Incorporation of Nucleosides into the Nucleotide Pools of Human Erythrocytes. Adenosine and Its Analogs†

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ABSTRACT: The incorporation of adenosine analogs into the nucleotide pools of human erythrocytes has been examined by high-pressure liquid chromatography. With a number of the analogs, the corresponding nucleotides were formed in concentrations substantially greater than those of the normal nucleotides. The analogs 6-methylmercaptopurine ribonucleoside, 8-azaadenosine, 2-fluoro-2′-deoxyadenosine, and several N^6-substituted adenosines gave 5′-monophosphate nucleotides as did adenosine, inosine, and 2′-deoxyadenosine. No evidence of nucleotide formation was observed with 3′-deoxyadenosine, formycin A, formycin B, arabinosyladenine, or several other analogs with modifications in the sugar moiety. The formation of di- and triphosphate nucleotides was observed with 2-fluoroadenosine (F-Ado), tubercidin, toyocamycin, N^6-methyladenosine and 2,6-diaminopurine ribonucleoside. Since 2-fluoroadenosine is not deaminated by erythrocytic adenosine deaminase and its nucleotides are readily detected in the chromatograms, it was chosen as a model adenosine analog for detailed studies. The rate of formation of F-ATP from F-Ado is about 0.027 μmol/min per ml of packed erythrocytes and F-ATP can be formed in erythrocytes in amounts that are severalfold greater than the ATP concentration. In erythrocytes from blood that had been stored for 1 month, the rate of F-ATP synthesis from F-Ado was reduced, but the concentration of F-ATP formed was the same as in fresh erythrocytes although a longer incubation was necessary to achieve these levels. In both fresh and stored erythrocytes the syntheses of analog nucleotides occurred with little detectable effect on the concentrations of either normal nucleotides or 2,3-diphosphoglycerate. During the synthesis of F-ATP from F-Ado the rate of lactate production increased from 2.4 to 5.9 μmol per hr per ml of erythrocytes. The rate of F-ATP synthesis was significantly inhibited by incubation with several N^6-substituted adenosines. However, coincubation with the inhibitor of nucleoside transport, 6-[(2-hydroxy-5-nitrobenzyl)thio]guanosine, or preincubation with uridine did not affect the rate of F-ATP synthesis in the presence of 1.0 mM fluoroadenosine. In the presence of high concentrations of nucleoside (>0.5 mM) the rate-limiting step in the conversion of fluoroadenosine to F-ATP appears to be the reaction catalyzed by the enzyme adenosine kinase rather than nucleoside transport. It is proposed that the relative K_m values of adenosine deaminase and adenosine kinase in the erythrocyte play a determining role in whether adenosine will be salvaged by phosphorylation to AMP or degraded by deamination to inosine.

Mature mammalian erythrocytes have several features that make them uniquely suited for the study of certain facets of purine metabolism. They are not nucleated and do not synthesize RNA and DNA. Although they do not perform *de novo* synthesis of purines, erythrocytes have active pathways for purine base and nucleoside salvage and for nucleotide synthesis (Bishop, 1964). Therefore, it is possible to study the metabolic pathways of adenosine in human erythrocytes by examining the incorporation of adenosine analogs[1] into nucleotides without the complications of competing synthetic or draining reactions.

The recent development of high-pressure liquid chromatography has made it possible to examine, rapidly and reproducibly, alterations in the concentrations of naturally occurring nucleotides concurrently with the formation of analog nucleotides in small amounts of tissue (Horvath *et al.*, 1967; Brown, 1970; Brown and Parks, 1971).

Reported in this paper are the effects of alterations in the adenosine molecule on the incorporation of these nucleosides into the nucleotide pools of fresh human erythrocytes. It was found that some adenosine analogs lead to substantial accumulations of 5′-monophosphate nucleotides. Other analogs such as 2-fluoroadenosine, which are not substrates for adenosine deaminase, are converted to 5′-triphosphate nucleotides.

Materials and Methods

Compounds. The chemicals used in this study were all reagent grade. Adenosine and inosine were purchased from P-L Biochemicals. 6-Methylmercaptopurine ribonucleoside, 2′-deoxyinosine, 2′-deoxyadenosine, cordycepin, and hexokinase were bought from Sigma Chemical Co. The N^6-hexyl, N^6-phenyl, N^6-amyl, N^6-allyl, and N^6-ethoxyethyl analogs of adenosine were graciously supplied by Dr. M. H. Fleysher of Roswell Park Memorial Institute and the 2-fluoroadenosine, 2′-deoxy-2-fluoroadenosine, the α anomer of 2′-deoxy-2-fluoroadenosine, 9-β-D-arabinofuranosyl-2-fluoroadenine, and 9-β-D-xylofuranosyl-2-fluoroadenine by Dr. John Montgomery of the Southern Research Institute (Montgomery and Hewson, 1960). The N^6-methyladenosine was purchased from Terra-Marine Bioresearch. Tubercidin, 9-β-D-xylofuranosyladenine, 9-β-D-arabinofuranosyladenine, 9-β-D-xylopyranosyladenine, and 9-β-D-fructopyranosyladenine were obtained through the courtesy of Dr. Harry B. Wood, Jr., from the Drug Development Branch, Division of Cancer Treatment, of the National Cancer Institute. Toyocamycin was kindly supplied by the Royal Netherlands Fermentation Industries, Ltd., and the formycin A and B by Dr. Hamao Umezawa of the Institute of

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[1] Chemical names, trivial names, and abbreviations of nucleosides referred to in this article are listed in Table I.

Microbiological Chemistry, Tokyo. The 6-[(2-hydroxy-5-nitrobenzyl)thio]guanosine was sent to us through the courtesy of Dr. A. R. P. Paterson of the University of Alberta.

Preparation of Erythrocytes. Fresh blood in acid–citrate–dextrose from which the platelet-rich plasma had been removed was obtained from the Division of Hematologic Research of the Pawtucket Memorial Hospital.

In the preliminary experiments, human erythrocytes were separated from blood collected in acid–citrate–dextrose medium. The buffy coat and supernatant fluid were removed from the blood and the erythrocytes were washed twice in isotonic saline solution. In addition, blood that had been stored in acid–citrate–dextrose medium for 4 weeks was used as indicated. The erythrocytes employed in later experiments were obtained from fresh blood drawn in heparin-containing Vacutainer tubes (from Becton-Dickinson and Co.). The heparinized blood samples were centrifuged for 5 min in a clinical centrifuge (3600*g*) and the plasma and buffy coat were discarded. The erythrocytes were washed twice by suspension in 0.9% NaCl and centrifugation. After the second washing, the sedimented erythrocytes were resuspended in an equal volume of isotonic saline solution.

Incubation Procedure. Two different incubation media were employed. Medium I contained 128 mM NaCl, 1.2 mM $MgCl_2$, 18 mM potassium phosphate buffer (pH 7.4), and 16 mM glucose. Medium II contained 108 mM NaCl, 30 mM potassium phosphate buffer (pH 7.4), and 10 mM glucose. Adenosine and various adenosine analogs were added in the appropriate concentrations. Washed human erythrocytes were added in the concentrations indicated. All incubations were carried out in a Dubnoff shaking water bath (80 oscillations/min) at 37°, with air as the gas phase.

To determine the role of a mediated transport system in the phosphorylation of F-Ado, two experiments were run. A 20% suspension of washed human erythrocytes was incubated for 1 hr in medium II containing 10 mM uridine. After 1 hr, the uridine-containing medium was removed by centrifugation and replaced by a similar medium containing 1.0 mM F-Ado.[2] In the other investigation, 10 μM 6-[(2-hydroxy-5-nitrobenzyl)-thio]guanosine and 1 mM F-Ado in medium II were incubated with fresh washed erythrocytes for 1 hr.

Sample Preparation. Two methods of sample preparation were employed. In one method a 33% suspension of erythrocytes was incubated in medium I. To extract the nucleotides, 1-ml samples of the suspension were pipetted dropwise into 2 ml of cold 12% trichloroacetic acid stirred rapidly on a Vortex mixer (Miech and Tung, 1970). After centrifugation, 0.5-ml samples of the supernatant fluid were neutralized with 2.0 M Tris (Brown and Miech, 1972).

In the other method, 20% suspensions of erythrocytes were incubated in medium II. Samples (1 ml) of the suspensions were added dropwise to 1 ml of cold 15% trichloroacetic acid stirred rapidly on a Vortex mixer. After thorough mixing, the samples were twice frozen in an acetone–Dry Ice mixture and thawed with thorough mixing. Following this freeze–thaw procedure, the mixtures were centrifuged for 5 min in a clinical centrifuge and 1-ml samples were taken. The samples were extracted three times with ten volumes of water-saturated diethyl ether which removed most of the trichloroacetic acid and brought the pH to the range of 6.0–6.5. No essential differences were observed with the different incubation, sample preparation, or extraction procedures employed.

Nucleotide Analysis. The high-pressure liquid chromatographic procedure used was that described previously (Brown, 1970). Samples (20 μl) were injected into a Varian LCS 1000 which was equipped with a 1 mm × 3 m Reeve Angel column packed with an anion-exchange resin AS-Pellinex-SAX or a 1 mm × 3 m Varian Aerograph column packed with an anion-exchange resin LFS. When the Reeve Angel column was used, the low concentration eluent was 0.001 M KH_2PO_4 and with the Varian column, 0.015 M KH_2PO_4. The high concentration eluent was the same for both columns, 0.25 M KH_2PO_4 in 2.2 M KCl. The column flow rate was 12 ml/hr and the gradient flow rate 6 ml/hr. The full scale absorbance was 0.04 ODU. The peaks were identified by use of internal standards, by comparison to chromatograms of known standards and by enzymic peak-shift techniques. The concentrations of the nucleotides were determined by comparing peak areas (peak-height times width at half-height) with those of standard solutions of known concentrations. It should be noted that the liquid chromatograph used in these studies records absorbancy at 254 nm; however, several of the analog nucleotides examined vary greatly in their absorbancy at this wavelength. Therefore, one may not draw conclusions on the relative quantities of nucleotide synthesized by simple inspection of the chromatograms.

Identification of F-ATP by an Enzymic Peak-Shift Technique. In order to identify the F-ADP and F-ATP peaks, an enzymic peak-shift method was used (Brown, 1970). To 0.1 ml of erythrocytic extracts containing F-ADP and F-ATP, six units of crystalline yeast hexokinase were added. The solution was stirred on a Vortex mixer and allowed to stand at room temperature for 10 min. Then 50 μl of cold 12% trichloroacetic acid was added to the solution to precipitate the enzyme. The solution was freed of trichloroacetic acid by repeated extractions with water-saturated diethyl ether. For a control, a 0.1-ml sample of the erythrocytic suspension was treated identically with 5 μl of isotonic saline solution replacing the hexokinase reaction mixture.

Glucose, 2,3-Diphosphoglycerate, and Lactate Determinations. The glucose content of reaction mixtures was determined by the glucose oxidase method, a modification of the Raabo and Terkildsen (1960) method, which employs reagents purchased from the Sigma Chemical Co.

Lactate concentrations were determined by a reaction with lactate dehydrogenase employing reagents purchased from the Sigma Chemical Co. (Sigma Technical Bulletin 826, 1968). The concentrations of 2,3-P_2-glycerate were determined by Dr. Norman Fortier of the Rhode Island Hospital who used the method of Keitt (1971).

Results

Incorporation of Natural Nucleosides into Erythrocytic Nucleotides. To examine the synthesis of nucleotides from exogenous naturally occurring nucleosides present in large concentrations, the natural nucleosides (adenosine, 2′-deoxyadenosine, or inosine) were incubated in 0.5 mM concentrations with 33% suspensions of washed human erythrocytes in medium I. Large peaks appeared in the monophosphate nucleotide region of chromatograms in all cases. No new peaks appeared in the di- and triphosphate nucleotide region and the amounts of ATP and ADP were not increased over control (endogenous) levels. Figure 1 presents a typical chromatogram obtained when adenosine was incubated for 2 hr with human

[2] Abbreviations used are: F-AMP, F-ADP, F-ATP, 2-fluoroadenosine 5′-mono-, 5′-di-, and 5′-triphosphate; IMP, inosine 5′-monophosphate; P_2-glycerate, 2,3-diphosphoglycerate; HONBzlsG, 6-[2-hydroxy-5-nitrobenzyl)thio]guanosine.

TABLE I: Formation of Nucleotides from Adenosine and Adenosine Analogs.[a]

Chemical Name	Trivial Name or Abbrev	Mononucleotide Phosphate Synthesized		
		Mono-phos-phate	Di-phos-phate	Tri-phos-phate
Adenosine	Ado	+	−	−
Inosine	Ino	+	−	−
6-Methylmercaptopurine ribonucleoside	MMPR	+	−	−
8-Azaadenosine	8-AzaAdo	+	−	−
2,6-Diaminopurine ribonucleoside	2,6-DAPR	+	+	+
7-Deazaadenosine	Tubercidin (Tu)	−	−	+
7-Deaza-7-cyanoadenosine	Toyocamycin (To)	−	−	+
7-Amino-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine	Formycin A	−	−	−
7-Hydroxy-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine	Formycin B	−	−	−
2-Fluoroadenosine	F-Ado	+	+	+
2′-Deoxy-2-fluoroadenosine	F-dAdo	+	−	−
α Anomer of 2′-deoxy-2-fluoroadenosine	α-F-dAdo	−	−	−
9-α-D-Arabinofuranosyl-2-fluoroadenine	α-Ara-FAde	−	−	−
9-β-D-Xylofuranosyl-2-fluoroadenine	Xylo-F-Ado	−	−	−
2′-Deoxyadenosine	dAdo	+	−	−
3′-Deoxyadenosine	Cordycepin	−	−	−
9-β-D-Arabinofuranosyladenine	AraAde	−	−	−
9-β-D-Xylopyranosyladenine	XyloPAde	−	−	−
9-β-D-Fructopyranosyladenine	FructoPAde	−	−	−
9-β-D-Xylofuranosyladenine	XyloFAde	−	−	−
N^6-Methyladenosine	N^6-MeAdo	+	+	+
N^6-Allyladenosine	N^6-AllylAdo	+	−	−
N^6-Amyladenosine	N^6-AmylAdo	−	−	−
N^6-Ethoxyethyladenosine	N^6-EthoxyethylAdo	+	−	−
N^6-Hexyladenosine	N^6-HexylAdo	−	−	−
N^6-Phenyladenosine	N^6-PhenylAdo	−	−	−

[a] Column 1 gives the chemical name of the adenosine analog used in an incubation experiment, column 2 the trivial name or abbreviation, and column 3 denotes whether mono-, di- or triphosphate nucleotides were synthesized when this analog was incubated with fresh human erythrocytes for periods up to 2 hr. + means that the nucleotide was formed and − means that no nucleotide formation could be observed. The identity of the nucleotides formed from 2,6-DAPR is not established since the new peaks occur in the chromatograms with retention times similar to those of the guanine nucleotides.

erythrocytes. These results support the observations of Manohar *et al.* (1968) that incubation of adenosine with fresh human erythrocytes does not increase the level of ATP. Bartlett and Buccolo (1968) and Lerner and Rubinstein (1970) noted, however, that the ATP levels of outdated human blood (stored 6 weeks in a blood bank) can be greatly increased by incubation with adenosine. The identity of the large peaks in the monophosphate region, when erythrocytes were incubated with inosine, adenosine, or 2′-deoxyadenosine, was not definitely established since AMP, IMP and 2′-dAMP are not well resolved under the chromatographic conditions used. However, it is likely that these monophosphate peaks may be IMP because, under the incubation conditions employed, significant amounts of adenosine and 2′-deoxyadenosine are deaminated by adenosine deaminase. Inosine and deoxyinosine are formed, which, in turn, are hydrolyzed by purine nucleoside phosphorylase to hypoxanthine and ribose 1-phosphate and deoxyribose 1-phosphate (in the case of deoxyinosine). The hypoxanthine thus liberated can react with phosphoribosyl pyrophosphate, and the enzyme, hypoxanthine–guanine phosphoribosyltransferase (HGPRTase), to form IMP. In other studies, it has been shown that incubation of human erythrocytes with adenosine results in the intracellular accumulation of IMP (Manohar *et al.*, 1968). Although rabbit erythrocytes can convert IMP to AMP, human erythrocytes are unable to make this conversion (Lowy and Williams, 1966; Lowy *et al.*, 1958, 1962; Meyskens and Williams, 1971). It may be noted that incubation of human erythrocytes with guanosine leads to the synthesis of substantial quantities of the guanine nucleotides, GMP, GDP, and GTP (Brown and Parks, 1973).

Formation of 5′-Monophosphate Nucleotides from Adenosine Analogs. As seen in Table I, large peaks in the mononucleotide region of the chromatograms with no new peaks in the diphosphate or triphosphate regions were noted after incubation with a number of adenosine analogs. This observation implies that these analogs are capable of serving as substrates for adenosine kinase but that the respective monophosphate nucleotides are inactive with adenylate kinase in the erythrocyte. A number of analogs with modifications on C-6 of the purine ring formed monophosphate nucleotides. This finding is consistent with the observation of Loo *et al.* (1968, 1969) that the analog nucleotide, MMPR-5′-phosphate, can accumulate to very high concentrations in erythrocytes after incubation with MMPR. Several other C-6-substituted analog nucleosides listed in Table I gave substantial accumulation of 5′-monophos-

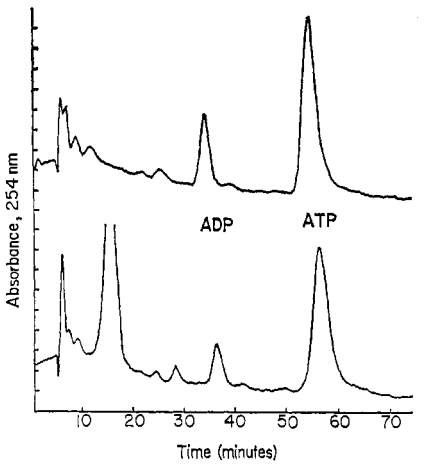

FIGURE 1: Incorporation of adenosine into the nucleotide pools of human erythrocytes. A 33% suspension of fresh, washed human erythrocytes was incubated for 2 hr in 5 ml of medium I containing 0.5 mM adenosine. The suspension (1 ml) was added dropwise into 2 ml of 12% trichloroacetic acid with rapid stirring. After neutralization with 2 M Tris, a 20-μl sample was analyzed by high-pressure liquid chromatography as described in Methods. The upper chromatogram is a chromatogram of the nucleotides in fresh human erythrocytes. The lower one is a chromatogram of the nucleotides of fresh human erythrocytes that had been incubated with adenosine.

phate nucleotides. Therefore, erythrocytic adenosine kinase is relatively nonspecific for substitutions on C-6 of the purine ring. This result is in accord with the observations of Bennett *et al.* (1966) and Schnebli *et al.* (1967). Modifications in the sugar moiety, however, markedly change the ability of the analog nucleoside to form 5′-monophosphate nucleotides. No evidence of nucleotide formation was observed with 9-β-D-arabinofuranosyl, β-xylopyranosyl, β-fructopyranosyl- or β-xylofuranosyladenine, and 3′-deoxyadenosine.

The effects of incubating 2-fluoro-2′-deoxyadenosine with erythrocytes was examined and a somewhat unexpected observation was the formation of only the 5′-monophosphate derivative. Like the ribonucleoside, F-Ado, this analog is not a substrate for erythrocytic adenosine deaminase (Agarwal *et al.*, 1973). Therefore, the fact that a monophosphate nucleotide was formed suggests the occurrence in erythrocytes of an adenosine or deoxyadenosine kinase which is capable of reacting with 2′-deoxyadenosines. Since no di- and triphosphate nucleotides of this analog were found, it seems apparent that 2-fluoro-2′-deoxyadenosine 5′-phosphate is not a substrate for erythrocytic adenylate kinase.

Formation of Triphosphate Nucleotides from Adenosine Analogs. The nucleotide extracts were examined by high-pressure liquid chromatography to monitor the incorporation of the analogs listed in Table I into the nucleotide pools of erythrocytes. Of these analogs, tubercidin, toyocamycin, 2,6-diaminopurine ribonucleoside, and N^6-methyladenosine, in addition to F-Ado, yielded distinctive nucleotide chromatograms with evidence of formation of polyphosphate nucleotides as shown in Figure 2. The di- and triphosphate nucleotides formed from 2,6-diaminopurine ribonucleoside have retention times comparable to those of GDP and GTP, respectively. In accord with observations with adenosine deaminase preparations from other sources (Kornberg and Pricer, 1951; Cory and Suhadolnik, 1965), it has recently been found that 2,6-diaminopurine ribonucleoside is a good substrate for human erythrocytic adenosine deaminase and is converted to guanosine by this enzyme in the erythrocyte (Agarwal *et al.*, 1973). Both GDP and GTP are formed

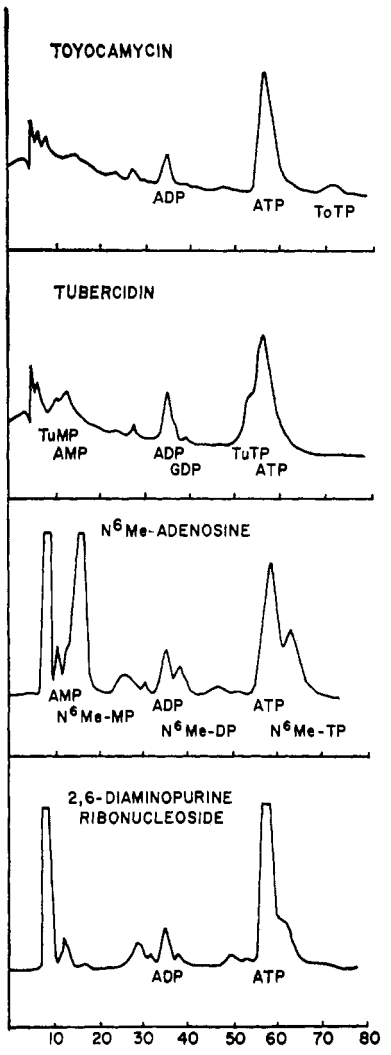

FIGURE 2: Synthesis of polyphosphate nucleotides. Samples (20 μl) of acid-soluble extracts of fresh human erythrocytes that had been incubated for 2 hr with tubercidin, toyocamycin, N^6-methyladenosine, and 2,6-diaminopurine ribonucleoside were analyzed by high-pressure liquid chromatography as described in Methods. The identity of the nucleotides formed from 2,6-DAPR is not established since the new peaks occur in the chromatograms with retention times similar to those of the guanine nucleotides.

rapidly in erythrocytes incubated with guanosine (Brown and Parks, 1973). Therefore, perhaps the new peaks that are formed from 2,6-DAPR are guanine nucleotides. It is also possible that they represent nucleotides of 2,6-DAP, alone or in combination with guanine nucleotides. Consequently isolation of substantial quantities of these new substances will be necessary to enable definitive characterization.

Since N^6-methyladenosine is an excellent substrate for adenosine kinase isolated from liver (Lindberg *et al.*, 1967), it is not surprising that nucleotides of this compound are formed in erythrocytes as shown in Figure 2. However, previous workers did not observe the formation of polyphosphate nucleotides with Ehrlich ascites cells (Shigeura *et al.*, 1966). The formation of nucleotides, including the di- and triphosphates of tubercidin in mouse fibroblasts (Acs *et al.*, 1964) and in human erythrocytes (Smith *et al.*, 1970), has been described elsewhere.

The fact that these analogs are capable of conversion to the triphosphate nucleotide level suggests that the 5′-monophosphate nucleotides can serve as substrates for erythrocytic

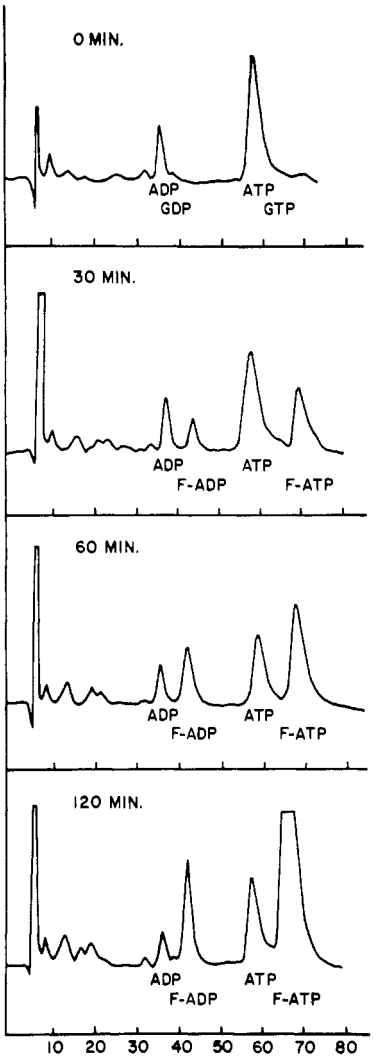

FIGURE 3: Progressive synthesis of polyphosphate nucleotides of 2-fluoroadenosine in human erythrocytes. A 20% suspension of fresh washed erythrocytes was incubated at 37° in 10 ml of medium II containing 1 mM F-Ado. A 1-ml sample was added dropwise with rapid stirring to 1 ml of cold 15% trichloroacetic acid. After freezing and thawing twice, the trichloroacetic acid was removed from the solution by ether extraction and 20 μl was analyzed by high-pressure liquid chromatography as described in Methods

adenylate kinase and that the resulting diphosphate nucleotides can serve as substrates for nucleoside diphosphokinase, as well as for other enzymes capable of converting ADP to ATP. Of the N^6-substituted adenosines examined, only N^6-methyladenosine formed polyphosphate nucleotides.

Incorporation of 2-Fluoroadenosine into the Nucleotide Pools of Intact Human Erythrocytes. F-Ado is a close structural analog of adenosine and is not degraded by adenosine deaminase but is a good substrate for adenosine kinase. The nucleotides of this analog have distinctive peaks in the chromatograms that are well separated from those of the naturally occurring nucleotides; the fluoroadenine nucleotides have retention times about 10 min longer than their adenine nucleotide counterparts. In contrast to results with adenosine, large amounts of F-ADP and F-ATP were synthesized over a 2-hr period when fresh human erythrocytes were incubated with F-Ado. The ready conversion of F-Ado to the polyphosphate nucleotides has been previously observed with Ehrlich ascites cells (Shigeura *et al.*, 1965). F-ATP was synthesized in quantities that exceeded the ATP concentration by two- to

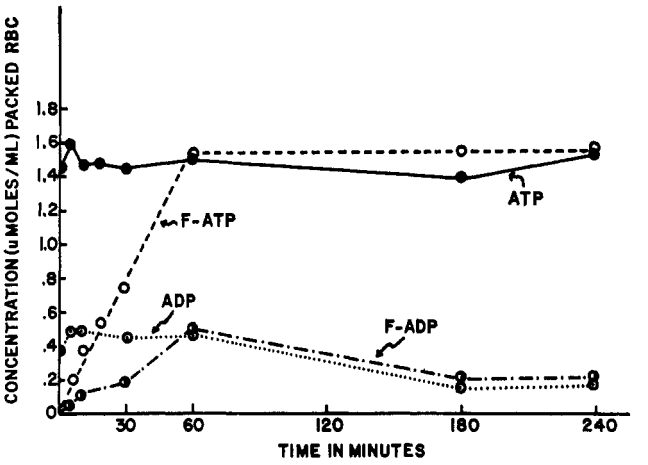

FIGURE 4: Rate of synthesis of fluoroadenine-containing nucleotides. A 33% suspension of fresh, washed erythrocytes was incubated in medium I containing 0.5 mM F-Ado. At the times indicated, 1-ml samples were extracted with 12% trichloroacetic acid and analyzed by high-pressure liquid chromatography. The concentrations of ADP, ATP, F-ADP, and F-ATP were estimated from the peak areas of the chromatograms. The ADP is represented by ⊙, the F-ADP by ◑, the ATP by ● and the F-ATP by ○.

threefold when a 20% suspension of fresh erythrocytes was incubated in medium II containing 1 mM F-Ado (Figure 3). Since F-Ado is not a substrate for human erythrocytic adenosine deaminase (Agarwal *et al.*, 1973) most, if not all, of the analog nucleoside that enters the erythrocytes becomes phosphorylated by the enzyme, adenosine kinase.

Figure 4 presents the results of an experiment in which a 33% suspension of fresh human erythrocytes was incubated in medium I with 0.5 mM F-Ado over a 4-hr time period. It may be seen that the synthesis of F-ATP was linear with time during the first hour and was terminated when virtually all of the F-Ado was converted to nucleotides in the erythrocyte. The concentration of ATP was unchanged over the 4-hr period of study and the F-ATP level remained constant after reaching a peak of about 1.5 μmol/ml of erythrocytes. The concentrations of ADP and F-ADP decreased after the first hour of study. It was estimated from this experiment that F-ATP was synthesized at a rate of about 0.027 μmol/min per ml of packed erythrocytes. From the data of Lerner and Rubinstein (1970) similar rates of adenosine incorporation in human erythrocytes were calculated.

Synthesis of F-ATP by Outdated Blood. On storage of whole blood in acid–citrate–dextrose, the ATP levels decrease by two-thirds after storage for 1 month. Therefore, to determine whether a low concentration of ATP has any effect on the phosphorylation of F-Ado, a 33% suspension of erythrocytes from human blood that had been stored for 1 month was incubated with F-Ado (incubation concentration 0.5 mM) under the same conditions as were used with the fresh blood. Figure 5 shows a chromatogram of the nucleotide profile before and after the incubation period. The nucleotide pattern of stored erythrocytes was markedly different from that of fresh human erythrocytes. The concentration of ATP in these cells was about 0.5 mM, a value about one-third of that usually obtained with fresh erythrocytes. Despite the lowered levels of the normal nucleotides, the analog nucleotide, F-ATP, was synthesized to levels of approximately 1.5 mM, a value comparable to that obtained with fresh blood. The rate of F-ATP formation with the stored blood, however, was slower (Figure 3). In the case of the stored erythrocytes, the rate of F-ATP

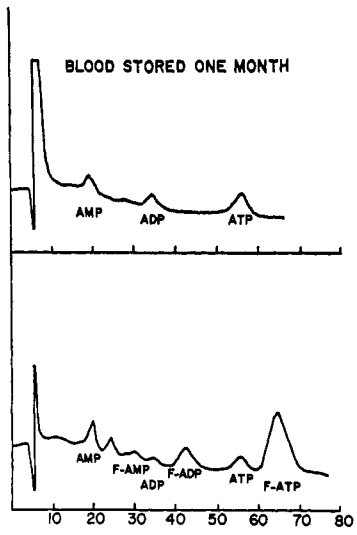

FIGURE 5: Synthesis of fluoroadenosine-containing nucleotides in outdated blood. A 33% suspension of washed human erythrocytes isolated from blood stored for 1 month in acid–citrate–dextrose medium was incubated in medium I containing 0.5 mM F-AdR. Chromatograms of 12% trichloroacetic acid extracts taken at zero time (A) and after 2-hr incubation (B) are shown. The concentration of ATP in the erythrocytes estimated from the peak area is about 0.5 mM and of F-ATP after 2-hr incubation (B) is about 1.5 mM.

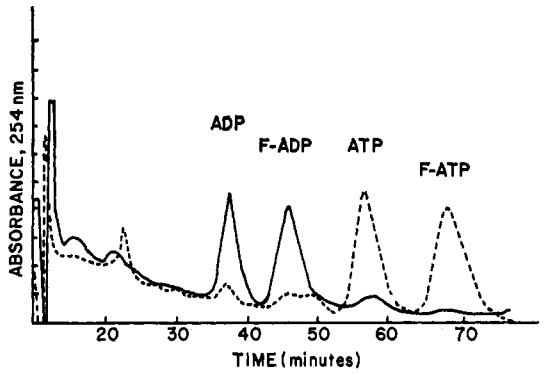

FIGURE 6: Characterization of fluoroadenine ribonucleotides by the enzymic peak-shift technique. An 0.1-ml sample of an erythrocytic extract containing F-ADP and F-ATP was incubated at room temperature for 10 min with 5 μl of yeast hexokinase (about 6 units) after which time the reaction was stopped by the addition of 50 μl of 12% trichloroacetic acid. The reaction mixture was centrifuged and the trichloroacetic acid was extracted with water-saturated diethyl ether. A 20-μl sample was analyzed by liquid chromatography. A control sample was run simultaneously with 5 μl of saline replacing the hexokinase solution. The dotted line indicates the nucleotides in fresh human erythrocytes that had been incubated with 2-fluoroadenosine. The solid line indicates the nucleotides after reaction with hexokinase and glucose.

synthesis was decreased to about 0.009 μmol/min per ml of packed erythrocytes. It is of interest that in erythrocytes with ATP concentrations about one-third of normal, the rate of F-ATP synthesis is decreased to about one-third of the rate measured with fresh erythrocytes.

Verification of F-ATP Synthesis. In order to establish that the nucleotide peaks that have the retention times about 10 min longer than ADP and ATP are actually F-ADP and F-ATP, an enzymic peak-shift technique was employed. When the acid-soluble extract was incubated with yeast hexokinase as described in Methods, the ATP and F-ATP peaks at 59 and 70 min, respectively, disappeared and the peaks of ADP and F-ADP at 37 and 47 min, respectively, were substantially increased (Figure 6). The high specificity of yeast hexokinase for ATP and its close analogs establishes that the peaks in question are F-ADP and F-ATP.

Effect of F-ATP Synthesis on Lactate Production, Glucose Consumption, and 2,3-Diphosphoglycerate Levels. In order to investigate the source of energy for the phosphorylation of F-Ado, glucose consumption, lactate production and 2,3-P_2-glycerate levels were determined. Erythrocyte suspensions were preincubated in medium II for 60 min before F-Ado was added. A control suspension of erythrocytes was incubated simultaneously in medium II. At various times throughout the experiment, 1-ml samples of the suspension containing cells and medium were extracted with 15% trichloroacetic acid and the concentrations of lactate, glucose, and 2,3-P_2-glycerate were determined. The amount of glucose in the incubation medium was too great to permit the accurate measurement of the rate of glucose consumption. Lactate was produced during the preincubation period and by the control sample at a rate of 2.4 μmol/hr per ml of packed erythrocytes. This value agrees well with a normal erythrocytic lactate production rate of approximately 2.0 μmol/hr per ml of cells reported elsewhere (Bartlett and Marlow, 1953). After the addition of F-Ado, the rate of lactate production was markedly increased to a value of 5.9 μmol/hr per ml of erythrocytes. In accord with the observation that the concentrations of ATP and ADP remain relatively constant, the concentration of 2,3-diphosphoglycerate was not affected by F-ATP synthesis. Throughout the course of the F-ATP synthesis, the 2,3-P_2-glycerate level remained at about 3.5 ± 0.1 μmol/ml of packed erythrocytes.

Role of Mediated Nucleoside Transport in F-ATP Synthesis. The recent work of Paterson and his colleagues (Paterson and Oliver, 1971; Oliver and Paterson, 1971; Cass and Paterson, 1972) has demonstrated the existence of a mediated transport system for nucleosides in human erythrocytes. After preincubation with uridine, a nonmetabolizable pyrimidine nucleoside in human erythrocytes, the efflux of uridine is greatly accelerated by the addition of other nucleosides such as adenosine, which suggests the existence of an exchange-transport mechanism. Furthermore, several highly potent and specific inhibitors of the transport system have been identified (Paterson and Oliver, 1971). One of these, 6-[(2-hydroxy-5-nitrobenzyl)thio]guanosine (HTG), causes a complete inhibition of the mediated transport system when added in concentrations as low as 10 μM. In order to test the possible role of this mediated transport system in the overall pathway of F-ATP synthesis, the effects of preincubation with uridine and coincubation with HTG were examined. The amount of F-ATP synthesized after 1 hr's preincubation with uridine and incubation for 30 and 60 min with F-Ado was essentially indistinguishable from that of a control sample examined simultaneously. Also, no difference in F-ATP synthesis was detected with erythrocytes coincubated with 10 μM HTG and F-Ado. Therefore, it appears that the mediated transport system for nucleosides is not rate limiting in the pathway of conversion of F-Ado to F-ATP under the conditions of these experiments. It is possible, however, that for conversion under other conditions, such as lower concentrations of F-Ado, the mediated transport system may be rate limiting.

Inhibition of F-ATP by Synthesis by N^6-Substituted Adenosines. Divekar and Hakala (1971) have demonstrated that several N^6-substituted adenosines are both inhibitors and substrates for adenosine kinase purified from Sarcoma 180 cells and several of these compounds have shown interesting biological effects (Fleysher *et al.*, 1968, 1969; Fleysher, 1972). As

TABLE II: Inhibition of F-ATP Synthesis by N-Substituted Adenosine Analogs.[a]

	% Inhibn
F-Ado	0
F-Ado + N^6-ethoxyethyladenosine	36
F-Ado + N^6-allyladenosine	45
F-Ado + N^6-amyladenosine	76
F-Ado + N^6-methyladenosine	79
F-Ado + N^6-phenyladenosine	79
F-Ado + N^6-hexyladenosine	85

[a] Twenty per cent suspensions of fresh, washed human erythrocytes were incubated in medium II containing 1.0 mM F-Ado and the appropriate N^6-substituted adenosine in 1.0 mM concentration. Samples were taken at 30, 60, and 120 min and the amount of F-ATP synthesized was estimated by high-pressure liquid chromatography. The rate of synthesis of F-ATP was determined in the presence and absence of the N^6-substituted adenosines and the percentage of inhibition was calculated.

seen in Table II the percentage of inhibition of F-ATP synthesis in intact erythrocytes agreed well with the inhibitions observed with isolated Sarcoma 180 adenosine kinase preparations. This observation is further evidence that the rate-limiting step in the conversion of F-Ado to F-ATP is the reaction catalyzed by adenosine kinase. Since several N^6-substituted adenosine analogs form nucleotides when incubated with erythrocytes (see Table I), it is likely that the inhibitions noted above are caused by competition between alternative substrates for the enzyme, adenosine kinase.

Discussion

These studies demonstrate that human erythrocytes are capable of incorporating large amounts of certain analog nucleosides into their nucleotide pools. Earlier it had been shown by Loo *et al.* (1968) and Ho *et al.* (1968) that MMPR can accumulate in human erythrocytes at the 5′-monophosphate nucleotide level in amounts approaching 1 mM and that the presence of this large amount of analog nucleotide does not affect the survival of erythocytes infused into patients. In addition to MMPR, a number of adenosine analogs now have been identified that accumulate at the 5′-monophosphate nucleotide level indicating that they are substrates for erythrocytic adenosine kinase. Other analogs, such as F-Ado, tubercidin, toyocamycin, and N^6-methyladenosine, which are not substrates for erythrocytic adenosine deaminase (Agarwal *et al.*, 1973), are converted to the triphosphate nucleotide level. These observations indicate that the mono- or diphosphate nucleotides of these analogs may serve as substrates for erythrocytic adenylate kinase and for enzymes such as pyruvate kinase, 3-phosphoglyceric acid kinase, and nucleoside diphosphokinase. Formycin A, a close structural analog of adenosine (Robins *et al.*, 1966; Ishizuka *et al.*, 1968; Ward *et al.*, 1969), is rapidly converted to the 5′-triphosphate nucleotide in Ehrlich ascites tumor cells (Caldwell *et al.*, 1966, 1967; Henderson *et al.*, 1967). With human erythrocytes, no nucleotide formation was detected. Formycin A is deaminated by purified erythrocytic adenosine deaminase about four times faster than the natural substrate, adenosine (Agarwal *et al.*, 1973). In recent studies in this laboratory, with a preparation of human erythrocytic adenosine deaminase purified about 3000-fold, a K_m value for adenosine of about 1.2×10^{-4} M was determined (Agarwal *et al.*, 1973). The apparent K_m of erythrocytic adenosine kinase with adenosine is about 1.9×10^{-6} M (Meyskins and Williams, 1971), the K_m of adenosine with the Sarcoma 180 adenosine kinase is 5×10^{-7} M (Divekar and Hakala, 1971), and K_m values of about 2.0×10^{-6} M have been reported for adenosine kinases from Ehrlich ascites cells (Murray, 1968), H. Ep. 2 cells (Schnebli *et al.*, 1967) and rabbit liver (Lindberg *et al.*, 1967). The activity of adenosine deaminase in human erythrocytes is about 0.15 μmolar unit/ml of cells (Agarwal *et al.*, 1973) and on the basis of the rate of F-ATP synthesis and the average rate of adenosine uptake calculated from the data of Lerner and Rubinstein (1970), the activity of adenosine kinase is about 0.027 μmolar unit/ml of cells. Since the K_m for adenosine with adenosine kinase is about 60-fold lower than with adenosine deaminase (*e.g.*, 1.9×10^{-6} M *vs.* 1.2×10^{-4} M), adenosine entering the erythrocyte in low concentrations (*e.g.*, less than 10^{-5} M), as might occur under normal physiological conditions, would be preferen tially salvaged by conversion to AMP. On the other hand, if adenosine enters the erythrocyte in high concentrations (*e.g.*, greater than 10^{-4} M), as might occur during tissue breakdown, most of the adenosine would be degraded to inosine and hypoxanthine which cannot be reconverted to AMP by human erythrocytes (Lowy *et al.*, 1958, 1962; Lowy and Williams, 1966; Manohar *et al.*, 1968). Therefore it is suggested that the relative K_m values of adenosine for the enzymes, adenosine kinase and adenosine deaminase, play a crucial role in determining whether adenosine will be salvaged or degraded in the erythrocyte. It is also likely, however, that allosteric regulation of these enzymes plays a role. The possible importance in the regulation of erythrocytic purine metabolism of the interrelation between the K_m values of adenosine with adenosine kinase and adenosine deaminase has also been noted by Meyskens and Williams (1971). A factor difficult to evaluate on the basis of present information is the function of 5′-AMP-deaminase in the control of nucleotide synthesis and content in erythrocytes, since this enzyme appears to play a major role in a number of other tissues (Lowenstein, 1972). This enzyme occurs in human erythrocytes, and evidence has been presented that it is inhibited by 2,3-diphosphoglycerate (Askari and Rao, 1968). It is of interest that 2-fluoroadenosine 5′-phosphate does not serve as a substrate for 5′-AMP-deaminase from rabbit muscle (O. Haavik and R. E. Parks, Jr., unpublished data).

On first consideration the activity (about 0.027 μmol/min per ml of packed erythrocytes) of adenosine kinase reported above appears low in comparison with the activity of other enzymes of purine metabolism and of glycolysis in these cells. If one assumes, however, that the red cell mass in an adult human is about 2400 ml, there is sufficient adenosine kinase present in these cells to accomplish the salvage of approximately 1.04 g of adenosine/hr or about 25 g/day, an impressive quantity when one considers that the normal human adult excretes less than 500 mg of uric acid/day. This observation again emphasizes the potentially important role of erythrocytes in the whole body economy of purines in man.

An unexpected and provocative finding is the occurrence of a new peak in the mononucleotide region of the chromatograms in erythrocytes incubated with the analog, 2-fluoro-2′-deoxyadenosine. Since this analog is not a substrate for adenosine deaminase (Agarwal *et al.*, 1973), it could not have been converted to the corresponding inosine analog. Therefore, it must have been converted to the 5′-monophosphate nucleo-

tide by an adenosine kinase. Possible explanations of this observation are that erythrocytic adenosine kinase is able to react with both ribonucleosides and deoxyribonucleosides or that in erythrocytes, as in other mammalian cells, there is a separate deoxyadenosine kinase (Krygier and Momparler, 1971). No new peaks appeared in the di- and triphosphate nucleotide regions of the chromatograms, suggesting that this analog of 2′-dAMP does not serve as a substrate for erythrocytic adenylate kinase. The latter is not consistent with the findings of Bartlett (1968) that dADP and dATP are formed after incubation of human erythrocytes with deoxyadenosine. In our experiments, if di- and triphosphate nucleotides are formed at all, the amounts are such that they are not detected. Therefore, work is in progress to isolate sufficient quantities of this new analog nucleotide to permit definitive characterization for further study.

The evidence above suggests that the enzyme, adenosine kinase, is the rate-limiting step in the conversion of F-Ado to F-ATP under the conditions used in the present study. The velocity of F-ATP synthesis, 0.027 μmol/min per ml of erythrocytes, is substantially lower than the activities in human erythrocytes of adenylate kinase (1.3 EU/ml) and nucleoside diphosphokinase (76.0 EU/ml) (Agarwal *et al.*, 1971). Also, a number of N^6-substituted adenosines, which were shown to inhibit adenosine kinase from Sarcoma 180 cells (Divekar and Hakala, 1971), similarly inhibited F-ATP synthesis in erythrocytes (Table II). In the present studies, which employed relatively high concentrations of F-Ado (0.5 and 1.0 mM) and long incubation periods (30–60 min), it was not possible, through the use of uridine preincubation or the inhibitor HTG, to demonstrate that the nucleoside-mediated transport system of Paterson and colleagues (Oliver and Paterson, 1971; Paterson and Oliver, 1971; Cass and Paterson, 1972) is rate limiting. One may not conclude, however, that nucleoside transport would not be a major factor under different experimental conditions. In the recent report of Roos and Pfleger (1972), it was suggested that nucleosides, such as adenosine, can enter erythrocytes through two processes, a saturable transport mechanism and diffusion. Perhaps under our experimental conditions the rate of diffusion was sufficiently great and the incubation period sufficiently long that stimulation or inhibition of the mediated transport mechanism could not be detected. It is entirely possible that if the concentration of F-Ado were lowered to 10^{-5}–10^{-6} M, the nucleoside transport mechanism might then become rate limiting in the synthesis of F-ATP. Firm conclusions on this interesting question must await studies with labeled F-Ado. The possibility that F-AMP might have been formed from fluoroadenine *via* the adenine phosphoribosyltransferase reaction seems highly unlikely. It was shown in studies of rat erythrocytes with uniformly labeled adenosine that virtually all of the adenosine that entered the nucleotide pools was incorporated *via* the adenosine kinase reaction and that cleavage of adenosine to liberate free adenine was negligible (Miech and Santos, 1969).

An interesting question raised by these studies relates to the source of energy consumed during the synthesis of large amounts of analog nucleotides. For example, each mole of F-ATP synthesized requires the utilization of three moles of high-energy phosphate. Surprisingly, during the synthesis of relatively large amounts of analog nucleotides little change was observed in the concentrations of the normal nucleotides. Also, the concentration of 2,3-P_2-glycerate, a presumed storage form of high-energy phosphate in mammalian erythrocytes, was unchanged during the conversion of F-Ado to the nucleotide level. However, a substantial increase in the rate of lactate production from a normal value of 2.4 μmol/hr per ml of cells to 5.9 μmol/hr per ml of cells occurred. Since the production of 1 mol of lactate from glucose by erythrocytic glycolysis results in the production of 1 mol of high-energy phosphate, the rate of lactate production could account for the formation of about 6 μmol of high-energy phosphate/hr per ml of cells, an amount sufficient for the synthesis of 2 mol of F-ATP/hr per ml of erythrocytes. Therefore, the rate of glycolysis observed here can explain the observed rate of F-ATP synthesis. Apparently, the erythrocyte can increase glycolysis enough to provide the required high-energy phosphate without causing a significant depletion of normal nucleotides or of 2,3-P_2-glycerate.

These studies demonstrate that better understanding of human erythrocytic metabolism can be obtained from studies of the incorporation of adenosine analogs into nucleotide pools. An intriguing possibility raised by these studies is that the incorporation of analog nucleosides into the nucleotide pools of erythrocytes may be employed in chemotherapy (Smith *et al.*, 1970; Grage *et al.*, 1970; Jaffee *et al.*, 1971; Stegman *et al.*, 1973; Senft *et al.*, 1973a). It has been demonstrated recently that certain intravascular parasites, such as *Plasmodium berghei* (Büngener and Nielson, 1964) and *Schistosoma mansoni* (Senft *et al.*, 1972), are deficient in the *de novo* pathway for purine biosynthesis and therefore must be dependent upon exogenous sources of purines for the synthesis of such vital substances as coenzymes and nucleic acids. Schistosomes have been shown capable of incorporating both purine bases and nucleosides into their nucleotide pools and to possess the enzymes, adenosine kinase, adenine phosphoribosyltransferase, and one or more phosphoribosyltransferases for guanine and hypoxanthine (Senft *et al.*, 1973a). In addition, they are capable of converting analog nucleosides, such as F-Ado and tubercidin, to the triphosphate nucleotide level (Stegman *et al.*, 1973). Promising chemotherapeutic results were obtained when tubercidin-loaded erythrocytes were infused into mice bearing *Schistosoma mansoni* (Jaffee *et al.*, 1971). The present study demonstrates that a large number of nucleoside analogs are readily incorporated into the nucleotide pools of erythrocytes. Therefore, they are candidates for chemotherapeutic study and it would be of interest to determine whether erythrocytes containing large quantities of these analog nucleotides have normal survival time. There has been considerable speculation that erythrocytes may be capable of delivering nucleotides to peripheral tissues (Henderson and LePage, 1959; Pritchard *et al.*, 1970). Obviously the question of the possible transfer of nucleotides from erythrocytes to other tissues must be resolved before this potential chemotherapeutic approach can be brought to the level of practice.

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