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PHOSPHORYLATED CARBOHYDRATE INTERMEDIATES OF THE HUMAN ERYTHROCYTE DURING STORAGE IN ACID CITRATE DEXTROSE. III. EFFECT OF INCUBATION AT 37° C WITH INOSINE, INOSINE PLUS ADENINE, AND ADENOSINE AFTER STORAGE FOR 6, 10, 14, AND 18 WEEKS *

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It has been found that a short incubation with inosine at 37° C will bring about a marked resynthesis of adenosine triphosphate and other metabolites in human erythrocytes which had been stored in the cold in acid citrate dextrose (ACD) for 4 weeks (1). It was felt desirable to extend these observations to longer periods of storage.

Nakao, Nakao, Tatibana and Yoshikawa (2) have reported on the intermediate metabolites of the human erythrocyte after cold storage in ACD plus inosine, and have made the important discovery that when stored blood was incubated with inosine plus adenine there was regeneration of a large amount of adenosine triphosphate, whereas incubation with inosine alone was much less effective. In the present study, experiments have been carried out to see whether this effect of adenine could be demonstrated by incubation of the blood with the purine for 1 hour at 37° C after extended periods of storage.

The results of Mollison and Robinson (3) suggest that adenosine is more effective than inosine in extending the survival of stored erythrocytes and in causing regeneration of adenosine triphosphate. In view of the favorable influence of adenine on adenosine triphosphate formation, this superiority of adenosine, if true, might arise from a partial splitting of the compound to adenine, although it is generally agreed that adenosine is very rapidly deaminated to inosine by the erythrocyte. It seemed important to include in the present study a comparison of the effects of adenosine with those of inosine alone and with inosine plus adenine.

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The experiments reported here were performed by storing human blood at 4° C in ACD under the usual blood banking conditions for 6, 10, 14, and 18 weeks followed by a 1 hour incubation at 37° C with inosine, inosine plus adenine, or adenosine. The metabolic intermediates were then isolated by column chromatography on ion-exchange resins.

METHOD

From healthy donors, 480 ml blood was collected by gravity into 120 ml ACD (NIH formula B) and was stored at 4° C. After 6, 10, 14, and 18 weeks a unit of blood (600 ml ACD blood mixture) was removed from storage and divided into aliquots of 100 ml. Each aliquot was incubated for 1 hour at 37° C after the addition of 10-ml portions of inosine, inosine plus adenine, or adenosine, dissolved in normal saline. Controls were run after the addition of normal saline alone. The effect of the addition of adenine alone under the same conditions was studied only at the 6-week storage period. Adenine was added in an amount of 10 μmoles and nucleoside in an amount of 15 μmoles per ml of blood.

Adenine and adenosine were obtained from the California Corporation for Biochemical Research, and inosine from the Pabst Laboratories. The adenine was dissolved in dilute HCl and then neutralized with NaOH. The HCl and NaOH were added in amounts so that the resulting solution of NaCl was 0.9 per cent in concentration. The adenosine was examined by paper chromatography, using *n*-butanol saturated with boric acid as the solvent system, and only the spot of adenosine was found. The adenosine was also examined by column chromatography with Dowex-1 resin, and only 0.2 per cent of the total ultraviolet-absorbing material appeared in the position expected for adenine (4).

After the 1 hour incubation at 37° C, the blood was centrifuged, plasma and buffy coat removed, and the erythrocytes washed with cold normal saline. The erythrocytes were mixed with 2 vol of 10 per cent trichloroacetic acid (TCA), the mixture centrifuged, and the precipitate re-extracted with 5 per cent TCA. The TCA was removed with ether. The neutralized extract was

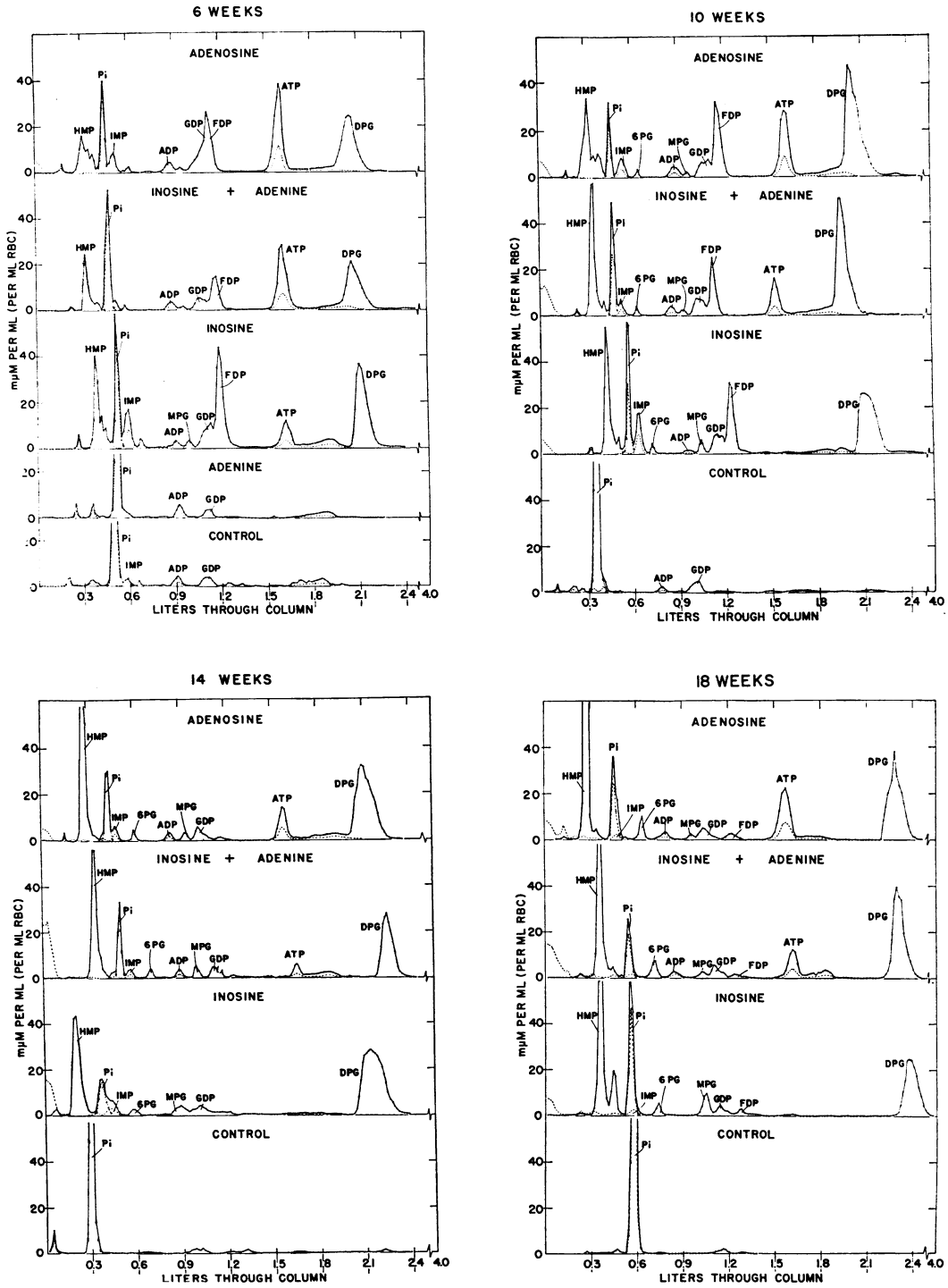

FIG. 1. ION-EXCHANGE CHROMATOGRAPHS OF TRICHLOROACETIC ACID EXTRACTS OF ERYTHROCYTES STORED IN ACID CITRATE DEXTROSE FOR 6, 10, 14, AND 18 WEEKS, FOLLOWED BY INCUBATION FOR 1 HOUR AT 37° C WITH NORMAL SALINE (CONTROL), INOSINE, INOSINE PLUS ADENINE, OR ADENOSINE. The data are plotted as millimicromoles per milliliter of eluate (calculated for 1 ml of erythrocytes). Total phosphorus, •———•; adenine (from absorbance at 260 mμ), ------. Abbreviations: HMP, sugar monophosphate; Pi, inorganic phosphate; IMP, inosine monophosphate; ADP, adenosine diphosphate; GDP, glucose-1,6-diphosphate; FDP, fructose-1,6-diphosphate; ATP, adenosine triphosphate; DPG, 2,3-diphosphoglycerate; MPG, monophosphoglycerate; 6 PG, 6-phosphogluconic acid.

TABLE I

Phosphate compounds of erythrocytes after storage in ACD, followed by incubation at 37° C with nucleoside, purine base, or both*

Compound†	6 Weeks‡					10 Weeks				14 Weeks				18 Weeks			
	C§	A	HR	A+HR	AR	C	HR	A+HR	AR	C	HR	A+HR	AR	C	HR	A+HR	AR
Pi	3.92	2.68	1.41	1.29	1.23	5.50	1.49	1.38	0.97	4.80	1.13	1.01	1.08	4.08	2.29	0.87	1.45
DPN	0.07	0.12	0.15	0.06	0.10	0.09	0.09	0.10	0.08	0.25	0.09	0.08	0.08	T	T	T	T
TPN	0.04	0.10	0.12	0.11	0.08	0.07	0.11	0.19	0.10	T	0.08	0.08	0.12	T	T	T	T
AMP	0.07	0.13	0.02	0.02	0.05	0.03	0	0.03	0.03	0	0	0	0	0	0.07	0	0
ADP	0.23	0.31	0.21	0.13	0.31	0.11	0.11	0.25	0.31	T	0.06	0.18	0.18	0	0	0.18	0.22
ATP	T	0	0.86	1.92	2.59	T	T	1.10	1.78	T	0	0.30	0.86	0	0	0.87	1.70
AXP	0.45	0.40	0.73	0.60	0.65	0.15	0.68	0.85	1.95	0	T	0.45	0.90	0	T	0.45	0.30
IMP	0.07	0.06	0.33	0.09	0.17	0.09	0.88	0.35	0.42	0	0.49	0.16	0.24	0	0.13	0.08	0.11
DAP	0	0	0.35	0.17	0.21	0	0.22	0.19	0.44	0				0			
R5P	T	0	0.38	0.31	0.24	T	0.23	0.33	0.22	0	0.17	0.20	0.17	0	0.15	0.24	0.16
G6P	T	0	0.51	0.42	0.34	T	0.83	0.95	0.60	0	1.23	0.89	0.72	0	1.28	1.50	1.01
F6P	T	0.02	0.43	0.23	0.21	T	0.44	0.43	0.31	0	0.52	0.47	0.25	0	0.58	0.34	0.31
S7P	T	0	0.12	0.05	0.02	T	0.15	0.20	0.17	0	0.51	0.69	0.59	0	0.31	0.56	0.62
6 PG	0	0	0.17	0.07	0.09	0	0.14	0.13	0.11	0	0.16	0.16	0.13	0	0.20	0.34	0.34
GDP	0.14	0.30	0.54	0.38	0.35	0.24	0.33	0.42	0.45	T	0.27	0.24	0.35	T	0.40	0.31	0.34
FDP	0	0	2.06	0.38	1.79	0	1.59	1.04	2.03	0	0.15	0	0.03	0	0.14	0.09	0.13
MPG	0	0	0.17	0.10	0.09	0.03	0.23	0.13	0.11	0	0.37	0.21	0.16	0	0.48	0.16	0.13
DPG	0	0	3.16	2.68	2.84	0	3.39	4.40	5.16	0	4.55	2.37	4.22	0	2.60	3.64	3.57
TP	4.99	4.48	11.90	9.06	12.10	6.30	11.20	12.6	15.3	5.05	9.79	7.15	10.1	4.08	8.63	9.63	10.4

* Expressed as μmoles P/ml erythrocytes.
† Abbreviations: see Figure 1; DAP, dioxyacetone phosphate; AXP, unknown nucleotide; TP, total phosphorus; T, trace.
‡ Storage period.
§ Incubation solution: C, normal saline; A, adenine; HR, inosine; A+HR, adenine plus inosine; AR, adenosine.

passed through a 1 × 20 cm column of Dowex-1-X8 formate (100–325 wet mesh) resin which was eluted with 4 L of a linearly increasing concentration of 5 N ammonium formate buffer, pH 3.0 (1 part ammonium formate to 4 parts formic acid), at a rate of 1 ml per minute; fractions were collected at 15-minute intervals.

Each fraction was analyzed for total phosphorus and for absorbance at 260 mμ. All compounds were identified tentatively by their elution position, as previously determined in this laboratory with reference compounds. Nucleotides were identified by their ratio of adenine: phosphorus: ribose and their absorption spectra. Sugar phosphates were analyzed by their reactions in the anthrone (5), carbazole (6), orcinol (7), and cysteine-sulfuric acid (8) tests. Phosphogluconic acid was identified by enzyme assay with 6-phosphogluconic acid dehydrogenase. Dihydroxyacetone phosphate was identified by its characteristic color in a modified carbazole reaction (9). For a more detailed description of the methods used, see Bartlett (9–12).

RESULTS

All chromatographs are reproduced in Figure 1, and the quantitative results are given in Table I. All data are presented as micromoles of phosphorus per milliliter of erythrocytes.

1. Six weeks' storage. After incubation at 37° C, the control sample of blood, which had been stored for 6 weeks, contained very little organic phosphate—0.8 μmole per ml erythrocytes as compared to a normal value of approximately 12 μmoles. Only a trace of ATP was found, ADP had decreased in concentration to about 50 per cent of normal, and adenosine monophosphate (AMP) was present in a normal amount. Of the sugar phosphates, the monophosphate fraction was barely detectable, fructose-1,6-diphosphate (FDP) was absent as is usual in ACD, and glucose-1,6-diphosphate (GDP) had decreased to about one-half its normal value. 2,3-Diphosphoglycerate (DPG) had completely disappeared, but monophosphoglycerate (MPG) was still present in a small amount. The pyridine nucleotides, DPN and TPN, were unaffected by these conditions of storage and incubation. A large amount of inorganic phosphate had accumulated.

The blood which was incubated with adenine alone showed no difference from the control; therefore, at the later storage periods, this incubation test was not repeated.

After incubation with inosine, there was resynthesis of 10 μmoles organic phosphate per ml erythrocytes and a decrease in inorganic phosphate. The concentration of ATP increased to about 40 per cent of normal; whereas, the amount of ADP was the same as in the control. A striking accumulation of ribose, glucose, fructose, and

sedoheptulose monophosphate was found. There was an increase in concentration of GDP to about twice normal and of FDP to six times normal after the incubation with inosine. DPG had increased from 0 to about 30 per cent of the amount found in the fresh erythrocyte. Phosphogluconic acid, which is not found in the fresh erythrocyte and was not present in the control blood, had accumulated in a concentration of 0.18 μmole. Inosine monophosphate was present in a concentration of 0.33 μmole.

The effects of incubation with inosine plus adenine or adenosine were quite similar to treatment with inosine alone except that there was a much greater increase in concentration of ATP, up to or slightly exceeding normal limits.

2. Ten weeks' storage. In the control sample, as found at the 6-week period of storage, only a small fraction of the total phosphate was organic. The same was true also at 14 and 18 weeks. Again only a trace of ATP was present, 50 per cent less ADP was found, and AMP had almost disappeared. The concentration of GDP had increased, and FDP was still absent. The oxidized form of the pyridine nucleotides was present in normal amounts.

After this period of storage, incubation with inosine resulted in the synthesis of sugar mono- and diphosphate as it did after 6 weeks of cold storage. However, these cells were no longer able to synthesize adenylate, and only a trace of ATP was found. A large amount of DPG accumulated during this incubation.

In contrast to the effect of treatment with inosine alone, when these cells were incubated with either inosine plus adenine or adenosine, there was an increase in the amount of adenylate. The concentration of ADP increased two- to threefold, and the level of ATP increased to 1.1 to 1.8 μmoles phosphorus after incubation with inosine plus adenine and with adenosine, respectively.

Incubation with either substance resulted in an even greater accumulation of DPG than was found after treatment with inosine. The sugar phosphate picture was much the same as that after incubation with inosine alone.

3. Fourteen weeks' storage. The small amount of organic phosphate found in the control blood sample consisted of traces of ADP, ATP, TPN, and GDP and a normal amount of DPN. Incubation with inosine resulted in the synthesis of 9 μmoles of organic phosphate, which consisted almost entirely of sugar phosphate and DPG. No ATP was present, and the concentration of ADP was lower than that found at the earlier storage periods. As compared to blood stored for 10 weeks, there was an even greater accumulation of mono- and diphosphoglycerate. The sugar monophosphate and glucose diphosphate pictures were similar; however, at this period, the concentration of FDP was much lower than that found at 6 or 10 weeks. The concentration of inosine monophosphate had fallen.

Incubation with inosine plus adenine or with adenosine resulted again in a picture similar to that found after treatment with inosine alone, except that there was a synthesis of a considerable amount of adenylate. Once again incubation with adenosine resulted in a greater increase in concentration of ATP than with inosine plus adenine.

4. Eighteen weeks' storage. At this period, the only organic phosphate found in the control was a trace of the pyridine nucleotides, DPN and TPN, and GDP. There was no evidence for the presence of adenylate.

Incubation with inosine resulted in the synthesis of only 3.1 μmoles of organic phosphate. No adenylate was present. Smaller amounts of sugar mono- and diphosphates were found than at 14 weeks. Both mono- and diphosphoglycerate accumulated, although there was less DPG than at 14 weeks.

In contrast, almost 9 μmoles organic phosphate was synthesized after incubation with inosine plus adenine or adenosine. There was a synthesis of both ADP and ATP; again adenosine was more effective than inosine plus adenine. More ATP was found at this storage period than at 14 weeks, a finding which we are unable to explain. Otherwise, the picture was similar to that found at the 14-week storage period.

DISCUSSION

We have reported previously (1) that erythrocytes stored in ACD in the cold for 4 weeks, when incubated with inosine at 37° C for a few minutes, were capable of synthesizing large amounts of organic phosphate, including ATP. In the present study, it was found that after 6 weeks of storage

followed by incubation with inosine alone, erythrocytes synthesized significant amounts of ATP. However, after longer periods of storage, the erythrocytes were unable to do this, presumably due to an inability to convert inosine monophosphate to adenosine monophosphate.[1] It is possible that the synthesis of adenylate after incubation with inosine alone at the 6-week period was due to the presence of a small amount of free adenine in the blood (see below).

When blood which had been stored for 6 weeks was incubated with adenine alone, there was no demonstrable effect on the metabolic intermediates. However, even after 18 weeks of storage, when the blood was incubated with a mixture of adenine plus inosine, the erythrocytes were able to synthesize adenylate. It would appear that after extended storage, synthesis of adenylate by the erythrocytes is dependent on the presence of a ribose donor (nucleoside) and adenine (the purine alone or in adenosine).

Most investigators have felt that adenosine would be so rapidly deaminated to inosine by the erythrocytes that the effect of these two nucleosides on the erythrocyte after storage would be identical. In the early experiments of Gabrio, Donohue and Finch (13), most of the studies demonstrating an increased post-transfusion survival of erythrocytes after extended storage with nucleoside were performed with adenosine. More recently, Mollison and Robinson (3) have emphasized that adenosine may be more effective than inosine in extending the "storage life" of the erythrocyte. Our findings show that erythrocytes which have been stored for 10 to 18 weeks are able to synthesize ATP after incubation with adenosine, but not with inosine. Studies with labeled nucleoside would help to provide information concerning the pathways involved in the conversion of adenosine to adenylate.

Phosphogluconic acid was found at all storage periods when the blood was incubated with nucleoside. This indicates that there was some shunting of hexose phosphate into or perhaps through the pentose oxidative pathway.

The pyridine nucleotides, DPN and TPN, were the most stable compounds in the erythrocyte during storage and were still found in trace amounts in the control blood after 18 weeks (14).

In future studies it would seem of special interest to determine whether there is any correlation between viability and the regeneration of intermediate metabolites (especially ATP) in the long-stored human erythrocyte which has been exposed briefly to purine, purine nucleoside, or both, and also to learn more about the optimal conditions for the synthesis of ATP as regards the concentration of added substrate, the time of incubation, and so forth.

[1] For a discussion of possible pathways for the conversion of inosine to adenylate, see Bartlett and Shafer (1).

SUMMARY

After storage at 4° C for 6, 10, 14, and 18 weeks in acid citrate dextrose, blood was incubated for 1 hour at 37° C with normal saline, inosine, inosine plus adenine, or adenosine, and the phosphorylated carbohydrate intermediates of the erythrocytes were separated on columns of ion-exchange resin. After 6 weeks of storage, very little organic phosphate remained in the control blood. When blood stored for 6 weeks was incubated with inosine, there was resynthesis of a large amount of organic phosphate including adenosine triphosphate; however, after longer periods of storage, incubation with inosine alone was not effective in producing a synthesis of adenylate. When blood which had been stored 18 weeks was incubated with inosine plus adenine or with adenosine, there was still a synthesis of a large amount of organic phosphate, including adenosine triphosphate.

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