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THE HIGH PERMEABILITY OF HUMAN RED CELLS TO ADENINE AND HYPOXANTHINE AND THEIR RIBOSIDES

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Human red blood cells are highly permeable to glucose, which is their normal substrate for glycolysis. Several nucleosides also yield lactic acid, however, and like glucose they support the active transport of sodium and potassium ions (Kahn & Cohen, 1957; Lowy, Jaffe, Vanderhoff, Crook & London, 1958). During an investigation of the effects of nucleosides on cation transport a number of questions arose about the permeability of the red-cell membrane to these compounds (Whittam, 1960). This paper describes the resulting study of the uptake of inosine, adenosine, hypoxanthine and adenine by human red cells. The main findings are the high membrane permeability to these compounds and the intracellular conversion of adenine to a non-diffusible form when a substrate for glycolysis was provided.

METHODS

Preparation of cells for incubation. Blood which had been stored for 4 weeks at 4° C in the 'acid-citrate dextrose' solution used in Blood Transfusion Centres (the following solution is mixed with 420 ml. of blood: anhydrous glucose 3·0 g, disodium hydrogen citrate hydrated 2·0 g, water 120 ml.) was obtained from the Radcliffe Infirmary, Oxford. The cells were washed five times with saline medium (mM: NaCl 150, KCl 5, sodium phosphate, pH 7·4, 10) to remove glucose, and the white cells and the upper layer of red cells were removed by suction. The remaining red cells were suspended in the saline medium (50% haematocrit) and shaken in air in stoppered conical flasks in a water-bath at 5 or 37° C. Samples of the suspension were removed after various times for chemical analysis. Precautions to ensure sterile conditions of incubation were not taken, but it is unlikely that bacterial growth during the longest period of incubation of 2 hr would affect the results.

Haematocrit determinations. Samples of the suspension were taken at the beginning of each incubation for haematocrit determinations, which were made by centrifuging the suspension in capillary tubing of uniform bore (13 cm long and 3 mm bore) for 30 min at 1500 *g*. The haematocrit value was taken to represent the actual cell volume, since the volume of trapped fluid under these conditions is probably not more than 2% (Jackson & Nutt, 1951).

Chromatography

Samples of the medium after various periods of incubation were dried on Whatman No. 1 filter paper in a stream of air at room temperature after previously washing the paper according to the directions of Eggleston & Hems (1952). A separation of inosine and adenosine from hypoxanthine and adenine respectively was required by using a solvent which

itself did not absorb in ultra-violet light. The most convenient solvent was found to be 0·1 M sodium borate, pH 10·0 (see Burke, 1954, for electrophoretic separation), with which an ascending solvent front for 6 hr at 20° C gave separations with the R_F values shown in Table 1. The separations of the compounds were reproducible and tailing was not encountered. Inosine was eluted from the paper with 4·0 ml. 0·1 M sodium phosphate (pH 3·0) and estimated from its absorption at 250 mμ, using an extinction coefficient of 11,800 (Kalckar, 1947).

Radioactive chemicals

8-^{14}C adenine sulphate and 8-^{14}C hypoxanthine were supplied by the Radiochemical Centre, Amersham, and standard solutions of these were added to the saline medium. The fall in the radioactivity in the medium was measured by drying known volumes (20–80 μl.) of the medium on Whatman No. 1 filter paper, which was then placed below an end-window G–M tube for the assay of the disintegration rate. A standard solution of the labelled purine, with respect both to radioactivity and to concentration, gave counts per minute proportional to the volume of the solution dried on the paper, and, therefore, allowed the concentration in samples of medium to be calculated from the disintegration rate. 1 μc gave 65,000 counts/min under the present conditions of counting.

TABLE 1. R_F values of purine-containing compounds after 6 hr ascending migration at 20° C

Compound	R_F	Compound	R_F
Inosine	0·86	Hypoxanthine	0·64
Xanthosine	0·85	Deoxyadenosine	0·53
Deoxyinosine	0·79	Uric acid	0·53
Guanosine	0·78	Guanine	0·43
Deoxyguanosine	0·67	Adenine	0·37
Adenosine	0·64		

Sodium borate solution (0·1 M, pH 10·0), in a glass tank (52 cm deep), was allowed to ascend Whatman No. 1 filter paper on which solutions of the compounds had been dried and which was supported from the lid of the tank. The chromatograms were then dried at room temperature and examined under a low-pressure mercury lamp (Chromatolite, Hanovia, Slough), when the compounds were located as dark areas on a light background. Photographs of the chromatograms under ultra-violet light were also made, essentially as described by Markham & Smith (1949).

RESULTS

The permeability of red cells to nucleosides and deoxynucleosides

Inosine. The uptake of inosine by human red cells was taken to be equal to the disappearance of inosine from the saline medium of a 50% cell suspension. Inosine was added to several portions of the suspension to give concentrations in the medium of 36, 18 and 9 mM in order to see in what way the uptake of inosine by the cells was proportional to the initial external concentration. Samples of the suspension were centrifuged after being shaken for 5, 10 and 20 min at 37° C, and inosine was analysed in the medium. The results (Table 2) show that already after 5 min the concentrations in the medium had fallen to 47, 58 and 68% of the initial concentrations, when the latter were 36, 18 and 9 mM respectively. Little further change occurred between 5 and 20 min. The percentage uptake by the

cells (68%) with the lowest concentration in the medium (9 mM) was greater than that (47%) when the concentration was 36 mM. Since a limited amount of phosphorolysis of inosine will lower the intracellular concentration of inosine, this result is to be expected in order to produce the same distribution of inosine between cells and medium with both low and high concentrations. The results are consistent with an uptake of inosine by the cells sufficient to produce approximately equal concentrations in the cells and medium.

TABLE 2. The fall in the inosine concentration in the medium during the incubation of red cells at 37° C

Time (min)	Inosine concn. in medium (mM)	Percentage fall in inosine concn. in medium	Minimum rate of uptake of inosine by cells (m-mole/l. cells/hr)
0	36·0	0	200
5	19·0	47	
10	21·5	40	—
20	18·0	50	—
0	18·0	0	120
5	7·5	58	
10	8·0	55	—
20	7·5	58	—
0	9·0	0	70
5	3·0	68	
10	3·0	68	—
20	2·0	78	—

Washed, cold-stored red cells were added to samples of saline medium containing inosine at concentrations of 9, 18 and 36 mM. The suspensions (50% haematocrit) were shaken in conical flasks in a water-bath at 37° C and samples were removed after 5, 10 and 20 min. The inosine concentration in the medium was measured after centrifugation to remove the cells.

The rates of net entry of inosine calculated from these results are minimum ones, because inosine may have reached equilibrium in less than 5 min. In view of the limitations of the technique of centrifugation for the sampling of the medium for short periods of incubation, it did not seem worth while to attempt to measure the exact rate of uptake. However, the minimum values for the rate of entry are 200, 120 and 70 m-mole/l. cells/hr for initial concentrations in the medium of 36, 18 and 9 mM respectively. These results show that, within this range of concentration, the minimum rate of net entry of inosine into the cells was directly proportional to the concentration in the medium.

Adenosine. Accurate measurement of the rate of uptake of adenosine is complicated, because this compound is deaminated to inosine in human red cells (Conway & Cooke, 1939), and two processes in series will, therefore, determine the rate of uptake of adenosine from the medium. The uptake

will be governed first by the permeability and secondly by the rate of lowering of the intracellular concentration owing to the deamination to inosine. To obtain an estimate of the uptake of adenosine by cells the absorption spectrum of the medium was measured after different periods of incubation when the initial concentration of adenosine in the medium was 20 mM. The fall in the height of the peak for adenosine at 260 mμ

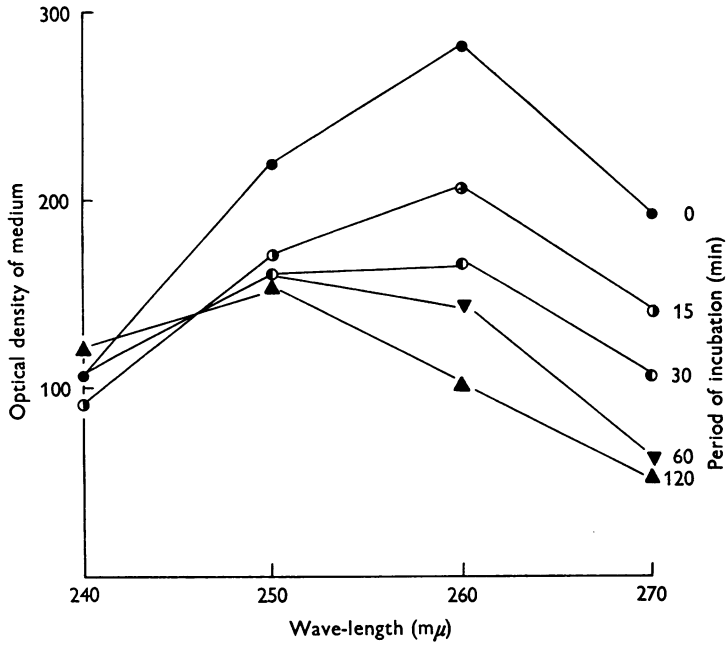

Fig. 1. The change in the peak of the absorption spectrum of the medium during the incubation of red cells with adenosine. Washed, cold-stored red cells were incubated at 37° C in a saline medium containing 20 mM adenosine. The cells were separated from the medium after various periods of incubation and the absorption spectrum of the medium in the range 240–270 mμ was measured.

within 15 min shows that adenosine was taken up from the medium (Fig. 1), and the minimum rate of uptake was 110 m-mole/l. cells/hr. Furthermore, the peak of absorption gradually changed from 260 to 250 mμ until, after 2 hr, the curve was characteristic of hypoxanthine or inosine to which adenosine is deaminated. This result shows that there is a rapid entry of adenosine into human red cells.

The permeability of red cells to hypoxanthine and adenine

In view of the high permeability of human red cells to inosine and adenosine it was of interest to see whether the purines hypoxanthine and adenine also readily crossed the cell membranes. In the following experi-

ments, the disappearance of either ^{14}C-labelled hypoxanthine or adenine was followed from the medium of a cell suspension with an approximately 50% haematocrit.

Hypoxanthine. Preliminary experiments showed that the hypoxanthine concentration in the medium fell to a constant level within 5 min of the addition of washed cells and that no further change occurred after incubation for 1 hr. A variation of the initial hypoxanthine concentration from 0·2 to 6·8 mM made no difference to the percentage of the medium hypoxanthine which entered the cells. Table 3 shows that about 50% (range 51–57) of the hypoxanthine initially present in the medium was taken up by the cells, and that the ratio of the concentrations in cells: medium must consequently have been between 1·0 and 1·3.

To see whether glycolysing cells took up more hypoxanthine than washed cells, guanosine was added as a convenient substrate for lactic acid production (Whittam, 1960). Table 3 shows that the addition of guanosine had no effect on the amount of hypoxanthine entering red cells, which was about 50% (range 46–52) of the initial amount present. It may be concluded that hypoxanthine reaches equilibrium across the red cell membrane within 5 min and that no appreciable concentration gradient was established between the cells and medium.

The fall in the concentration of hypoxanthine in the medium was also measured after incubation at 5° C and after the addition separately at 37° C of 0·4 mM uric acid, 3·8 mM allantoin and 7·0 mM adenine, as it was thought that these compounds might inhibit the uptake of hypoxanthine by the cells owing to their similar molecular structure. Within the limits of the present techniques, these conditions of incubation had no effect on the extent of the uptake of hypoxanthine.

Adenine. The fall in the adenine concentration in the medium was measured when the range of the initial concentration was 1·6–7·7 mM. Inosine was added to part of the suspension as a substrate for glycolysis to see whether it caused a greater uptake of adenine. Table 3 shows that after incubation at 37° C for 30–60 min, the fall in the adenine concentration in the medium without inosine was between 53 and 63%, which indicates that adenine must have reached approximately equal concentrations in the cells and medium. 53% of the adenine in the medium was also absorbed by the cells incubated with inosine when the initial adenine concentration was 7·7 mM, but at lower concentrations of adenine a greater percentage was removed. Thus, when the initial concentrations were 0·6–1·6 mM more than 95% of the adenine in the medium was taken up by the cells in the presence of inosine. More than 95% of the adenine in the medium was also taken up by the cells during incubation with either glucose, deoxyinosine or adenosine. It therefore seems that cold-stored

red cells provided with a substrate for glycolysis absorb more than 95% of the adenine in the medium when its concentration is 1·6 mM or lower. The uptake of 52–65% by glycolysing cells with the higher concentrations of adenine (3·0, 4·6 and 7·0 mM) suggests that the greater uptake of 95% at the lower concentrations was not the result of an active transport of adenine into the cells that depends on glycolysis, because an active uptake would be expected to operate over the whole range of concentration.

TABLE 3. The fall in the concentrations of hypoxanthine or adenine in the medium during the incubation of red cells at 37° C

Purine	Additions to suspension	Initial concn. in medium (mM)	Percentage fall in concn. in medium after 30–60 min
Hypoxanthine	None	6·8	51
		3·4	57
		2·2	55
		0·5	52
	Guanosine 10 mM	6·8	47
		3·4	52
		2·2	46
		0·5	50
Adenine	None	7·7	53
		4·6	56
		3·0	53
		1·6	63
	Inosine 10 mM	7·0	52
		4·6	64
		3·0	65
		1·6	> 95
		0·65	> 95
	Inosine 10 mM + iodoacetate 1 mM	0·59	63
	Glucose 10 mM	0·65	> 95
	Deoxyinosine 10 mM	0·55	> 95
	Adenosine 10 mM	0·66	> 95

Washed, cold-stored red cells were added to samples of saline medium containing either ^{14}C-labelled hypoxanthine or ^{14}C-labelled adenine at the concentrations shown. Further additions of various compounds were made to the suspensions (50% haematocrit) before they were incubated at 37° C for 30–60 min. After the separation of the cells by centrifugation the concentration of hypoxanthine or adenine in the medium was determined by the measurement of the decay of ^{14}C from Whatman No. 1 filter paper on which small volumes (0·02–0·08 ml.) of the medium had been dried.

The time course of the uptake of adenine both at 5 and 37° C is shown in Fig. 2. Equilibrium was reached after 5 min at each temperature, but only 67% of the adenine in the medium was taken up by the cells at 5° C, in contrast to 97% uptake at 37° C. The effect of lowering the temperature, therefore, was to prevent the extra uptake of adenine by the cells with inosine that was found during incubation at 37° C. Figure 2 shows that after a portion of the suspension was transferred from 5 to 37° C, an additional 30% of the adenine in the medium was taken up by the cells.

The converse experiment of transferring a sample of suspension from 37 to 5° C had no effect on the adenine concentration in the medium, suggesting that the adenine taken up at 37° C was not able to leak back into the medium on cooling.

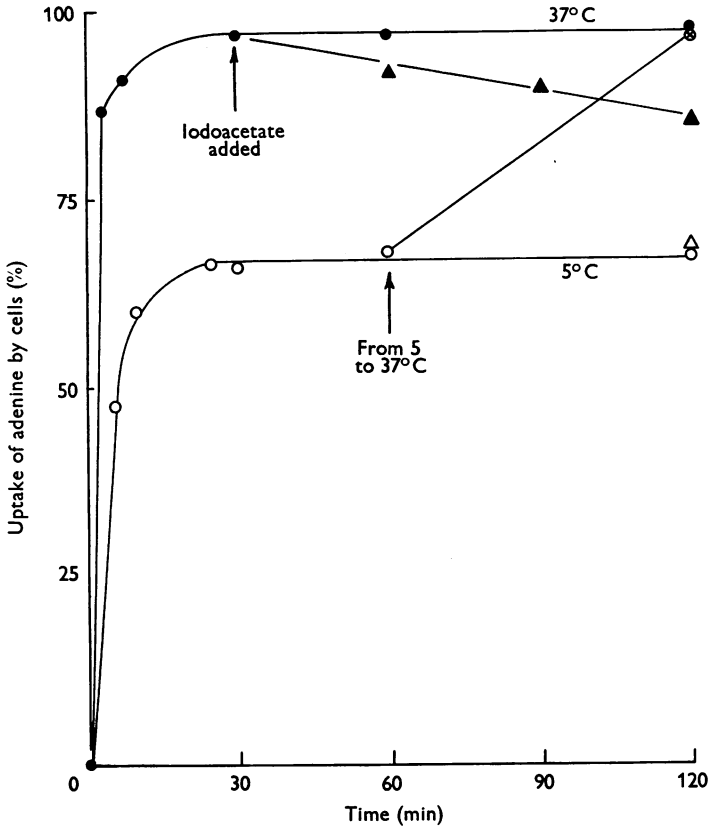

Fig. 2. The percentage uptake of adenine from the medium during the incubation of red cells at 5 or 37° C. Washed, cold-stored red cells were incubated either at 5 (○) or at 37° C (●) in a saline medium (50 % haematocrit) containing 20 mM inosine and about 0·6 mM ^{14}C adenine. The uptake of adenine by the cells was followed by measuring the fall in ^{14}C-adenine in the medium, from which the cells were separated by centrifugation. The decay of ^{14}C was measured from Whatman No. 1 filter paper on which samples of the medium (0·02–0·08 ml.) had been dried. 1 mM sodium iodoacetate was added to portions of the suspension either at the start of incubation (△) or after 30 min (▲). A sample of the suspension was transferred after 60 min from 5 to 37° C and left for a further 60 min (⊗).

The avid uptake of adenine at 37° C in the presence of a substrate for glycolysis suggested that iodoacetate, by its inhibition of glycolysis (triose-phosphate dehydrogenase), might counteract the effect of the substrate. This proved to be so (Table 3 and Fig. 2), for the uptake was 63 % instead

of 97 % after the addition of 1 mM iodoacetate. This compound was also added after 30 min incubation with inosine, and Fig. 2 shows that only a small amount (12 %) of the adenine initially taken up leaked from the cells into the medium.

DISCUSSION

Inosine and adenosine

The permeability of human red cells to nucleosides and purines does not appear to have been previously studied, and precise measurements of the rates of movement have not been possible in the present work owing to the high permeability that was found. The results with inosine and adenosine, however, show that these compounds were taken up by the cells from the medium and that the equilibrium of inosine between cells and medium in approximately equal concentrations occurred within 5 min. Because of the deamination of adenosine in human red cells (Conway & Cooke, 1939), adenosine did not reach a steady distribution across the membrane, but its uptake during the first 15 min of incubation indicated a high membrane permeability. Although only a qualitative description of the permeability is possible, the present study of the uptake of inosine and adenosine has shown that it is high enough not to be a limiting factor when these substances are used as sources of energy by human erythrocytes, which have been previously stored for 4 weeks at 4° C. The high membrane permeability to these compounds is consistent with their utilization as sources of energy to give the immediate increase in potassium influx which was found on their addition to cold-stored cells (Whittam, 1960).

Hypoxanthine and adenine

Hypoxanthine reached a roughly equal distribution between cells and medium within 5 min when it was initially present in the medium. No evidence was found for an active transport of hypoxanthine either into or out of the cells, for neither the temperature of incubation (5 or 37° C), the presence or absence of glycolysis nor the addition of compounds of a similar molecular structure like uric acid, adenine and allantoin had any effect on the extent of the uptake.

The uptake of adenine resembled that of hypoxanthine in being fast, but differed in being greater when a substrate for glycolysis was present at low concentrations of adenine in the medium. Several compounds that are converted to lactic acid by red cells (inosine, glucose, deoxyinosine and adenosine) caused an almost complete uptake of adenine from the medium (more than 95 %) when the initial concentration was 1·6 mM or lower. When glycolysis was suppressed, either by deprivation of substrate, by cooling to 5° C or by the addition of iodoacetate, the cells in a 50 % suspension took up only about 60 % of the adenine of the medium. Once

adenine had been taken up by the cells at 37° C, the addition of iodoacetate caused only a small leakage, and subsequent cooling to 5° C caused no release of adenine to the medium.

The results suggest that glycolysing human red cells may convert adenine into a form that is unable to pass readily out of the cell. At high concentrations of adenine (3–7 mM), the percentage of adenine taken up by the cells was the same both in the presence and absence of glycolysis, presumably because the amount of adenine that may be converted to a non-diffusible form was low relative to the absolute amount present. This observation makes it unlikely that adenine was actively transported into the cells. The most likely compounds that could arise from adenine and be unable to leave the cells are the adenine nucleotides, and it is of interest that Nakao, Nakao, Tatibana, Yoshikawa & Abe (1959) briefly described a synthesis of adenosine triphosphate by human erythrocytes, which has also been observed under the conditions of the present experiments (J. S. Wiley & R. Whittam, unpublished). Possible differences in purine metabolism that may explain the greater uptake of adenine than hypoxanthine by glycolysing cells are being investigated.

SUMMARY

1. A study has been made of the uptake of inosine, adenosine, hypoxanthine and adenine from a saline medium during the incubation of cold-stored human red cells under various conditions.

2. The inosine concentration in the medium fell within 5 min at 37° C to a constant level that was consistent with an even distribution of inosine between the cells and medium. Adenosine was quickly taken up by red cells and was deaminated to inosine.

3. Hypoxanthine reached an approximately even distribution between cells and medium within 5 min at 37° C; this was not changed by either cooling or the addition of guanosine as a substrate for glycolysis.

4. At concentrations of adenine in the medium of 3·0–7·7 mM, approximately equal concentrations between cells and medium were reached within 5 min at 37° C. At low concentrations (0·6–1·6 mM) adenine was almost completely taken up by the cells in the presence of substrates for glycolysis; cooling and iodoacetate abolished this metabolically dependent uptake. It was concluded that adenine may be converted into another form to which the membrane is impermeable.

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REFERENCES

BURKE, D. C. (1954). Ionophoresis of some nucleosides and their derived bases. *Chem. & Ind. (Rev.)*, 1510.

CONWAY, E. J. & COOKE, R. (1939). The deaminases of adenosine and adenylic acid in blood and tissues. *Biochem. J.* **33**, 479–492.

EGGLESTON, L. V. & HEMS, R. (1952). Separation of adenosine phosphates by paper chromatography and the equilibrium constant of the myokinase system. *Biochem. J.* **52**, 156–160.

JACKSON, M. D. & NUTT, M. E. (1951). Intercellular plasma and its effect on absolute red cell volume determination. *J. Physiol.* **115**, 196–205.

KAHN, J. B. & COHEN, S. B. (1957). Effects of ribosides and related compounds on cation transport in cold-stored human erythrocytes. *J. Pharmacol.* **120**, 9–19.

KALCKAR, H. M. (1947). Differential spectrophotometry of purine compounds by means of specific enzymes. 1. Determination of hydroxypurine compounds. *J. biol. Chem.* **167**, 429–443.

LOWY, B. A., JAFFE, E. R., VANDERHOFF, G. A., CROOK, L. & LONDON, I. M. (1958). The metabolism of purine nucleosides by the human erythrocyte in vitro. *J. biol. Chem.* **230**, 409–419.

MARKHAM, R. & SMITH, J. D. (1949). Chromatographic studies of nucleic acids. 1. A technique for the identification and estimation of purine and pyrimidine bases, nucleosides and related substances. *Biochem. J.* **45**, 294–298.

NAKAO, M., NAKAO, T., TATIBANA, M., YOSHIKAWA, H. & ABE, T. (1959). Effect of inosine and adenine on adenosine triphosphate regeneration and shape transformation in long-stored erythrocytes. *Biochim. biophys. acta*, **32**, 564–565.

WHITTAM, R. (1960). The support of active potassium transport in human red cells by nucleosides and deoxynucleosides. *J. Physiol.* **154**, 608–613.