Chemical Changes in Stored Blood, with Observations on the Effects of Adenosine

By T. A. J. PRANKERD
Medical Unit, University College Hospital Medical School, London

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In a recent paper (Prankerd & Altman, 1954) certain changes in phosphorus metabolism occurring in fresh cells incubated *in vitro* at 37° were described. It was shown (i) that adenosine and guanosine make possible the exchange of ^{32}P between cells and plasma in the absence of glucose, (ii) that ^{32}P entering the cells is incorporated into phosphate esters, and (iii) that ^{32}P-labelled cells, when incubated at 37° with an excess of glucose, begin to lose ^{32}P after 16 hr. owing to breakdown of phosphate esters, but that this change can be prevented by the addition of adenosine or guanosine. It was concluded that the effect of nucleosides arose from their cleavage by a phosphorylase, and that the ribose phosphate so formed was metabolized in the hexose monophosphate shunt. Recently, Gabrio, Donohue & Finch (1955) have shown that adenosine improves the storage of red cells at 4°, and Gabrio, Hennessey, Thomasson & Finch (1955) have reported that adenosine increases the content of easily hydrolysable phosphate in the stored red cells. The experiments described in this paper were made in order to obtain more precise information on changes in blood stored at 4° in the presence and absence of nucleosides.

MATERIAL AND METHODS

Whole blood was obtained under sterile conditions from healthy donors and mixed in 5:1 ratio with 'A–C–D medium' (disodium citrate 2·0 g., dextrose 3·0 g., water 120 ml.). To this mixture was added 0·1 vol. of 0·9% NaCl or 0·1 vol. of 0·05M adenosine in 0·9% NaCl, and the mixtures were kept in a refrigerator at 4°. Samples for analyses were taken with aseptic precautions at varying intervals.

Glucose. This was estimated in duplicate samples of whole blood by the method of Nelson (1944). The standard error of these estimations on five samples of blood was ±0·45%.

Phosphate esters. These were separated from trichloroacetic acid (10%) extracts of washed cells by the method of Caldwell (1955). The chromatograms were run in propanol–NH_3 soln.–water for 18 hr. to get better separation of adenosine triphosphate and adenosine diphosphate. The total phosphorus estimated in the esters thus separated accounts for about 70% of the total phosphorus of blood cells.

Phosphorus. This was determined by the method of Berenblum & Chain (1938).

Sodium and potassium. These were estimated in blood cells washed with 0·9% NaCl by means of an EEL flame photometer (Evans Electroselenium Ltd., Harlow, Essex), with known standards. The standard error of these estimations on five samples of blood was Na, ±0·75%; K, ±0·61%.

Lactic acid. This was determined by the method of Barker & Summerson (1941).

Nucleosides and purines. These were separated and estimated by methods described by Prankerd (1955). It has recently been found by paper electrophoresis that adenosine is first deaminated to inosine, which then undergoes phosphorylative cleavage to hypoxanthine and ribose phosphate. This method of separation only differentiated nucleosides from purines.

Cells were separated from blood samples by centrifuging at 3000 *g* for 10 min. in graduated tubes. Changes in cell volume were estimated by centrifuging samples under the same conditions in Wintrobe haematrocrit tubes. Intercellular fluid was taken to constitute 2% of the cell volume (Jackson & Nutt, 1951) and this was deducted to obtain true cell volume. Intracellular contents were corrected for changes in cell volume during storage.

Although in some experiments about 20 μc of carrier-free [^{32}P]orthophosphate was added to the blood after temperature equilibration had been obtained at 4°, in other experiments blood was incubated with the same amount of [^{32}P]-orthophosphate at 37° for 4 hr., then washed with saline, and resuspended in a previously separated portion of extracellular fluid (which was free from radioactivity) before storing at 4°. The radioactivity in phosphate esters and supernatant fluid was assayed by means of an end-window Geiger–Müller counter. For this purpose areas of paper containing the separated phosphate esters were counted, and 0·1 ml. of supernatant fluid was dried on filter paper and also counted. Counting rates of 25 000 counts/min./μc of ^{32}P were obtained; all counting was continued sufficiently long to give standard errors of less than 3%.

RESULTS

Table 1 shows changes in phosphate esters which occurred during storage of blood cells with and without adenosine.

Storage without adenosine. In the absence of adenosine the following sequence of events was observed. During the first 3 weeks 5·95 ± 0·23 m-moles of glucose/l. of blood disappeared (1·8 ± 0·29 m-moles of glucose/l./week), and 1·4 ± 0·2 m-moles of lactic acid/l. of blood appeared. The amount of lactic acid formed over this period was slightly, but

significantly, less than that which corresponds to the amount of glucose utilized ($P < 0.05$ by Student's t test). Within the cells there was a slow but fairly constant fall in the amount of 2:3-diphosphoglycerate (2:3-DPG) and of adenosine triphosphate (ATP). However, the rate of fall in ATP content was less than that of 2:3-DPG (0·11 μmole/ml. of cells/week compared with 0·23 μmole/ml. of cells/week of 2:3-DPG). The rise in cell orthophosphate during this period can be accounted for quantitatively by the breakdown of 2:3-DPG and ATP; this indicates that no significant loss of orthophosphate occurred from the cells during 3 weeks. In spite of the fall in ATP content there was no corresponding rise in the adenosine diphosphate (ADP) content, and presumably ADP must have broken down to adenylic acid (AMP). Fructose 1:6-diphosphate (F 1:6-P) increased slightly (t, 4·25; $P < 0.05$).

After 3 weeks of storage the cellular metabolism can be seen to alter profoundly. Glucose utilization showed a sharp fall (0·55 ± 0·17 μmole/l./week); lactic acid production almost ceased. About the end of the third week the 2:3-DPG content suddenly decreased and by the fourth week this ester disappeared altogether; its disappearance was associated with a further rise in cell orthophosphate. The fall in cell ATP continued and there was no corresponding rise in ADP. However, about the fourth week the breakdown of ATP appeared to come to a standstill in spite of there being about a quarter of the original content left. Fructose 1:6-diphosphate showed no further increase.

From about the end of the fourth week no further intracellular changes occurred, but haemolysis progressively developed. Over these 4 weeks concentrations of cell Na^+ ion and K^+ ion altered owing to the movement of these ions in the direction of their concentration gradients.

Storage with adenosine. In the blood stored with adenosine the changes occurring in the first 3 weeks closely resembled those described above, except with regard to 2:3-DPG. This ester showed no significant decrease over this period, and there was a significant difference between the amounts of 2:3-DPG in cells stored with and without adenosine (t, 4·35; $P < 0.05$). Consistent with the better preservation of 2:3-DPG in cells stored with adenosine was the finding that cell orthophosphate increased less in these cells. Glucose utilization appeared to occur faster in the presence of adenosine (2·0 m-moles of glucose/l./week), but this difference was not significant (t, 2·04; $P > 0.1$), and lactate production was not increased (t, 1·17; $P > 0.1$). The nucleoside disappeared from the extracellular fluid at the rate of 4·71 μmoles/ml. of cells/week, and was replaced by purine. Changes in electrolyte composition of cells stored in this way were no different from those stored in the A–C–D medium alone.

Table 1. *Chemical changes in blood stored in A–C–D with and without the addition of adenosine* (0·005 M)

For details see text.

Results are given as means of three experiments ± standard error. 2:3-DPG: 2:3-diphosphoglycerate; F 1:6-P: fructose 1:6-diphosphate; ATP: adenosine triphosphate; G 6-P: glucose 6-phosphate; ADP: adenosine diphosphate; IP: inorganic phosphate.

Days of storage	Glucose (mg./100 ml. of blood mixture)	Lactic acid (mg./100 ml. of blood mixture)	Phosphate esters (μg. of P/ml. of cells)					m-equiv./l. of cell water	
			2:3-DPG	ATP	ADP	IP	F 1:6-P	G 6-P	K^+	Na^+
					Without adenosine					
0	640 ± 12·3	—	165 ± 5·1	71 ± 12·0	21 ± 3·1	24 ± 6·1	15 ± 1·7	10 ± 1·4	102 ± 3·7	19 ± 2·0
7	606 ± 8·5	55 ± 12·0	155 ± 13·6	56 ± 6·8	24 ± 2·0	72 ± 10·2	17 ± 2·0	12 ± 1·7	82 ± 5·1	43 ± 3·1
14	570 ± 2·7	—	119 ± 9·2	47 ± 6·8	19 ± 2·4	85 ± 15·0	22 ± 1·7	15 ± 1·3	74 ± 2·7	59 ± 4·8
21	547 ± 6·2	110 ± 6·8	92 ± 17·8	37 ± 3·4	28 ± 3·1	112 ± 7·9	25 ± 2·7	16 ± 1·7	68 ± 2·7	67 ± 3·1
28	533 ± 4·1	126 ± 4·5	6 ± 3·4	31 ± 2·4	25 ± 4·8	204 ± 6·5	25 ± 2·4	0	60 ± 5·1	72 ± 2·4
35	522 ± 3·1	—	0	29 ± 3·7	23 ± 2·0	210 ± 4·5	20 ± 3·8	0	53 ± 2·4	79 ± 4·1
42	517 ± 3·4	138 ± 7·9	0	26 ± 3·7	20 ± 2·4	200 ± 6·5	20 ± 2·0	0	49 ± 3·1	80 ± 4·8
					With adenosine					
0	640 ± 12·3	—	165 ± 5·1	71 ± 12·0	21 ± 3·1	24 ± 6·1	15 ± 1·7	10 ± 0·68	102 ± 3·7	19 ± 2·0
7	592 ± 6·5	69 ± 11·6	168 ± 8·9	53 ± 3·8	20 ± 2·0	47 ± 6·8	19 ± 2·4	14 ± 2·7	92 ± 4·1	40 ± 2·0
14	571 ± 4·5	—	175 ± 8·5	33 ± 2·4	15 ± 2·4	43 ± 3·1	20 ± 2·4	17 ± 1·7	69 ± 4·4	56 ± 5·8
21	533 ± 4·1	128 ± 9·6	170 ± 7·5	35 ± 2·7	19 ± 1·7	52 ± 5·8	17 ± 2·0	17 ± 1·0	63 ± 2·0	69 ± 5·8
28	525 ± 4·1	149 ± 6·2	165 ± 3·8	30 ± 2·0	24 ± 1·7	57 ± 1·7	23 ± 2·7	5 ± 3·4	61 ± 4·8	71 ± 7·2
35	523 ± 5·1	—	149 ± 2·7	29 ± 1·7	24 ± 2·0	72 ± 1·7	23 ± 1·7	0	56 ± 3·1	78 ± 3·8
42	519 ± 3·1	189 ± 3·7	83 ± 27·0	25 ± 1·4	21 ± 2·4	130 ± 20·8	25 ± 2·7	0	45 ± 3·1	80 ± 4·4

Table 2. *Amounts of phosphate esters and their specific activities in cells stored with* 20 μC *of* ^{32}P *at* 4° *with and without adenosine* (0·005 M)

Content of phosphate esters (P) is expressed in μg. of P/ml. of washed blood cells. The specific activity (S.A.) of these esters is expressed in counts/min./μg. of P. (Abbreviations as in Table 1.)

	With adenosine								Without adenosine							
	2:3-DPG		ATP		ADP		IP		2:3-DPG		ATP		ADP		IP	
Days of storage	P	S.A.	P	S.A.	P	S.A.	P	S.A.	P	S.A.	P	S.A.	P	S.A.	P	S.A.
0	158	—	65	—	15	—	34	—	158	—	65	—	15	—	34	—
7	164	20	55	18	8	—	37	14	128	16	59	16	20	—	88	7
14	143	31	43	14	10	3	41	70	81	42	40	18	19	2	125	69
28	139	35	38	16	17	3	68	95	9	50	42	9	12	2	190	86
35	74	84	30	10	15	6	112	71	0	—	25	12	17	5	210	112

Table 3. *Amounts of phosphate esters and their specific activities in cells stored at* 4° *with and without adenosine* (0·005 M) *after incubation for* 4 *hr. with* 20 μC *of* ^{32}P *at* 37°

Content of phosphate esters (P) is expressed in μg. of P/ml. of washed blood cells. The specific activity (S.A.) of these esters is expressed in counts/min./μg. of P. (Abbreviations as in Table 1.)

	With adenosine								Without adenosine							
	2:3-DPG		ATP		ADP		IP		2:3-DPG		ATP		ADP		IP	
Days of storage	P	S.A.	P	S.A.	P	S.A.	P	S.A.	P	S.A.	P	S.A.	P	S.A.	P	S.A.
0	170	1480	70	1120	19	650	20	1200	170	1480	70	1120	19	650	20	1200
7	169	1150	63	1520	20	795	47	1380	164	1100	57	1100	21	790	74	1450
14	175	945	29	1450	19	940	43	1170	135	805	51	1350	22	870	90	1850
28	175	775	31	1400	11	1050	50	1290	23	775	30	1270	18	980	175	1650
35	113	760	26	1500	17	1100	120	1580	0	0	21	1090	17	1070	206	1510

After 3 weeks' storage there was a remarkable difference between cells stored with adenosine and those without, namely, 2:3-DPG persisted in considerable amounts in cells stored with adenosine until after the fifth or sixth week. The contents of cell orthophosphate differed (t, 3·9; $P<0·05$) and only rose in parallel with the fall of 2:3-DPG and ATP. However, in spite of the persistence of 2:3-DPG, ATP fell a similar extent in the presence as in the absence of adenosine. There was no difference in glucose utilization (t, 1·58; $P>0·10$) but lactic acid production was greater (t, 1·0; $P<0·01$). The continued production of lactic acid corresponded quantitatively to the amount of nucleoside disappearing over the same 3 weeks (6·8 m-moles of lactic acid/l.: 6·1 m-moles of nucleoside/l.). As pointed out later this can be explained by the formation of lactic acid from the pentose of the nucleoside. Cell electrolyte changes did not differ significantly in the two media (for K^+, t, 0·82; $P>0·10$; for Na^+, t, 0·43; $P>0·10$).

Turnover of ^{32}P. The results of experiments on ^{32}P turnover in stored cells are given in Tables 2 and 3. Table 2 shows changes which occurred when ^{32}P was added to the blood mixture at the beginning of storage. There appeared to be practically no flow of ^{32}P into the ATP pool after the first week. This applied equally to cells stored in either way. On the other hand, there appeared to be a continuous flow of ^{32}P into the pool of 2:3-DPG in both the presence and the absence of adenosine during storage for the first 4 weeks, as shown by the rise in specific activity of this ester. In the cells stored with adenosine, where 2:3-DPG persisted into the fifth week, the specific activity still rose at that time.

Table 3 records changes in cells which were first incubated with ^{32}P for 4 hr. at 37°, and then subjected to cold storage. In previous experiments (Prankerd & Altman, 1954) it was found that incubation for 4 hr. resulted in a radioactive equilibrium. In the experiments in Table 3 adenosine was not added until the blood mixture had been kept at 4° for 30 min. To a large extent the changes which occurred were a reverse of those shown in Table 2, since the cells were washed and resuspended in a ^{31}P-phosphate medium before storage. Thus the specific activity of the 2:3-DPG pool fell owing to dilution with unlabelled phosphorus, which must have come from the medium, whereas the specific activity of the ATP pool showed no significant change. The activity of the cell orthophosphate pool increased considerably in the first 2 weeks and the increase must have resulted from the breakdown of labelled esters. In the cells stored with adenosine, where the breakdown of esters was less marked, the rise in the

activity of the cell orthophosphate pool was less also; however, later in storage when phosphate esters had decreased, even in the presence of adenosine, then the activity of the cell orthophosphate pool rose markedly.

The very limited incorporation of ^{32}P into ATP in the experiments recorded in Table 2, considered together with the lack of dilution of labelled phosphorus by unlabelled phosphorus in the ATP of cells as shown in Table 2, indicated that relatively little synthesis of ATP was occurring in these cells compared with 2:3-DPG. Furthermore, in the presence of adenosine, synthesis of 2:3-DPG continued for a longer time than in its absence.

Reincubation at 37° after storage at 4°. In Table 4 are shown changes in phosphate esters and electrolytes which occurred in cells reincubated for 3 hr. at 37° after varying periods of storage at 4°. Both storage and reincubation occurred with and without the addition of adenosine. After 3–4 weeks of storage at 4° red cells lost their ability to utilize glucose on reincubation at 37°, whether they were stored with adenosine or not, and there was no reaccumulation of K^+ ion and no resynthesis of phosphate esters. Reincubation at 37° before this time enabled cells to reverse those changes that had occurred during cold storage. If, however, adenosine was added to the reincubating mixture to a final concentration of 0·005 M, then, even after storage for 6 weeks uptake of K^+ ion and resynthesis of phosphate esters occur, and after incubation for 1 hr. cells started reutilizing glucose. At this stage the ATP content had risen to 0·54 μmole/ml. of cells. A considerable amount of adenosine is utilized on reincubating stored cells; about 3·1 μmoles/ml. of cells/hr.; an amount much greater than was found with fresh cells (Prankerd, 1955). These changes on reincubating stored cells appeared to occur just as readily whether adenosine is present during storage at 4° or not.

DISCUSSION

It is clear from the experiments described above that for about 3 weeks of cold storage the red cells retain their ability to metabolize glucose. At 4° the rate of synthesis of phosphate esters is less than their rate of breakdown, and the levels of these esters consequently decrease progressively. This fall is most marked in ATP, which decreases at a fairly constant rate from the start of storage, whether or not the cells are utilizing glucose; the rate of fall is similar to that observed by Maizels (1943).

From the experiments in which ^{32}P-phosphate was added to the storage mixture it appears that the incorporation of ^{32}P into ATP is very small in

Table 4. *Changes in phosphate esters and cations on reincubation of blood at 37° with and without adenosine* (0·005 M) *after varying periods of storage at 4°*

For explanation of abbreviations see Table 1. A: adenosine.

Days of storage ±adenosine	Conditions of incubation ±adenosine		Glucose (mg./100 ml.)	Phosphate esters. μg. of P/ml. of cells				m-equiv./l. of cells	
				2:3-DPG	ATP	ADP	IP	K^+	Na^+
21 −A	−A	Before incubation	510	95	43	28	102	61	45
		Incubated 1 hr.	501	—	—	—	—	60	44
		Incubated 3 hr.	485	70	49	2	80	66	39
21 −A	+A	Before incubation	510	95	43	28	102	61	45
		Incubated 1 hr.	497	—	—	—	—	61	44
		Incubated 3 hr.	475	95	58	6	65	69	38
21 +A	−A	Before incubation	501	165	35	19	54	70	31
		Incubated 1 hr.	493	—	—	—	—	70	29
		Incubated 3 hr.	466	184	47	10	34	72	26
21 +A	+A	Before incubation	501	165	35	19	54	70	31
		Incubated 1 hr.	490	—	—	—	—	72	27
		Incubated 3 hr.	465	197	54	12	27	77	25
42 −A	−A	Before incubation	485	0	25	21	194	50	71
		Incubated 1 hr.	487	—	—	—	—	50	71
		Incubated 3 hr.	484	0	10	5	79	49	76
42 −A	+A	Before incubation	485	0	25	21	194	50	71
		Incubated 1 hr.	483	36	40	5	113	51	72
		Incubated 3 hr.	471	112	65	9	54	57	64
42 +A	−A	Before incubation	499	75	27	19	131	47	67
		Incubated 1 hr.	495	—	—	—	—	47	69
		Incubated 3 hr.	489	0	7	9	75	49	68
42 +A	+A	Before incubation	499	75	27	19	131	47	67
		Incubated 1 hr.	499	90	45	8	104	47	65
		Incubated 3 hr.	481	158	62	12	36	51	61

comparison with the incorporation into 2:3-DPG, and this raises the question whether significant synthesis of ATP occurs during cold storage. In contrast 2:3-DPG synthesis continues during the first 3 weeks of storage and is increased, and continues longer during storage, in the presence of adenosine. The continued synthesis of 2:3-DPG, and rise in concentration of F 1:6-P, suggest that the glycolytic steps below the triose phosphate dehydrogenase stage are defective at 4°, and this would account for the lack of ATP synthesis observed. This defect is reversible on reincubation at 37°. The disappearance of 2:3-DPG after glucose utilization ceases and in the absence of adenosine must be due to uncompensated breakdown, perhaps as a result of phosphatase activity.

The fall in content of glucose 6-phosphate in cells after 3 weeks' storage coincides with their diminished ability to utilize glucose, and implies that the reaction which fails is the conversion of glucose into glucose 6-phosphate. The failure of this reaction would appear to be due to depletion of cell ATP since it is reversible when sufficient ATP has been synthesized in the presence of adenosine.

In the presence of adenosine, even after 3 weeks' storage, the synthesis of 2:3-DPG and the formation of lactic acid still occur. The formation of triose phosphates from adenosine by the activity of red-cell nucleoside phosphorylase was first described by Dische (1951), and it is likely that triose phosphate formed in this way contributes to the synthesis of 2:3-DPG. In spite of these reactions, however, no increase in ATP synthesis appears to occur, and the presence of adenosine during storage does not increase the ability of the red cell to metabolize on reincubation in a medium devoid of this nucleoside. If, however, adenosine is present during reincubation then resynthesis of intracellular phosphate esters occurs and the cell regains its ability to move cations against their concentration gradients, and cell potassium rises.

The ability of cells to metabolize adenosine after they can no longer utilize glucose may be attributed to the fact that ATP would not be required for the phosphorylation of the ribose fraction of adenosine, which occurs as a result of phosphorolysis, whereas ATP is required for the phosphorylation of glucose. Once ATP has been synthesized to a sufficient extent in the presence of adenosine the ability of cells to phosphorylate glucose is restored. It should be stated that there is considerable variability in the response of blood to incubation after storage, and many samples fail to revive their metabolism at all. When revival does occur the changes are consistent.

SUMMARY

1. Changes in phosphate ester composition occurring in red cells stored at 4° have been investigated.

2. During the first 3 weeks of storage the content of 2:3-diphosphoglycerate and adenosine triphosphate decreases. The flow of ^{32}P added as phosphate into 2:3-diphosphoglycerate exceeds by several times that into adenosine triphosphate.

3. In the presence of adenosine the concentration of 2:3-diphosphoglycerate is maintained for periods up to 6 weeks at 4°.

4. On reincubation at 37° after storage for 3 weeks at 4° red cells lose their ability to utilize glucose. This can be restored by adenosine; the ability to utilize glucose appears to be related to levels of adenosine triphosphate within the cell. Restoration can occur after 6 weeks of storage in the presence of adenosine.

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