ERYTHROCYTE PRESERVATION. VIII. METABOLIC DEGRADATION OF NUCLEOSIDES *IN VITRO* AND *IN VIVO* [1]

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It has been shown previously that the addition of inosine to ACD preservative [2] prolongs the effective period of *in vitro* storage of erythrocytes (1). Inosine is utilized by the red cell after a phosphorolytic cleavage to ribose-l-phosphate and hypoxanthine, mediated by a nucleoside phosphorylase (2, 3). Ribose-l-phosphate enters the "aerobic shunt pathway" of glucose metabolism after conversion to ribose-5-phosphate, and a subsequent effect is a generation of ATP [3] and the resultant maintenance of the energy reserve of the red cell (4). Inasmuch as the red cell lacks the enzyme, xanthine oxidase (5), it is evident that the other cleavage product, hypoxanthine, is not metabolized further but remains in the red cell and the plasma during storage.

The present investigation is concerned with the rate of conversion of inosine to hypoxanthine by the red cells and the ratio of these substances in the plasma of blood stored in ACDI, as well as the *in vivo* metabolism of inosine after infusion. Related studies on the nucleosides, adenosine and guanosine, will be presented also.

METHODS

Human blood was collected in ACD, and the nucleoside, dissolved in 0.9 per cent NaCl, was added with sterile precautions.

Inosine and adenosine were obtained from Schwarz Laboratories; hypoxanthine and guanosine from Nutritional Biochemicals Corporation.

Hemolysis during storage was determined by the measurement of plasma hemoglobin as the pyridine hemochromogen according to the method of Flink and Watson (6).

Paper chromatography (one-dimensional) on Whatman No. 1 filter paper was performed with the following solvent systems: isobutyric acid: concentrated ammonia: water (66:1:33), n-butanol: water (86:14), and water adjusted to pH 10 with NH_4OH, according to the methods described previously (1). After identification of the purine-containing compounds with the use of an ultraviolet light (Mineralite), the materials were eluted from the paper with water and measured spectrophotometrically. These substances were estimated also by quantitative densitometry [4] of the paper chromatograms.

The hypoxanthine content of the plasma and red cells was determined enzymatically, using xanthine oxidase (7), except that the assays were performed on neutralized PCA filtrates of the various fractions.

Acid filtrates of plasma were prepared in the following way: 2 ml. of plasma were added to 2 ml. of cold 0.6 N PCA, mixed well, and centrifuged at 4° C for 5 minutes at 15,200 × g.[5] The precipitate was washed once with 2 ml. of cold 0.3 N PCA, and the centrifugation was repeated. The supernatant fluid and the washing were combined and neutralized with cold 20 per cent KOH, followed by centrifugation at 4° C for 10 minutes at 15,200 × g. The $KClO_4$ precipitate was washed once with 2 ml. of cold distilled water and centrifugation repeated. This second supernatant fluid and the washing were combined for analysis. Acid filtrates of the red cells were prepared in essentially the same way. Approximately 4 ml. of cells were washed twice with an equal volume of cold 0.9 per cent NaCl each time with centrifugation for 20 minutes at 1,700 × g.[6] The washings were discarded, since they contained only negligible amounts of ultraviolet absorbing materials.

A 50 per cent cell suspension was prepared with 0.9 per cent NaCl. Hematocrit determinations were made on this cell suspension. One ml. of cell suspension was added to 3 ml. of cold 0.6 N PCA, and the neutralization of the acid filtrate proceeded as described above.

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[2] Acid-citrate-dextrose, National Institutes of Health, Formula B.

[3] The following abbreviations have been used: ATP = adenosine triphosphate; PCA = perchloric acid; ACDI, ACDA, ACDG, = acid-citrate-dextrose, inosine, adenosine or guanosine, respectively.

[4] Photovolt densitometer, Model 301A, phototube B, filter 5265, wave-length 253 mμ.

[5] Twelve thousand rpm; International Centrifuge Model PRI; high speed attachment; radius from bottom of tube equals 9.5 cm.

[6] Three thousand rpm; International Centrifuge Model PRI; rotor No. 269, radius from center of tube equals 17 cm.

Absorption spectra measurements in the region of 230 to 300 mμ were obtained with the use of the Beckman DU spectrophotometer on the PCA filtrates of plasma or red cells. Since both hypoxanthine and inosine were present in the plasma of blood stored in ACDI or ACDA (1, 4, 8), the relative amounts of each substance could not be estimated directly from light absorption measurements alone. However, after the amount of hypoxanthine had been determined by means of xanthine oxidase, it was possible from the light absorption data on the plasma filtrates, to correct the extinction values at 249 mμ (peak light absorption of hypoxanthine, millimolar extinction coefficient = 10.5) for hypoxanthine concentration. The readings were also corrected for the light absorption of appropriate control samples, *i.e.*, untreated with nucleoside. Thus a value was obtained which was due to inosine. Approximately 400-fold dilutions were made of all samples for spectrophotometric measurement, and the light absorption of the control samples during storage was less than 2 per cent of the nucleoside-treated samples.

Uric acid was determined on plasma and urine by the uricase method of Dubbs, Davis, and Adams (9).

RESULTS AND DISCUSSION

Storage of blood in ACDI

The ratio of hypoxanthine to inosine during the storage of blood in ACDI at 4° C was determined in two experiments. In the first investigation, only the ratio in the plasma was estimated, while the second study involved the ratio of the two compounds in both plasma and red cell fractions.

Experiment 1. Human blood was collected in ACD and divided into two 100-ml. aliquots. After 24 hours of storage at 4° C, 20 ml. of 0.9 per cent NaCl were added to one aliquot, and 20 ml. of inosine solution in 0.9 per cent NaCl (1300, μmoles, *i.e.*, 3,000, μmoles per 100 ml. red cells) were added to the other. Analyses were performed subsequently on days 4, 9, 15, 23, 37, and 57.

There is progressive uptake of inosine by the red cell (*cf.*, Table I), so that by 57 days there are only 14 μmoles of inosine remaining in the plasma, while 1,286 μmoles have been taken up by the cells. Of this amount absorbed, 440 μmoles have returned to the plasma as hypoxanthine, leaving 846 μmoles inside the cells as inosine and hypoxanthine. Paper chromatographic experiments revealed that only two ultraviolet absorbing materials, *i.e.*, hypoxanthine and inosine, were present in the plasma fractions throughout storage. Furthermore, quantitative densitometry of the chromatograms confirmed the ratios of hypoxanthine to inosine given in Table I. The enzyme responsible for the phosphorolytic cleavage of the nucleoside, nucleoside phosphorylase, has been shown previously to be in the soluble portion of the red cells (3).

After phosphorolytic cleavage of inosine, the ribose moiety is metabolized further by the red cell during storage (4), while the nitrogenous base, hypoxanthine, remains unchanged. As the time of storage progresses, hypoxanthine diffuses from the red cell into the plasma fraction in increasing amounts.

Experiment 2. Human blood was collected in ACD and divided into two aliquots: 1) 100 ml. blood + 20 ml. saline; 2) 100 ml. blood + 20 ml. inosine solution (1,272 μmoles, *i.e.*, 3,500 μmoles per 100 ml. red cells). Both aliquots were stored at 4° C for 36 days. The data are reported in Table II. After 36 days of storage there were 742 μmoles of hypoxanthine and 361 μmoles of inosine distributed between the plasma and red cell fractions, or a total of 1,103 μmoles of material. This leaves 169 μmoles (13 per cent of the original amount of inosine) unaccounted for. This loss of material is unexplained, at present, although paper chromatography indicated the presence of an unknown compound capable of absorbing ultraviolet light in the nucleoside-treated sample which was not present in the control erythrocytes.

TABLE I

Plasma hypoxanthine:inosine ratio of blood stored in ACDI

Days' storage	Plasma fraction*,† Inosine (μmoles)	Hypoxanthine (μmoles)	Hypoxanthine: Inosine ratio	Inosine "absorbed" (μmoles)	% Inosine "absorbed"
1	1,300‡				
4	928	143	0.15	372	29
9	709	241	0.34	591	46
15	538	296	0.55	762	59
23	277	298	1.08	1,023	79
37	150	376	2.51	1,150	89
57	14	440	31.40	1,286	99

* The plasma fraction represents the plasma, ACD, and the saline diluent for inosine added to the 100-ml. aliquot of blood (20-ml. inosine solution).

† All values have been corrected for the values of the control sample (100 ml. aliquot of blood stored in ACD + 20 ml. saline).

‡ Thirteen hundred μmoles inosine added after 24 hours' storage.

TABLE II

The relationship of hypoxanthine to inosine in plasma and red cells of blood stored in ACDI

Human blood was stored for 36 days in ACD (100 ml. blood + 20 ml. saline) and in ACDI (100 ml. blood + 20 ml. inosine = 1272 μmoles).

	36 days stored (ACDI)		
	μmoles Is*	μmoles Hx*	Hx:Is ratio
Plasma fraction	330	529	1.6
RBC fraction	31	213	6.9
Total†	361	742	

* Hx = Hypoxanthine; Is = Inosine.
† Total recovery of 1,103 μmoles (361 + 742) is 87% of the original amount. All values are corrected for those of the control (ACD).

Calculations of the concentration of hypoxanthine in either plasma or red cells revealed that at 36 days of storage there was an equilibrium established (approximately 6 μmoles hypoxanthine per ml.).

Storage of blood in ACDA and ACDG

Similar experiments were carried out on blood stored in ACD with the addition of either 1,200 μmoles of adenosine or 1,300 μmoles of guanosine under conditions identical to those described in Experiment 1.

In the study of blood stored in ACDA it was apparent from paper chromatographic analysis that, after 4 days of storage, there was no adenosine in the plasma fraction, and that inosine and hypoxanthine were the only ultraviolet absorbing substances present. In the plasma filtrates throughout storage (*i.e.*, from 4 to 57 days) maximum light absorption occurred between 247 and 249 mμ. By direct measurement, the amounts and ratios of hypoxanthine and inosine were similar to those described for blood stored in ACDI, *e.g.*, on days 4, 9, 15, 23, 37, and 57, the ratios of hypoxanthine to inosine in the plasma were 0.09, 0.23, 0.76, 1.60, 2.20, and 35.3, respectively. Thus, it appears as if storage of blood with adenosine is quite similar to storage with inosine except that the enzymatic conversion of adenosine to inosine *via* the adenosine deaminase (10) results in the liberation of ammonia which accumulates in the blood during storage (1). This conversion of adenosine to inosine is complete after one-hour incubation of stored cells with adenosine (1). The above evidence is confirmatory to previous work from this laboratory (4, 11) and in accordance with the observations of Rubinstein, Kashket, and Denstedt (8).

TABLE III

Hemolysis during storage of blood in ACDI and ACDA

Days' storage	Mg. hemoglobin per 100 ml. plasma fraction		
	Control*	ACDI*	ACDA*
4	29	14	15
9	37	24	35
15	59	34	42
23	121	56	61
37	292	102	107
57	1,296	362	329

* Control (ACD) = 100 ml. blood + 20 ml. saline, ACDI = 100 ml. blood + 20 ml. inosine (1,300 μmoles), ACDA = 100 ml. blood + 20 ml. adenosine (1,200 μmoles).

The degree of hemolysis in blood stored in ACDI and ACDA is compared in Table III. The similarity is apparent, inasmuch as the presence of either nucleoside suppresses hemolysis during storage, although all values are relatively high due to the fact that at each time of sampling, the blood was agitated by thorough mixing.

Analyses of plasma removed from blood stored in ACDG show that guanine is the purine base liberated from guanosine as a result of phosphorolytic cleavage. Guanine is not degraded further during *in vitro* storage. Studies employing paper chromatography reveal that the increasing concentration of plasma guanine during storage is of the same order of magnitude as the amount of plasma hypoxanthine found in blood stored in ACDI. Neither adenosine nor inosine were detected in blood stored in ACDG.

The fate of hypoxanthine and guanine after infusion of their ribosides

It was of interest to investigate the metabolism of purine moieties of inosine and guanosine after intravenous administration of these purine nucleosides into human recipients.

Two experiments were performed in which normal subjects received about 7,000 μmoles of inosine intravenously in a 500-ml. saline infusion. This amount of inosine is equivalent to that required for the preservation of one unit of blood.

TABLE IV

Serum uric acid after inosine infusion

Experiment 1. Normal male subject (99 Kg.) received intravenously 6,960 μmoles inosine over a period of 1.5 hr.

Experiment 2. Normal male subject (103 Kg.) received intravenously 7,470 μmoles inosine over a period of 1.25 hr.

Time after end of infusion	Mg. uric acid per 100 ml. serum
Expt. 1	
0	6.1
5 min.	8.8
45 min.	9.8
3 hr.	7.4
5 hr.	8.4
Expt. 2	
0	6.1
8 min.	11.5
1 hr.	10.0
3 hr.	8.9
5 hr.	8.7
8 hr.	8.5
27 hr.	6.1

Levels of uric acid in serum and urine were determined at various time intervals after the infusion. A transient rise in serum uric acid was produced soon after the infusion, but the value returned to normal in about 24 hours (Table IV).

Uric acid excretion was measured over a period of about 2 days after the infusion and was compared to the normal excretion levels of the subjects. Approximately 32 per cent of the dose of inosine was excreted as uric acid during a period of 24 hours after the infusion (Table V). Subsequent sampling indicated that an additional 10 per cent of the dose was excreted over the next 12 hours at which time the uric acid levels had returned to normal.

Although these experiments are preliminary in nature, it is probable that the series of events which occur *in vivo* after the infusion of inosine are those shown in equation (*1*).

TABLE V

Uric acid excretion after inosine infusion

Experimental conditions as in Table IV

	μmoles uric acid excretion above normal in 24-hour post infusion period*	% dose of inosine excreted as uric acid in 24 hr.
Expt. 1	2,427	34.9
Expt. 2	2,344	31.4

* Normal urinary uric acid was established in the subjects as 3,993 μmoles per 24 hours.

Guanosine (7,060 μmoles) was administered similarly to a patient with leucopenic leukemia, and the data recorded in Table VI indicate that the metabolic end product of the purine base in this case was the same, *i.e.*, uric acid. It is probable that the phosphorolysis and oxidation proceed according to equation (*2*).

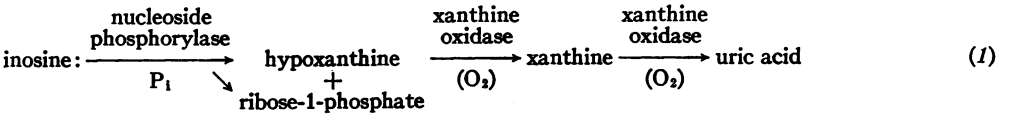
Previous studies on the subcutaneous injection of guanosine have demonstrated increments of uric acid excretion in the urine (12). While neither these previous results nor the data presented in this communication permit any conclusions as to the urinary yield of converted nucleoside, they would appear to be within the range observed after the intravenous administration of uric acid alone, *i.e.*, about 60 per cent (13).

TABLE VI

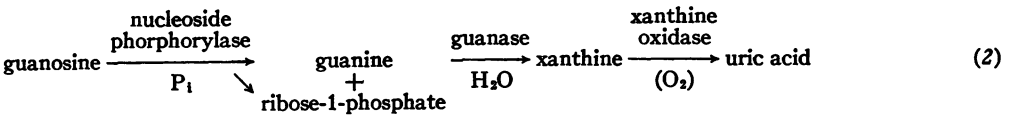
Serum uric acid after guanosine infusion

Patient (58 Kg.) with leucopenic leukemia received intravenously 7,060 μmoles guanosine over a period of one hour.

Time after end of infusion	Mg. uric acid per 100 ml. serum
0	4.4
10 min.	10.9
2 hr.	11.9
3 hr.	12.0
5 hr.	13.3
12 hr.	10.8
20 hr.	6.5



(*1*)



(*2*)

SUMMARY

After being taken up by erythrocytes, inosine undergoes enzymatic phosphorolysis to yield ribose-l-phosphate and hypoxanthine. The hypoxanthine diffuses outward into the plasma during storage until an equilibrium is reached with that inside the cells.

Adenosine is converted rapidly to inosine during storage and is utilized through the same metabolic pathway, with the resultant increasing concentration of hypoxanthine in the plasma. Guanosine is utilized similarly with the exception that guanine is the purine base which accumulates in the plasma.

The intravenous administration of inosine indicates that the hypoxanthine is oxidized further *in vivo* to uric acid. Likewise, guanosine infusion results in increased concentrations of uric acid in the blood serum.

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