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EFFECTS OF TRIS AND HISTIDINE ON HUMAN ERYTHROCYTES AND CONDITIONS INFLUENCING THEIR MODE OF ACTION

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SUMMARY

1. The incubation of human erythrocytes in 0.172 M Tris · HCl, pH 7.6 buffer at 37° leads to (1) a pronounced cellular volume increase, (2) a preferential release of Na^+, and (3) if continued sufficiently long, hemolysis. These effects are pH dependent and also are influenced to a considerable degree by such diverse reagents as NaCl, glucose, and histidine. In each instance, increasing levels of the latter compounds in a Tris · HCl incubation mixture led to diminished cellular volume increase and prolonged time of onset of hemolysis.

2. Histidine solutions of 0.31 M, pH 7.5 caused a rapid and dramatic decrease in cellular volume of human erythrocytes and a concomitant rapid exit of cations. However, in a prolonged incubation, human erythrocytes slowly regained their cell volume as a result of histidine entry into the cell. Of considerable interest: Tris swollen cells undergo immediate shrinkage to far below the initial cell volume when incubated in histidine at 37 °C. Through repetition of this process two additional times, as much as 90–95 % of the total cellular Na^+ and K^+ was removed without hemolysis.

3. Human erythrocytes washed in 0.12 M $MgCl_2$ and then suspended in 0.31 M histidine, pH 7.5, lost upwards of 60 % of their total Na^+ and 30 % of their total K^+ after a 40 min incubation at 37 °C. However, when increasing amounts of 0.172 M Tris · HCl, pH 7.6 were added to the histidine suspension of cells, the release of K^+ was reduced to 5 % but the release of Na^+ decreased only to 40 % of the total cellular level. On the basis of these observations, it is evident that Tris exerts a preferential activity towards the efflux of Na^+ from the human erythrocyte, whereas histidine results in high efflux of K^+ and Na^+ from the cell.

The permeability of the human erythrocyte has presented a challenging problem for many years, particularly as regards its anion and cation permeability [1–5], selectivity of sugar transport [6], and processes involved in the initiation

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Abbreviations: Tris buffer: 0.172 M Tris · HCl, pH 7.6; Histidine: 0.31 M histidine, pH 7.5; NaCl: 0.155 M NaCl; $MgCl_2$: 0.120 M $MgCl_2$

of hemolysis [7]. In the latter situation, osmotic swelling of erythrocytes in hypotonic solutions has provided considerable insight into the biophysical and biochemical behavior of the erythrocyte [8, 9]. An equally important, but infrequently applied approach to investigation of the stability and behavior of the erythrocyte resides in the use of isotonic solutions of specific compounds under carefully controlled conditions. An example of the latter is illustrated in a report by Omachi et al. [10] who first noted that aliphatic amines such as Tris (2-amino-2-hydroxymethyl-1,3-propanediol) at isotonic levels could penetrate the human erythrocyte resulting in an increased cellular volume. Recently, Hanahan et al. [11] reported that human erythrocytes suspended in 0.172 M Tris · HCl buffer, pH 7.6 and incubated at 37 or 44 °C underwent a time-dependent hemolysis and that 92 % of the cells could be hemolyzed without exposing $Na^+/K^+/Mg^{2+}$ and Ca^{2+}-sensitive ATPase activities.

During the course of further study of the Tris effect on human erythrocytes it was discovered that histidine behaved in a manner opposite to that of Tris. Specifically, histidine caused a shrinkage of Tris-swollen, as well as normal erythrocytes, with no evidence of hemolysis, but with a definitive change in the ionic composition of the cells. The present communication describes the results of experiments designed to explore in detail the Tris and histidine effects, especially their mode and possible site of action on the erythrocyte, the reversibility of any changes, and those substances or conditions affecting the behavior of these compounds toward the intact erythrocyte.

EXPERIMENTAL

Blood samples

Blood was collected from healthy human adult donors, males and females, ranging in age from 21 to 40, directly into heparinized vacutainers (Becton-Dickinson), and centrifuged at 1000 × *g* at 4 °C. The cells were then washed with the desired buffer or reagent three times unless otherwise stated and finally stored at 4 °C, if necessary. Normally, the washed erythrocytes were used within 3 h.

Materials

Tris base and derivatives, L-histidine, imidazole, were Sigma (St. Louis) products. [U—^{14}C]Histidine (270 Ci/mole) was obtained from New England Nuclear Co. (Boston). Deuterium oxide, 99.8 % (Cat. No. 7101003) was a Bio-Rad product (Richmond, Calif.). All other reagents were of C. P. grade.

In order to effect solubilization of L-histidine in water, it was necessary to warm the mixtures to allow complete solution. On occasion, certain histidine solutions developed a yellow color, probably due to photo-oxidation since storage in the dark diminished or inhibited this color development.

Analytical procedures

Hematocrit values, hemoglobin levels, and cation content were determined as described before [11]; chloride was assayed by the method of Cotlove et al. [12], using a Chloridometer (Buchler). Radioactivity was measured in a Beckman Model LS 250 Liquid Scintillation Spectrometer, using Aquasol (New England Nuclear Co., Boston) as the scintillant fluid. Infrared spectra were recorded on a Beckman IR-12 Infrared Recording Spectrophotometer. Variable path length cells equipped with

Irtran windows, were used for the sample examination and were purchased from Precision Cells, Inc., Hicksville, New York.

Uptake of histidine in cells was measured as follows: 0.40 ml aliquots were removed at specific times and layered on 1.0 ml dibutylphthalate in a glass capillary tube, 0.4×15 cm. After centrifugation at room temperature for 20 min at 2000×*g*, the cells were recovered under the phthalate, leaving the incubation medium above the phthalate layer. The tube was cut just above the packed cell miniscus and phthalate remaining on the pellet was removed by careful use of tissue paper. The section of the tube containing the cells was placed in 1 ml distilled water and mixed thoroughly for hemolysis. Then, 2 ml chloroform/methanol (2 : 1, v/v), containing 0.10 ml 12 M HCl, were added to the hemolysate and mixed well. The addition of HCl was necessary since the presence of any phthalate prevented complete precipitation of hemoglobin. Aliquots of the clear upper phase containing the water-soluble compounds were removed for total radioactivity measurement. The observed counts were related to the original packed cell volume and plotted against time of incubation.

RESULTS

*I. Changes in cells upon incubation in Tris buffer**

Previously it was noted [11] that human erythrocytes incubated in Tris buffer at 44 °C underwent nearly complete hemolysis within 2 h. Not only was this a time- and temperature-dependent phenomenon, but it was also a pH-sensitive event since there was little or no hemolysis in the cells on incubation at pH 6.0 in a Tris—maleate or Tris · HCl buffer.

In a typical experiment, human erythrocytes were washed three times with Tris buffer, resuspended in the same buffer, and incubated at 37 °C. At specific times, aliquots were assayed for hematocrit value, hemoglobin release, and cation levels. During this incubation the cells swell as indicated by change in cell volume (increase in hematocrit) and also start to release hemoglobin at a relative hematocrit value near 1.6 (Fig. 1A). It was interesting to note that the relative hematocrit was still 1.6, even after 40 % hemolysis, and this suggested that different erythrocyte populations swell at different rates and possibly to a different volume prior to hemolysis. This overall

* The degree of swelling of erythrocytes and subsequent hemolysis by 0.172 M Tris · HCl, pH 7.6, at 37 °C is very slow in a number of mammalian species. This is reflected in the time and relative hematocrit at which cells undergo hemolysis, as shown by the following data:

Animal	Relative hematocrit	Start of hemolysis, (min)
Pig	1.21	180
Rabbit	1.48	120
Rat	1.75	120
Guinea Pig	1.37	80

In the course of this study, an interesting situation was found in the cow red cells. Both Tris and histidine caused hemolysis of the calf but not the adult cow red cell. While the Tris buffer induced swelling of the calf erythrocyte to a critical hemolytic volume of 1.25, histidine mediated hemolysis takes place unaccompanied by the cell swelling.

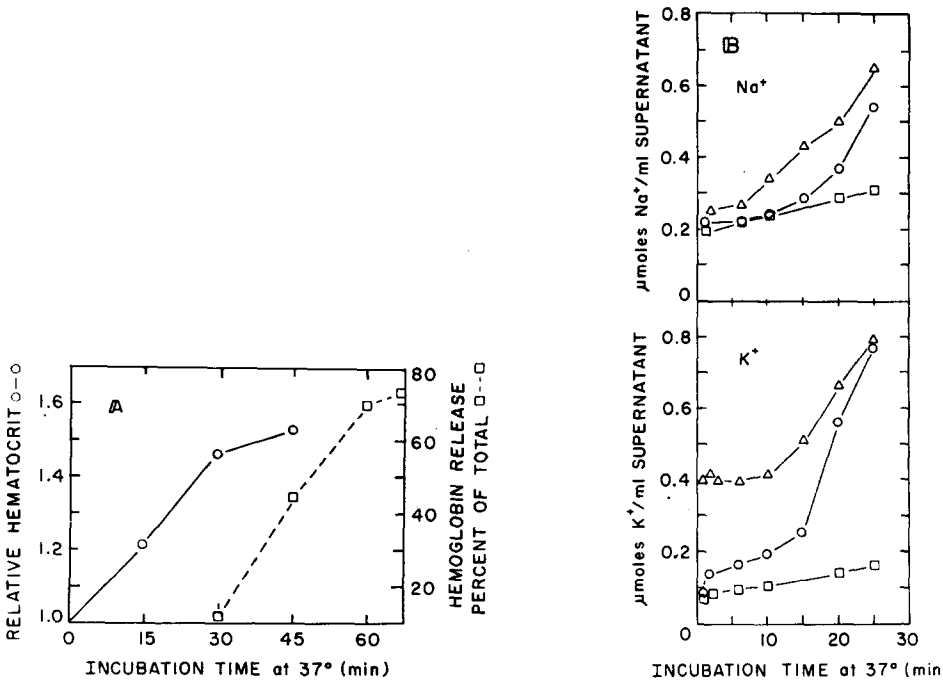

Fig. 1. Changes in human erythrocytes incubated at 37 °C. (A) Influence of 0.172 M Tris · HCl, pH 7.6 on relative hematocrit (cell volume) values and hemoglobin as a function of time. Relative hematocrit is the ratio of final cell hematocrit (cell volume) to the hematocrit at the start of incubation. The latter value was 15 %. (B) Influence of washing media on Na^+ and K^+ release from human erythrocyte incubated at 37 °C. Cells were treated as follows: □—□, washed and resuspended in 0.12 M $MgCl_2$ only; ○—○, washed in 0.12 M $MgCl_2$ and resuspended in 0.172 M Tris · HCl, pH 7.6; and △—△, washed and resuspended in 0.172 M Tris · HCl, pH 7.6, only. The total cell Na^+ and K^+ were 11 μmol and 100μmol/ ml packed cells, respectively.

phenomenon was examined further in cells: (a) washed and resuspended in $MgCl_2$; (b) washed in $MgCl_2$ and resuspended in Tris buffer; and (c) washed and resuspended in Tris buffer. The initial hematocrit in all cases was the same, 16.0 %. Upon incubation at 37 °C, there was no observed swelling in (a) but a significant increase of cell volume in (b) and (c). The swelling rate in the latter was identical to that depicted in Fig. 1A. Aliquots of each incubate were taken at different time intervals and immediately centrifuged for 30 s at room temperature and the supernatant analyzed for Na^+ and K^+. The rate of loss of these cations was expressed as μmol/ml supernatant and results are presented in Fig. 1B. It is apparent that Na^+ is preferentially released from Tris-swollen cells since the K^+/Na^+ molar ratio in the supernatant was in the range of 1–2 whereas the value for the intact cell was near 10–11. In no instance was cation release measured after the onset of hemolysis.

Some Na^+ and K^+ leaked to varying degrees from the cells on resuspension (zero time of incubation) in the different media. For example, $MgCl_2$-washed cells lost a very small amount of K^+ on resuspension either in Tris buffer (pH 7.6) or in $MgCl_2$, whereas in the case of Tris buffer washed cells (not treated with $MgCl_2$), resuspended in Tris buffer, four times more K^+ (approx. 2.4 μmol K^+/ml packed

cells) was lost. No significant differences were noticed in Na^+ release during resuspension of washed cells in different media (Fig. 1B). On the other hand, cells washed and incubated in 0.12 M $MgCl_2$ did not swell and leaked only a minimal amount of K^+ and Na^+, as indicated by their molar ratio of less than 0.5 in the supernatant (Fig. 1B). This is in distinct contrast to cells washed and resuspended in Tris buffer.

It should be emphasized that the observations recorded here were consistent and reproducible within a particular sample of blood. However, there were slight differences noted in erythrocytes obtained from different donors and different batches of Tris. Nonetheless, these differences were primarily on a quantitative basis and did not alter the general qualitative characteristics of the responses noted.

II. Influence of other compounds on Tris effect

In their investigation of the swelling of erythrocytes in Tris buffer, Omachi et al. [10] reported that the penetration of Tris into human erythrocytes could be decreased significantly by the addition of NaCl solutions. It seemed appropriate to re-investigate the effects of different inorganic salt solutions, as well as a typical

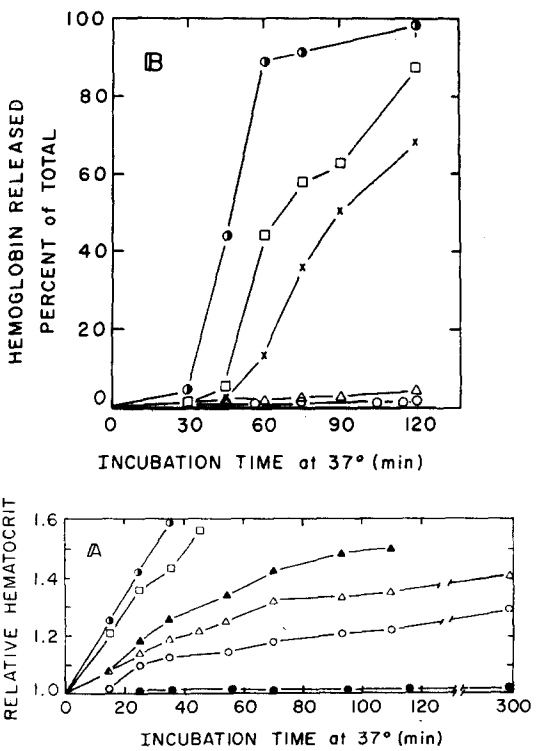

Fig. 2. Influence of varying concentrations of sodium chloride on Tris-induced changes in relative hematocrit (A) and hemoglobin release (B). Human erythrocytes were incubated at 37 °C for the indicated periods of time in the following mixtures (v/v) of 0.172 M Tris · HCl, pH 7.6 and 0.155 M sodium chloride (NaCl): ◑—◑, 100 Tris · HCl; □—□, 4 Tris · HCl: 1 NaCl; ×—×, 7 Tris · HCl: 3 NaCl; ▲—▲, 3 Tris · HCl: 2 NaCl; △—△, 1 Tris · HCl: 1 NaCl; ○—○, 2 Tris · HCl: 3 NaCl; ●—●, 100 NaCl. Starting hematocrit values ranged from 14 to 16 %.

non-electrolyte, glucose, on the action of Tris on erythrocytes. In addition, as will be discussed later (see below), histidine was included as a test reagent since it was shown to have unusual activity towards these cells.

A. Inorganic salts and glucose. Cells were washed three times with 0.155 M solutions of monovalent salts, such as NaCl, LiCl, KCl, and CsCl, and with a divalent salt, $MgCl_2$, centrifuged at 4 °C, resuspended in Tris buffer, pH 7.6 and incubated at 37 °C. The rate of hemolysis under these conditions was very comparable to that recorded in Fig. 1A and hence the swelling was not affected by the initial inorganic salt wash procedure. The inclusion of varying proportions of any of these salts in Tris incubation medium significantly decreased the rate of swelling of the cells and the time of onset (and magnitude) of hemolysis. The results with NaCl are presented in Figs 2A and 2B. It is clear that the change in the rate of swelling and hemolysis at 37 °C depended on the relative amount of NaCl present. Lithium chloride and magnesium chloride exhibited a very similar pattern whereas KCl was somewhat less effective in prevention of hemolysis. Of special interest: cells suspended in a mixture of Tris buffer · NaCl, 1 : 1 v/v, and incubated at 37 °C for 2 h showed no hemolysis (Fig. 2B). In another experiment, cells suspended in a mixture of Tris buffer · NaCl, 3 : 2, v/v and 1 : 1, but incubated at 44 °C for 2 h, showed 50 % and 5 % hemolysis, respectively.

Inclusion of 5 mM potassium phosphate buffer (pH 7.5) and 5 mM glucose in any of the above mixtures of salts and Tris · HCl buffer produced further delay in hemolysis. The effect was mainly attributed to the presence of glucose since 5 mM potassium phosphate buffer (pH 7.5) alone did not show any effect on the Tris hemolysis rate. Increasing levels of glucose caused a decided decrease in the rate (and extent) of Tris-induced hemolysis (Fig. 3). In fact, the time for 50 % hemolysis of a mixture of Tris buffer/0.31 M glucose, 9 : 1 v/v, was 120 min as compared to 45 min for Tris buffer alone. This effect was simply not the result of dilution of Tris

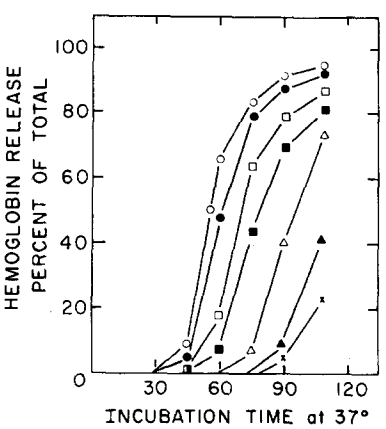

Fig. 3. Effect of glucose level on Tris-induced hemolysis. Human erythrocytes were washed 3 times with 0.172 M Tris · HCl, pH 7.6, then suspended and incubated at 37 °C in the following mixtures (v/v) of 0.172 M Tris · HCl and 0.31 M Glucose: ○—○, 100 Tris · HCl; ●—●, 99.7 Tris · HCl: 0.3 glucose; □—□, 99 Tris · HCl: 1 glucose; ■—■, 98.4 Tris · HCl: 1.6 glucose; △—△, 97 Tris · HCl: 3.0 glucose; ▲—▲, 94 Tris · HCl: 6 glucose; ×—×, 90 Tris · HCl: 10 glucose. Starting hematocrit, 14–16 %.

in the incubation mixture, since replacement of glucose with NaCl shifted the 50 % hemolysis time value to 60 min.

B. Histidine. It was noted that histidine consistently caused a reduction in the erythrocyte cell volume. Thus, whereas erythrocytes suspended in NaCl gave a hematocrit of 20 %, the same cells suspended in histidine gave a hematocrit of 16 %. On the basis of these observations, it was considered of value to explore the possible effects of histidine on the Tris-induced swelling of erythrocytes at 37 °C and on the erythrocytes per se. Human erythrocytes were allowed to swell up to a relative hematocrit of 1.5 (in Tris buffer). The cells then were quickly recovered by centrifugation at $1000 \times g$ at 4 °C and immediately resuspended in a comparable volume of histidine and further incubated at 37 °C. After specific time intervals, the cells were again recovered by centrifugation and resuspended in Tris buffer and re-incubated at 37 °C until a relative hematocrit of $\approx$ 1.5 (actually 1.3 times that of the starting hematocrit) was reached and the treatment with histidine repeated as above.

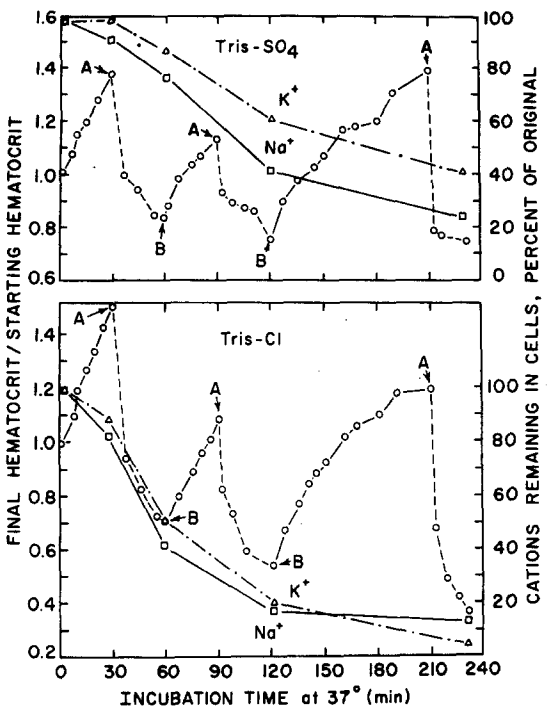

Fig. 4. Cation removal from human erythrocytes incubated successively in 0.172 M Tris · HCl or 0.172 M Tris · SO_4, pH 7.6, and in 0.31 M histidine, pH 7.5. Human erythrocytes were washed and suspended in 0.172 M Tris · HCl or Tris · SO_4, pH 7.6 and incubated at 37 °C until the hematocrit had reached a value near 1.4–1.5 (A), without hemolysis. At that point, the incubate was centrifuged at $1000 \times g$ for 5 min at 4 °C, resuspended in 0.31 M histidine, pH 7.5 and then re-incubated at 37 °C until the cells had reached a relative hematocrit near 0.7 (B), again with no hemolysis. At this point, the cells were centrifuged as above, suspended in the appropriate 0.172 M Tris buffer and again incubated at 37 °C. This entire cyclic process was performed a total of three times. The cells were assayed for their Na^+ and K^+ content and the relative hematocrit determined at the times indicated. The starting hematocrit values were 15 %.

TABLE I

RELEASE OF Na^+, K^+, Cl^- DURING HISTIDINE OR SUCROSE SHRINKAGE OF HUMAN ERYTHROCYTES PREVIOUSLY SWOLLEN IN 0.172 M TRIS · HCl, pH 7.6 OR 0.172 M TRIS · SO_4, pH 7.6

Cells were suspended in 0.172 M Tris · HCl, pH 7.6[a] or 0.172 M Tris · SO_4[a], pH 7.6, at a hematocrit of 15.0 % and 13.5 %, respectively, and incubated at 37 °C until a hematocrit value of 24.5 % and 20 %, respectively, was reached. Samples were then centrifuged at 1000 × *g* for 5 min at 4 °C and resuspended in 0.31 M histidine, pH 7.5 or 0.31 M sucrose, pH 7.6 and reincubated at 37 °C. Aliquots were removed at the indicated times, centrifuged at 1000 × *g* at 4 °C and the supernatant analyzed for Na^+, K^+, and Cl^-. Data expressed as millimolar concentration of ions in the supernatant at different times of reincubation. The volume of the cells and the supernatant measured by the hematocrit values at different times of incubation is expressed as percentage of the total suspension.

	0.172 M Tris · HCl swollen cells reincubated in:										0.172 M Tris · SO_4 swollen cells reincubated in:									
	0.31 M Histidine					0.31 M Sucrose					0.31 M Histidine					0.31 M Sucrose				
Reincubation time (min)	Cell vol. (%)	Supernatant vol. (%)	Na^+	K^+	Cl^-	Cell vol. (%)	Supernatant vol. (%)	Na^+	K^+	Cl^-	Cell vol. (%)	Supernatant vol. (%)	Na^+	K^+	Cl^-	Cell vol. (%)	Supernatant vol. (%)	Na^+	K^+	Cl^-
0[b]	24.6	75.4	0.69	2.26	N.D.[c]	24.6	75.4	0.69	2.26	N.D.[c]	20.0	80.0	0.55	2.02	N.D.[c]	20.0	80.0	0.55	2.02	N.D.[c]
1	21.6	78.4	0.41	0.57	9.23	21.4	78.6	0.32	0.57	12.89	16.9	83.1	0.25	0	0.14	16.2	83.8	0.24	0.32	0.14
10	17.2	82.8	0.55	3.23	19.29	17.3	82.7	0.80	0.07	15.77	15.9	84.1	0.50	2.18	0.14	15.2	84.8	0.33	2.02	0.14
20	14.9	85.1	0.62	4.68	23.45	14.9	85.1	0.79	4.60	19.93	14.2	85.8	0.48	2.66	0.07	13.1	86.9	0.69	4.68	0.07
30	13.8	86.2	0.58	4.36	24.5	13.4	86.6	0.62	5.57	23.59	13.1	86.9	0.44	2.18	0.07	12.2	87.8	0.42	3.23	0.14
50	12.7	87.3	0.66	5.65	25.2	13.4	86.6	0.68	6.54	26.19	12.3	87.7	0.51	3.87	0.14	11.6	88.4	0.55	4.60	0.22

[a] Na^+ and K^+/ml packed cell prior to Tris swelling was 9.5 μmol and 120 μmol, respectively.
[b] Zero time represents the cells swollen in different buffers (i.e., Tris · HCl and Tris · SO_4).
[c] Not determined.

This overall procedure was repeated one additional time. The results of these experiments are presented in Fig. 4. It is evident that histidine can rapidly reduce the volume of a Tris-swollen cell, with a significant attendant efflux of Na^+ and K^+. In fact, as much as 95 % of the cell K^+ and 90 % of the cell Na^+ could be released in this manner without any evidence of hemolysis. In addition to histidine, 0.31 M sucrose also could cause a reduction in volume of Tris-swollen cells and also influence the loss of Na^+, K^+, and Cl^- from these cells. A comparison of the pattern of release of these latter ions from Tris · HCl buffer or 0.172 M Tris · SO_4 (pH 7.6) swollen cells re-incubated in 0.31 M histidine or 0.31 M sucrose solutions is presented in Table I. In essence, these results illustrate that in a single Tris buffer or Tris-SO_4 swelling and histidine or sucrose shrinkage cycle, the loss of total Na^+ and K^+ was near 55–60 % and 30–35 % resp. Chloride flux was always higher than total K^+ and Na^+ efflux from Tris buffer swollen cells during either isotonic histidine or isotonic sucrose treatment whereas no detectable amount of chloride was released from Tris · SO_4 cells under similar conditions (Table I). These results strongly suggested that swelling of the cells in Tris medium was due to the uptake of Tris buffer under these conditions.

In order to explore these various effects further, cells washed in Tris buffer were incubated in mixtures of histidine/Tris buffer and the hematocrit values, hemoglobin (Figs 5A and 5B) and monovalent cation released (Fig. 6) followed over a 180 min period. These results clearly show that the increasing levels of histidine in the buffer mixture will retard the swelling of the cell and also the release of hemo-

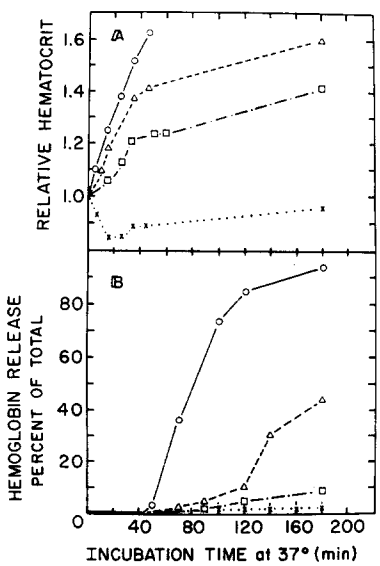

Fig. 5. Influence of histidine on Tris-induced swelling (A) and hemoglobin release (B) of human erythrocytes. Cells washed (× 3) in 0.172 M Tris · HCl, pH 7.6, were incubated in mixtures (v/v) of 0.172 M Tris · HCl, pH 7.6 : 0.31 M histidine, pH 7.5 at 37 °C as follows: ○—○, 100 Tris · HCl; △. . .△, 80 Tris · HCl: 20 histidine; □- . -□, 70 Tris · HCl: 30 histidine; ×- - -×, 100 histidine. The relative hematocrit and hemoglobin release were determined at the indicated times. The starting hematocrits were in the range 14–16 %.

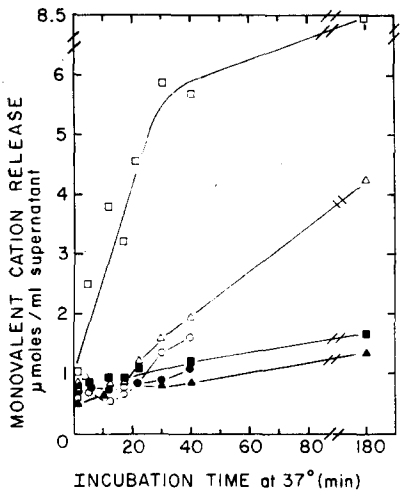

Fig. 6. Cation release from human erythrocytes washed (× 3) in 0.172 M Tris and incubated at 37 °C in solutions of 0.31 M histidine, pH 7.5, and 0.172 M Tris · HCl, pH 7.6; ■—■ and □—□, 100 histidine only; ▲—▲ and △—△, 1 Tris · HCl: 1 histidine; ●—● and ○—○, 100 Tris · HCl. Na^+ and K^+ are represented by closed and open symbols, respectively. The starting hematocrits were 14 %. The total cells K^+ and Na^+ were 100 μmol and 11 μmol per ml packed cells.

globin. Whereas the 50 % hemolysis time for cells incubated in Tris alone was 75 min, a value of 180 min was found in the mixture of Tris buffer/histidine, 4 : 1, v/v and an infinite value for cells in a mixture of Tris buffer/histidine, 7 : 3, v/v. On the other hand, Tris apparently did enter the cells, even in the latter mixture as shown by an increase in the relative hematocrit from 1.0 to 1.4, but with no hemolysis. Interestingly, cells incubated in histidine underwent a decrease in cell volume during incubation at 37 °C, to a relative hematocrit value of near 0.85 but slowly returned to near 1.0 at the end of 180 min. Electrolyte loss was calculated from supernatant values after correcting for the incubated hematocrit. The rate of Na^+ loss was found to be slightly higher in histidine than in Tris buffer incubated cells. At the end of a 40 min incubation the cells in Tris buffer and in histidine lost 6.5 μmol (56 % of cell Na^+) and 7.4 μmol (64 % of cell Na^+) per ml packed cells, respectively. Cells incubated over 3 h at 37° in histidine sustained a loss of 8.34 μmol, or 72 % of total cell Na^+ without hemolysis. On the other hand, when cells were incubated at 37 °C in mixtures of histidine and Tris buffer (2 : 3 and 1 : 1, v/v), initially the loss was similar to that noted in Tris buffer incubated cells. At the end of 3 h (no hemolysis) the loss was equivalent to 8.14 μmol/ml packed cells (70 %) in histidine/Tris buffer, 1 : 1, v/v and 7.7 μmol/ml packed cells (66 %) in histidine/Tris buffer, 2 : 3, v/v.

The K^+ release from cells incubated in Tris buffer was much lower than that from cells incubated in histidine, (Fig. 6). At the end of a 40 min incubation at 37 °C, the K^+ loss in Tris buffer was 9.2 μmol/ml (or 9.2 %) packed cells compared to 35.2 μmol/ml (or 35.7 %) packed cells in histidine. Even though the histidine incubated cells did not hemolyze, the K^+ loss after a 3 h incubation at 37 °C was 50.5 μmol/ml packed cells, or 50.5 % of the total K^+ level. Negligible differences were observed in release of K^+ from cells which were incubated for 40 min at 37 °C in varying mixtures of histidine · Tris buffer and in Tris buffer alone. However,

on further incubation at 37 °C in histidine/Tris buffer, 2 : 3, v/v and histidine/Tris buffer, 1 : 1, v/v, cells lost K^+ to the extent of 28.6 μmol/ml packed cells and 24.7 μmol/ml packed cells, respectively.

Cellular ATP levels did not appear to be of importance in the Tris effect. In a limited series of experiments, cells were washed with and incubated in NaCl for 12 h at 37 °C. Adenosine and phosphate were added to a final concentration of 5 mM each and the cells re-incubated at 37 °C for 2 h. Aliquots were taken at specified times, centrifuged, and the cells were suspended in Tris buffer and incubated at 37 °C. There was no apparent difference in the hemolysis rate in the "ATP depleted-repleted" cells as compared to untreated fresh cells incubated in Tris buffer.

III. Changes in cells upon incubation in histidine

The initial dramatic decrease in the volume of erythrocytes in histidine, followed by a slow return to original volume suggested histidine influx into the cell (Fig. 7A). Consequently, this question was explored as described below.

Freshly drawn erythrocytes were washed with Tris buffer and resuspended in (0.31 M) [^{14}C]histidine to a hematocrit of 13.8 %. The amount of Tris buffer contributed from packed cells into the suspension was equivalent to 3.5 % of the total histidine present in the suspension (calculated from the dilution of histidine radioactivity after mixing with erythrocytes). The cell suspension was incubated for 3 h at 37 °C and hematocrit value (Fig. 7A) and histidine uptake (Fig. 7B) were monitored. The counts obtained at zero time reflects the extracellular contamination. The radioactivity was found to increase in the cells with time and supported the conclusion that histidine was taken up by the cells during incubation. However, the amount of histidine taken up by the cells in the first 40 min of incubation was lower than the cation efflux thus causing a net cell volume decrease.

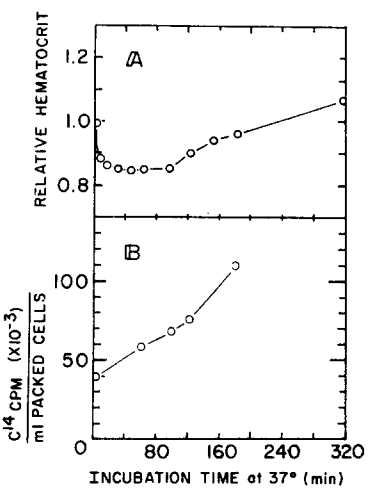

Fig. 7. Histidine uptake into human erythrocytes. Human erythrocytes were washed 0.172 M Tris · HCl, pH 7.6 and suspended in [^{14}C]histidine. The specific activity of the final suspension was 613 cpm/μmol of histidine. The sample was incubated at 37 °C. At indicated times, aliquots were withdrawn and analyzed for cell volume change (A) and for [^{14}C]histidine level (B). The starting hematocrit was 13.8 %. Further details are provided in the text.

In confirmation of previous observations, cells were found to shrink to 0.85 of the original volume in 40 min at 37 °C and then return slowly to a relative hematocrit of 0.95 after 3 h (Fig. 7A). After 6.5 h at 37 °C, the cells increased in size to 1.06 the original value.

IV. Compounds influencing the "histidine effect"

A. Imidazole. Omachi et al. [10] also reported that isotonic imadazole and isotonic 2, 4, 6-trimethylpyridine caused a rapid, pH-dependent hemolysis of human erythrocytes. Indeed human erythrocytes suspended in 0.31 M imidazole, pH 7.6, at room temperature hemolyzed almost completely within seconds. There was severe damage to the cell, as expressed mostly by the very significant loss of Ca^{2+}-ATPase activity. The membranes recovered from this treatment were very irregular in size and shape, and appeared quite fragmented. On the other hand, cells washed and suspended in a 0.31 M histidine/0.31 M imidazole mixture, 1 : 1, v/v, then incubated at 37 °C showed complete retention of hemoglobin, Ca^{2+}-ATPase, $Na^+ + K^+$, and exhibited no overt change in (phase) morphology. The initial hematocrit was 22 %, and it remained unchanged on incubation for 60 min at 37 °C. In addition, incubation of cells in histidine/imidazole mixtures ranging from 1 : 0, 4 : 1, 3 : 2, 2 : 3 (v/v) showed no hemolysis or change in cell volume (hematocrit). However, at a histidine-imidazole (v/v) ratio of 1 : 4, significant hemolysis ($\approx$ 25 %) was observed and in imidazole alone, hemolysis occurred within a few seconds at room temperature.

A highly interesting observation centered on the effectiveness of certain levels of NaCl in preventing imidazole-induced hemolysis. Upon incubation of human erythrocytes at 37 °C in NaCl/imidazole, 7 : 3, v/v, pH 7.6, the hematocrit value remained constant (at 19 %) and there was no hemolysis at the end of 35 min.

B. Tris. It was evident that the initial wash in Tris resulted in the leaky erythrocytes with respect to Na^+ and K^+ (Figs 1 and 6). Thus erythrocytes were washed in $MgCl_2$ and subsequently the effects of Tris on histidine induced ion loss was studied. The rate of release of Na^+ and K^+ in various mixtures of Tris and histidine is shown in Fig. 8A. These data show that the inclusion of as little as 20 % Tris buffer cells suspended in histidine dramatically lowered the histidine-stimulated K^+ loss from 30 % to $\approx$ 5 %. Under the same conditions, there was a definitive, but less pronounced decrease, from 60 % to 40 %, in Na^+ efflux from the cells. These responses can best be seen by displaying the data obtained (Fig. 8A) from one time interval, i.e., 40 min, as percent loss of Na^+ and K^+ as a function of the volume ratio of Tris to histidine in the incubation mixture (Fig. 8B). These observations suggested a selective effect of Tris on Na^+ permeability or towards some specific localized pool of Na^+.

In an attempt to delineate the mode of action of Tris and the reason for the effects of such diverse reagents as NaCl and histidine on modification of its behavior, it seems reasonable to consider the relation of the intra-molecular hydrogen bonded structure of Tris [13] to its activity. An examination of the infrared spectra of Tris and mixtures of Tris and NaCl and histidine, respectively, in ^2H_2O in the region from 4000–3000 $\cdot$ cm^{-1}, revealed that Tris did possess an intramolecular hydrogen-bonded structure and that NaCl and histidine solutions had no effect on this structure. These results would support a proposition that the main effects of NaCl or histidine in counteracting the effects of Tris must be on or at specific sites in the cell membrane.

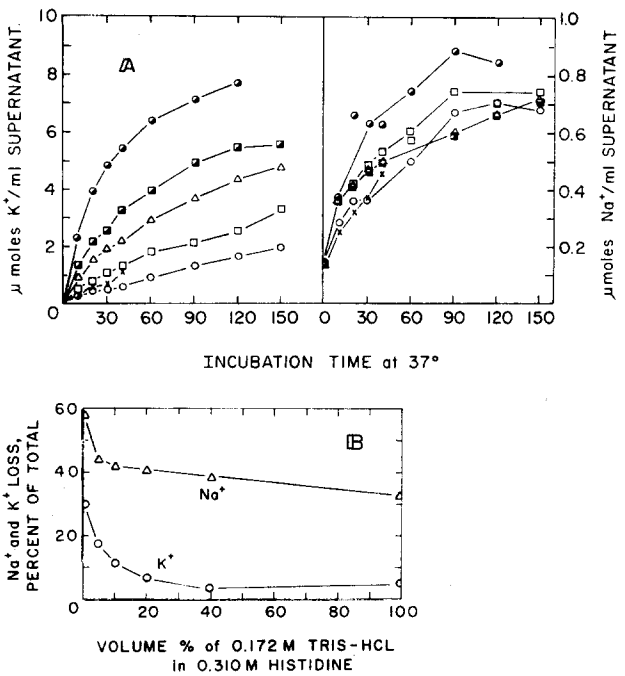

Fig. 8. K^+ and Na^+ release from human erythrocytes washed ($\times$ 3) in 0.12 M $MgCl_2$ and incubated at 37 °C in varying mixtures of 0.31 M histidine, pH 7.5 and 0.172 M Tris · HCl. (A) Pattern of cation release as a function of incubation time at 37 °C. The following mixtures (v/v) of histidine: Tris were used: ◑—◑, 100 histidine; ◧—◧, 19 histidine: 1 Tris · HCl; △—△, 9 histidine: 1 Tris · HCl; □—□, 4 histidine: 1 Tris · HCl; ○—○, 3 histidine: 2 Tris · HCl; ×—×, 100 Tris · HCl. Starting hematocrits ranged from 15.7% in 0.31 M histidine to 16.0% in 0.172 M Tris · HCl solutions. (B) Loss of K^+ and Na^+ after 40 min at 37 °C as a function of ratio of isotonic Tris to isotonic histidine in the incubation mixture. Data obtained from Fig. 8A.

DISCUSSION

Tris has been widely used to control pH and has been utilized [11] to create a "*zero trans*" condition [14] with respect to Na^+ and K^+ cations. Although the present data do not allow us to draw a definite conclusion regarding the exact mode of action of either Tris or histidine, data obtained in this study clearly illustrate some unique effects of these agents on human erythrocytes.

Tris buffer was found to cause quite a specific efflux of Na^+ and cell swelling which eventually leads to osmotic lysis.* These events were favored by pH values above 6.0 and temperatures above 25 °C. In part, our findings confirm those reported by Omachi et al. [10] who noted a cellular volume increase of erythrocytes suspended in Tris buffer. While Tris is known to be phosphorylated in human erythrocytes in the presence of 17 mM Tris augmented with inosine, glucose, and phosphate for an incubation period of 96 h at 37 °C [16], it is not known to what extent Tris phosphorylation occurs under our experimental conditions.

* The Tris uptake as measured by the procedure of Kanarek and Moshe [15] was greater than the total ion loss, the result of which will be presented elsewhere.

In sharp contrast to Tris, histidine solutions had an almost opposite effect in that this amino acid caused an immediate and pronounced decrease in cellular volume, and an efflux of relatively sizable quantities of both Na^+ and K^+. However, on further incubation at 37 °C, there was a regain of cell volume, without hemolysis even after 5 h. These findings are reminiscent of the well-documented observations in which human erythrocytes are suspended in non-electrolytes such as sucrose or a salt of impermeable anions [17, 18]. Since the membrane potential in erythrocytes is determined by the chloride ratio, when human erythrocytes are suddenly suspended in non-electrolyte or impermeable anion medium, a large driving force is created which induced the efflux of cations resulting in the loss of salt and water.

It is of interest to comment on approaches to understanding the mode of Tris buffer action on human erythrocytes and the factors affecting its activity. If conditions are monitored closely during the initial phases of Tris-induced swelling, the cellular volume can be reduced, without hemolysis by inclusion of rather high levels of sodium chloride or histidine. On the other hand, low levels of glucose (31 mM) can also delay cell volume increase and hemolysis. Even though the shrinkage of the Tris-swollen cells by histidine, as an example, was dramatic and reproducible, alteration of the cellular composition did occur and was characterized by a continued removal of Na^+ and K^+. If, in fact, the Tris swelling and histidine shrinkage cycle was repeated three times, as much as 95 % of the total monovalent cations could be removed from these cells. Interestingly these Na^+ and K^+ deficient cells still possessed enzymatic activities, such as Ca^{2+}-ATPase and acetylcholinesterase, had a normal phospholipid content, and showed hemoglobin levels comparable to untreated cells. As noted above, sodium chloride and histidine can prevent the Tris-induced hemolysis and significantly delay swelling and yet, the level at which this effect was noted under our experimental conditions was indeed puzzling. A complete inhibition of any Tris buffer hemolysis by sodium chloride, for example, was not achieved until the osmolar ratio of NaCl to Tris was close to 1. Similarly the osmolar level of histidine necessary to effect a similar inhibition of Tris buffer action was close to 1. Inasmuch as these reagents are so decidedly different in their chemical nature, it is difficult to rationalize or explain their seemingly comparable inhibitory properties. Yet it is possible that they are competing for or affecting sites similar or contiguous to those at which Tris buffer exerts its influence. Another facet of the Tris buffer effect is that it exhibits a pH dependence with values below pH 6.5 completely diminishing the cellular volume effect of this amine. It is clearly evident that these interactions are complex.

Isomolar histidine solutions had an effect on human erythrocytes quite opposite to that of Tris buffer. That histidine and Tris were acting differently with respect to the monovalent cations was evident from experiments in which increasing amounts of Tris buffer were added to cells suspended in iso-osmolar histidine and incubated at 37 °C. At an iso-osmolar ratio of histidine to Tris of 4 : 1, the efflux of K^+ was largely prevented while the exit of Na^+ was only slightly affected. These results emphasize not only the differences in the behavior of these two reagents on the erythrocyte per se, but also argue for different locations or accessibility sites for Na^+ and K^+ in these cells.

Other amino acids, such as glycine, proline, and alanine at iso-osmolar levels also exhibited characteristics similar to those of histidine in that they caused almost immediate shrinkage of the human erythrocyte and release of large amounts of K^+

on incubation at 37 °C. However, there was a significantly faster regain of the original cell volume with these amino acids as compared to histidine. Whether the latter is a significant difference is not clear at this time.

In the current study, a closer examination of the early phases (within 20 min at 37 °C) of cell swelling in Tris buffer revealed a significant cation release with a decided preference for Na^+ over K^+. This was supported by the repeated finding that the K^+/Na^+ molar ratio, in the extracellular fluid of the incubated cells was in the range of 1–2, whereas the molar ratio of these cations in untreated cells ranged from 10–12. These results emphasized the unique effectiveness of Tris as a reagent capable of distinguishing between Na^+ and K^+ as such in the human erythrocyte or of differential reactivity towards their transport systems per se.

In summary, it is interesting to speculate that Tris buffer may exhibit differential reactivity towards the high affinity and low affinity Na^+ sites in the human erythrocyte recently proposed [14, 19]. Thus, in this framework, Tris could prove of considerable advantage in exploring the location and importance of potential Na^+ pools in this cell. The ease with which Tris buffer causes quantum Na^+ release is reminiscent of the findings of Clarkson and Maizels [20] who first showed the presence of the rapidly exchangeable Na^+ pool presumably confined at the surface of the red cell membrane. Since, under our experimental conditions unless otherwise noted, all cell preparations are made by washing cells in Na^+-free medium of Tris buffer, it is conceivable that a part or all of the rapidly exchangeable Na^+ pool could have been removed. Studies are in progress to determine the extent to which the initial washings in Tris buffer result in the depletion of the rapidly exchangeable Na^+ pool in human cells. Concomitantly, the action of histidine, as well as other amino acids, such as alanine, glycine, and proline, in eliciting a high efflux of Na^+ and K^+, coupled with entry of these amino acids into the depleted cells, provides an exciting avenue to the elucidation of many biochemical parameters influencing the metabolism of the human erythrocyte. In the latter instance, reloading of these cells with various types of inorganic cations as well as other low molecular weight organic compounds assumes considerable potential in probing the physiological behavior of the erythrocyte.

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