated as follows: The culture medium was centrifuged or filtered. The filtrate was extracted with one volume of 1-butanol (three times). The butanol extract was evaporated to dryness; the residue was dissolved in 10 ml water and filtered. The filtrate was added to a Dowex 1-X8 (OH^-) column (13 ml, 1.5 cm diameter) and washed with 350 ml of methanol–water (70:30, v/v). Ara-A was eluted with 500 ml of 100% methanol, crystallized at 0° from water, water–methanol, or methanol, and further purified by paper chromatography in one or more of the solvents described here. In all cases, the ara-A was purified to constant specific activity before hydrolysis to adenine and arabinose. The yield was 27 mg/l. The physical and chemical properties of ara-A were identical with those properties for the chemically synthesized ara-A.

For the isolation of RNA components the culture broth was centrifuged and cells were washed thoroughly with water. The cells (20 g wet weight) were suspended in 30 ml of water, broken in the french press at 16000 psi. Trichloroacetic acid (200 ml) was added. After stirring for 10 min at 0°, the mixture was centrifuged (10,000*g*, 10 min), and the pellet was washed twice with 10% trichloroacetic acid (200 ml each time). This was followed by successive washings with 95% ethanol (150 ml, two times) and with ethanol–ether (3:1, v/v; 120 ml, three times). The pellet was dried *in vacuo*. The RNA was hydrolyzed in 300 ml of 1 N potassium hydroxide, 21 hr at 37° with shaking. The mixture was centrifuged. The brown supernatant, containing the nucleotides, was neutralized with 6 N HCl (40 ml), and cooled to 0°. Trichloroacetic acid was added until the concentration was 5%. The precipitate was removed by centrifugation (10,000*g*, 10 min) and was washed with an additional 100 ml of 5% trichloroacetic acid at 0°. The combined supernatants were extracted four times with 400-ml portions of ether, evaporated to remove traces of ether, and adsorbed onto 6 g of Norit A at pH 5.5. The charcoal was filtered and the nucleotides were eluted with 200 ml of ethanol–concentrated ammonium hydroxide–water (7:1:2, v/v). After removal of the solvent, the residue was digested with alkaline phosphatase (1 mg/ml), Tris buffer 0.08 M (pH 8.8), and magnesium chloride (1×10^{-2} M) at 25°, 90 min. Adenosine and cytidine were isolated by the method of Dekker (1965) and purified to constant specific activity by either crystallization or paper chromatography (solvents A and B). The yield of crystalline adenosine was 5.5 mg. Cytidine was not crystallized but was obtained in a yield of 24 μmoles.

Biosynthesis of ara-A from ^{14}C-Labeled Compounds. Sterile solutions of [U-^{14}C]adenosine, [U-^{14}C]adenine, D-[1-^{14}C]-ribose, D-[6-^{14}C]glucose, L-[1-^{14}C]arabinose, and D-[1-^{14}C]-arabinose were added to the culture medium when ara-A biosynthesis was first detected. The cultures were stopped either 12 min or 3 hr later. Ara-A was isolated as described above.

Distribution of ^{14}C in ara-A, Adenosine, and Cytidine. Ara-A and adenosine were hydrolyzed by Dowex 50 (H^+) (90°, 60 min). Adenine remained on the resin; the pentoses remained in solution. The Dowex 50 (H^+) 1-X8 was removed by filtration and washed with 5-ml portions of water five times. The adenine was displaced from the ion-exchange resin with 3 N ammonium hydroxide. The adenine, arabinose, or ribose was further purified by paper chromatography using solvents A, B, and C.

The distribution of ^{14}C in cytidine was determined by hydrolysis in 11.7 N perchloric acid (90°, 30 min). The cytosine was purified by Whatman No. 1 paper and solvent B. Ribose and arabinose were determined quantitatively (Dische, 1962).

Distribution of ^{14}C in D-Ribose and D-Arabinose from ^{14}C-Labeled Adenosine and ara-A. Radioactive ribose and arabinose were isolated by hydrolysis of ^{14}C-labeled adenosine and ara-A as described above. The ribose and arabinose were purified to constant specific activity by paper chromatography (Whatman No. 3MM) using solvents C and D. The sugars were detected on the paper chromatograms by 2-aminobiphenyl (Gordon *et al.*, 1956). The pentoses were converted to the osazones and degraded with periodate. C-1, -2, and -3 were isolated as the dimedone derivative (Suhadolnik *et al.*, 1964). The melting point of the mesoxaldehyde 1,2-bisphenylosazone was 186–190°; the melting point of the formal dimedone was 175–180°.

Solvents for Paper Chromatography. The solvents used for paper chromatography were as follows: solvent A, water; solvent B, water-saturated 1-butanol; solvent C, 1-butanol–pyridine–water (6:4:3, v/v); solvent D, ethyl acetate–acetic acid–formic acid–water (18:3:1:4, v/v).

[U-^{14}C]Adenosine was purchased from International Chemical and Nuclear Corp.; [U-^{14}C]adenine from Schwarz–Mann; D-[1-^{14}C]ribose and D-[6-^{14}C]glucose from Calatomic; D-[1-^{14}C]arabinose and L-[1-^{14}C]arabinose from New England Nuclear Corp. All other compounds used for these studies were the purest commercially available. Alkaline phosphatase (chicken intestine) was purchased from Worthington Corp.

Results

Incorporation of Labeled Compounds into ara-A. ^{14}C from [U-^{14}C]adenine, [U-^{14}C]adenosine, D-[6-^{14}C]glucose, and D-[1-^{14}C]ribose is incorporated into ara-A (Table I). D-[1-^{14}C]-Arabinose and L-[1-^{14}C]arabinose are not incorporated. D-[1-^{14}C]Arabinose is not taken up by the cells (Figure 1). All other radioactive compounds are taken up by *S. antibioticus*. To show that uptake of [U-^{14}C]adenosine by *S. antibioticus* was necessary for ara-A biosynthesis, experiments were done in which the filtrate and washed cells were incubated with [U-^{14}C]adenosine. While the washed cells gave rise to radioactive ara-A, the filtrate did not.

Incorporation and Distribution of ^{14}C in ara-A from the [U-^{14}C]Adenosine Experiments. ara-A and adenosine were hydrolyzed to adenine, D-arabinose, and D-ribose as described under Materials and Methods. The adenine was purified to constant specific activity. The ratio of the ^{14}C in the adenine:ribose moieties of the [U-^{14}C]adenosine added to the cultures of *S. antibioticus* was 46:54, respectively. Two experiments were performed to determine if adenosine would serve as the direct precursor for ara-A biosynthesis (Table II). These were short- and long-term experiments (12 min and 3 hr) in which the effect of unlabeled adenine or unlabeled D-ribose on the incorporation of [U-^{14}C]adenosine into ara-A and the AMP of the RNA was studied. The ratios of ^{14}C in the adenine:arabinose of ara-A and the adenine:ribose of the AMP isolated from the RNA of *S. antibioticus* isolated 3 hr after the addition of [U-^{14}C]adenosine (Table II, expt 1) were 58:42 and 60:40, respectively. When unlabeled adenine (1×10^{-3} M) was added simultaneously with the [U-^{14}C]adenosine (expt 3), the ratios of ^{14}C in the adenine:ribose and adenine:arabinose (from a 3-hr experiment) were both 54:46. The close agreement of the relative distributions of ^{14}C in the adenine and arabinose of ara-A and adenine and ribose of the AMP from the RNA (with and without added unlabeled adenine) suggest that the *N*-ribonucleoside bond of adenosine is not cleaved in the biochemical conversion of adenosine to ara-A by *S. antibioticus*. If there were hydrolysis of the *N*-ribonucleoside bond of adenosine, then the adenine and ribose must remain enzyme bound and can not equilibrate with the unlabeled adenine and ribose in the cell pool. Although uptake is observed it may be that there is a membrane-bound epimerase for ara-A biosynthesis that does not involve adenosine transport. Similarly, a comparison of the relative distribution of ^{14}C in the adenine and arabinose of ara-A and the adenine and ribose of the AMP from the RNA for the 12-min experiment (with added adenine, expt 2) was 52:48 and 51:49, respectively. Again, the relative distribution of the ^{14}C in the aglycone and pentoses in ara-A and AMP from the RNA were the same. When unlabeled D-ribose and [U-^{14}C]adenosine were added to the cultures, the relative distribution of the ^{14}C in the adenine and pentoses of ara-A and the AMP from the RNA were 60:40 and 54:46, respectively (expt 4). The results as shown in Table II, expt 1–4), strongly suggest that an intact purine ribonucleoside is the direct precursor for ara-A biosynthesis by *S. antibioticus*. The exact structure of the adenosine derivative that is required for the C-2′-epimerization is not known. Additional proof that there is very little hydrolysis of the *N*-ribonucleoside bond of the [U-^{14}C]adenosine added to the cultures of *S. antibioticus* was obtained by comparing the specific activities of the adenosine and cytidine isolated from the RNA. If there were appreciable hydrolysis of the exogenously supplied [U-^{14}C]-adenosine by *S. antibioticus*, then there would be a considerable amount of radioactivity in the ribose moiety of the cytidine. Experimentally, this is not observed. The specific activities of the adenosine were 2000, 1500, and 372 times greater than the specific activities of the cytidine (Table II, expt 1, 2, and 3). Proof that exogenously supplied D-[1-^{14}C]-ribose can be utilized is shown by the equal incorporation into AMP and CMP of the RNA of *S. antibioticus* (expt 5).

TABLE I: Incorporation of Radioactive Compounds into Ara-A by *S. antibioticus*.

Compound Added	Amount/Flask		Sp Act.	Ara-A Isolated	
	μCi	μmoles	(μCi/μmole)	Sp Act. (nCi/μmole)	Incorp Efficiency[d] (× 10^3)
[U-^{14}C]Adenine[a]	4.92	0.021	231	6.21	2.7
[U-^{14}C]Adenosine[a]	4.49	0.018	250	5.65	2.3
D-[6-^{14}C]Glucose[a]	4.10	0.41	10.0	0.015	0.15
D-[1-^{14}C]Ribose[a]	11.4	1.28	8.9	0.231	2.6
D-[1-^{14}C]Arabinose[b]	3.5	0.07	50	0	0
L-[1-^{14}C]Arabinose[c]	6.1	0.62	9.8	0	0

[a] Administered 50 hr after inoculation of one flask with 2 ml of seed culture. [b] Administered 67 hr after inoculation of four flasks (by the agar straw method). [c] Administered 62 hr after inoculation of two flasks (by the agar straw method). Additions of the ^{14}C-labeled compounds were always made as soon as ara-A was detected in the medium. [d] Incorporation efficiency—this term is defined as 100 times the specific activity of ara-A divided by the specific activity of the compound tested.

TABLE II: Distribution of ^{14}C in ara-A and the AMP and CMP Isolated from the RNA of *S. antibioticus*.

Expt	Additions	ara-A		Adenine Isolated from ara-A		^{14}C in RNA			
		μmoles/ Flask	nCi/μmole	nCi/μmole	% ^{14}C in Adenine	Adenosine nCi/μmole	Adenine % ^{14}C	Cytidine nCi/μmole	Cytosine % ^{14}C
1	[U-^{14}C]Adenosine, 3-hr expt[a]	13.0	38	22.0	58.0	20.0	60.0	0.01	15
2	[U-^{14}C]Adenosine + adenine (1×10^{-3} M), 12-min expt[b]	9.8	3.2	1.6	52.0	9.1	51.0	0.006	
3	[U-^{14}C]Adenosine + adenine (1×10^{-3} M), 3-hr expt[b]	14.0	26.0	13.9	54.0	18.6	54.0	0.05	6
4	[U-^{14}C]Adenosine + ribose (1×10^{-3} M), 3-hr expt[c]	24.0	13.0	7.8	60.0	25.3	54.0		
5	[1-^{14}C]Ribose, 3-hr expt[d]		0.12	0.02	15	0.25	7	0.27	2

[a] [U-^{14}C]Adenosine, 4.5 μCi and 0.023 μmole (200 μCi/μmole), was added to one flask 60 hr after inoculation as described by Uematsu and Suhadolnik (1972). Ara-A was isolated 3 hr later. [b] [U-^{14}C]Adenosine, 4.4 μCi and 0.022 μmole (200 μCi/μmole), was added to each flask. Adenine was added simultaneously to the four flasks 48 hr after inoculation to a final concentration of 1×10^{-3} M. Ara-A was isolated from two flasks after 12 min and from the remaining two flasks after 3 hr. [c] [U-^{14}C]Adenosine, 6.7 μCi and 0.027 μmole (250 μCi/μmole), was added to two flasks 67 hr after inoculation. A solution of ribose was added simultaneously to the two flasks to a final concentration of 1×10^{-3} M. Ara-A was isolated after 3 hr. [d] [1-^{14}C]Ribose, 5.2 μCi and 0.59 μmole (8.9 μCi/μmole), was added to two flasks 67 hr after inoculation. Ara-A was isolated after 3 hr.

TABLE III: Distribution of ^{14}C in Ribose and Arabinose of ^{14}C-Labeled Adenosine and ara-A.[a]

Compound	Carbon Atoms	% ^{14}C Theor	Ribose		Arabinose	
			nCi/μmole	% ^{14}C Exptl	nCi/μmole	% ^{14}C Exptl
Pentose	1–5	100	2.02	100	0.037	100
Mesoxaldehyde 2,3-bisphenylosazone	1–3	60	1.34	66.4	0.025	67.5
Formal dimedone	5	20	0.43	21.3	0.0084	22.7
Formic acid[b]	4	20				

[a] In all cases the per cent ^{14}C determined experimentally was about 10% higher than the theroetical value. The discrepancy probably arises by comparing the specific activities of the ribose and arabinose (determined by the orcinol colorimetric method) to the specific activities of the mesoxaldehyde 1,2-bisphenylosazone and the formal dimedone (determined gravimetrically). However, the ratios of specific activities of C-5 to C-1 to C-3 were 1:3.1 and 1:3.0 for ribose and arabinose, respectively. [b] C-4 was not analyzed.

The specific activities of the adenosine and cytidine isolated from the RNA were essentially the same (0.25 and 0.27 nCi per μmole, respectively). In both cases only 7 and 2% of the ^{14}C from the [1-^{14}C]ribose resides in the adenine and cytosine, respectively.

To prove that all five carbons of the ribose moiety of [U-^{14}C]adenosine were utilized for ara-A biosynthesis, the per cent distribution of ^{14}C in C-1, -2, -3, -4, and -5 of ribose and arabinose was determined. Periodate oxidation of the osazones of the pentoses gave crystalline mesoxaldehyde 1,2-bisphenylosazone (C-1, -2, and -3) and formaldehyde (C-5). The formaldehyde was distilled and isolated as the dimedone derivative. The per cent ^{14}C in the mesoxaldehyde 1,2-bisphenylosazone from ribose and arabinose was 66.4 and 67.5%, respectively; the per cent ^{14}C in C-5 of ribose and arabinose was 21.3 and 22.7%, respectively. These data supply additional information that all five carbons of the ribose moiety of adenosine are involved in the biosynthesis of the arabinose moiety of ara-A (Table III).

Discussion

Four pathways were considered for the biosynthesis of ara-A by *S. antibioticus*. They were as follows: (1) epimerization of the C-2′ hydroxyl of a purine nucleoside or nucleotide, (2) conversion of a nucleotide diphosphate hexose or pentose

to the arabinose derivative and subsequent condensation of the arabinosyl moiety with adenine, (3) condensation of D-arabinose 1-phosphate with adenine, and (4) condensation of 1-pyrophosphorylarabinose 5-phosphate with adenine and subsequent hydrolysis of the 5′-phosphate to form ara-A. The data obtained on the incorporation and distribution of ^{14}C from [U-^{14}C]adenosine into ara-A strongly support the first proposal. This conclusion is based on the similarities of the ratios of ^{14}C in the adenine:arabinose of ara-A and the adenine:ribose of the AMP isolated from the RNA (Table II). These ^{14}C ratios remained constant under the following experimental conditions: (1) short-term experiments (12 min), (2) long-term experiments (3 hr), (3) experiments with added unlabeled adenine, and (4) experiments with added unlabeled ribose. Experimental proof that all five carbon atoms of the ribose of [U-^{14}C]adenosine were involved in the formation of the arabinose of ara-A is shown in Table III. The possibility that the arabinose of ara-A was formed from the ribose of hydrolyzed [U-^{14}C]adenosine seems unlikely.

If the D-arabinose moiety of ara-A were formed by a pathway involving the epimerization of D-[U-^{14}C]ribose following hydrolysis of [U-^{14}C]adenosine and subsequent condensation of this D-arabinose with adenine, the ratios of ^{14}C in the adenine:arabinose of ara-A would not be the same as the ratios of ^{14}C in the adenine:ribose of the AMP isolated from the RNA of *S. antibioticus*. This would be especially true in those experiments in which unlabeled adenine or unlabeled D-ribose were added simultaneously with the [U-^{14}C]adenosine. Therefore, an intact purine ribonucleoside or derivative is required as a precursor for ara-A biosynthesis.

The conclusion of the findings reported here are in contrast to those findings of Roberts *et al.* (1955), Hochstadt-Ozer and Stadtman (1971), Hochstadt-Ozer (1971), and Hoffmeyer and Neuhard (1971) in which they showed that exogenously supplied nucleosides must first undergo scission to purine bases before they can be converted to the nucleotides. This mechanism involves the purine phosphoribosyl transferases and not the purine nucleoside phosphorylases. With *S. antibioticus*, it appears that, in the stationary phase of growth, the production of ara-A utilizes adenosine without scission of the *N*-ribonucleoside bond.

The epimerization of the C-2′ hydroxyl group of a purine nucleoside or nucleotide in ara-A biosynthesis is unique in that it is the first example of this type reaction that occurs at the level of a pentose in a nucleoside or nucleotide. The data available to date on the 2- and 4-epimerizations show that these reactions occur as either the nucleoside diphosphate sugar (such as UDP-galactose 4′-epimerase and UDP-*N*-acetylglucosamine 2′-epimerase) or as the sugar phosphate (such as ribulose 5-phosphate 4-epimerase) (Nelsesteun and Kirkwood, 1970; Salo and Fletcher, 1970; Deupree and Wood, 1970)). In view of the data presented here on the epimerization of the C-2′ hydroxyl of the ribose moiety of adenosine to form ara-A, it will be of extreme interest to elucidate the mechanism of the epimerization in this nucleoside or nucleotide interconversion. Although the biosynthesis of adenosine and AMP is known to occur by the condensation of D-ribose 1-phosphate with adenine or PRPP with adenine, it does not appear that ara-A biosynthesis is occurring by a similar type mechanism in which the corresponding arabinose phosphates are utilized.

The biosynthesis of 15 nucleoside antibiotics has been studied (Seto *et al.*, 1968; Rao *et al.*, 1969; Suhadolnik, 1970; Elstner and Suhadolnik, 1971a; Suhadolnik and Chock, 1971; Suhadolnik *et al.*, 1971; Kunimoto *et al.*, 1971). With the exception of showdomycin and formycin, all nucleoside antibiotics are directly dependent on normal pathways of purine or pyrimidine biosynthesis.

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Phylogeny of Hemoglobins. β Chain of Frog (*Rana esculenta*) Hemoglobin*

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ABSTRACT: The amino acid sequence of the β chain of frog hemoglobin (*Rana esculenta*) has been studied. The five fragments produced by tryptic cleavage of the trifluoroacetylated, carboxymethylated β chain were first isolated. After removal of the trifluoroacetyl groups and further cleavage with trypsin, 15 peptides were purified and sequenced. The order of the tryptic peptides in each fragment was determined through chymotryptic peptides and the alignment of the fragments was established by isolating arginine-containing peptides from a chymotryptic digest of the β chain. The frog β chain comprises 140 residues. When the 26 known mammalian β, δ, and γ chains and frog β chain are compared, 54 positions out of 146 are invariant in all the proteins. Of 47 amino acids involved in the interactions of β chain with either α chains or heme group, 30 are invariant. Two long sequences (28–40 and 96–108) seem particularly stable. The comparison of β chains from eutherians, metatherians (kangaroo), and amphibians (frog), which have diverged approximately 80, 130, and 320 million years ago, respectively, reveals that the number of amino acid substitutions is dependent but not proportional to time. These results are confronted with current concepts of evolution.

Among the proteins which were chosen for a study on molecular evolution, the hemoglobin family is one of the most attractive. Because hemoglobin is particularly abundant and easy to purify from red cells, the protein seems amenable to structural investigations in almost all vertebrates. On the other hand, the "molecule" is generally built with polypeptide chains of 140–150 residues in length and determination of the complete amino acid sequence can be performed under rather good conditions with the current techniques of protein chemistry. However, separation of α and β chains turned out to be more difficult for lower vertebrates than for mammals and probably for this reason our knowledge on hemoglobins of lower vertebrates is very limited since to the present time only the α chains of the chicken (Matsuda *et al.*, 1970) and of the carp (Hilse *et al.*, 1966) have fully been sequenced.

The choice of hemoglobin as an evolutionary tracer is not only determined by practical reasons. In contrast to most enzymes, this protein takes in account a whole physiological function, the oxygen transport, and hence is directly subjected to selective pressure; the so-called phenotypic character on which selection acts is apparently related to a few structural genes and not to many genes as in a polymolecular function (Simpson, 1964). In another point of view, the vertebrates, except fishes, used dissolved oxygen in the first part of their life and aerial oxygen in the second part and the switch might be associated with a molecular change. This change has actually

* From the Laboratory of Biological Chemistry, University of Paris VI, 96, Boulevard Raspail, Paris (6e), France. *Received June 21, 1971.* This study was supported in part by a grant from the Délégation à la Recherche Scientifique et Technique (66-00-146). Maps of chymotryptic peptides from tryptic fragments, tables of tryptic peptides subjected to Edman degradation and to chymotryptic hydrolysis, and tables of amino acid compositions and of yields of chymotryptic from tryptic fragments and from β chain will appear following these pages in the microfilm edition of this volume of the journal. Singles copies may be obtained from the Business Operations Office, Books and Journals Division, American Chemical Society, 1155 Sixteenth St., N.W., Washington, D. C. 20036, by referring to code number BIO-11-916. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche.

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