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THE CONTROL OF MEMBRANE-BOUND Ca^{2+} BY ATP

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SUMMARY

1. It is known that the viscosity of venous blood is greater than that of arterial blood, and that an ATP-depleted erythrocyte loses its deformability. In order to test the hypothesis that these effects may be explained by an intracellular chelation of membrane-bound Ca^{2+} by ATP, the effect of ATP on the membrane-bound Ca^{2+} of erythrocyte ghost membranes was examined by adding $^{45}Ca^{2+}$ and ATP to the isolated membranes.

2. The membrane-bound Ca^{2+} was reduced to zero at 1.5 mM ATP, pH 7.

3. A complete adsorption isotherm for ATP at 21° indicated that there was a very small binding component of about 540 molecules of ATP per single erythrocyte membrane which became saturated at about 10^{-7} M ATP. The majority of the ATP binding sites, however, did not indicate any saturation up to 10^{-3} M ATP.

4. 10^{-5} M Ca^{2+} or Mg^{2+} depressed the membrane-bound ATP, but increased it at 1 mM.

5. ATP binding was increased at low pH.

6. Calculations indicated that the oxygenated erythrocyte would have a cytoplasmic free ATP level of 1.45 mM, and that the ATP concentration in the erythrocyte of venous blood (60 % oxygenated) would be 0.94 mmole per l water. The level of membrane-bound Ca^{2+}, therefore, would tend to be higher in the deoxygenated cell, explaining its greater rigidity and the higher viscosity of venous blood.

INTRODUCTION

The viscosity of venous blood is greater than that of arterial blood because of the different contents of O_2 and CO_2 (refs. 1–5). The physiological mechanisms underlying this arterio–venous difference in viscosity are not known, however.

The major component of the blood viscosity is that contributed by the erythrocyte membrane rather than by the hemoglobin[6,7]. WEED *et al.*[8] have clearly shown that the viscosity of an erythrocyte suspension and the rigidity of the erythrocyte membrane were both low in the presence of a normal intracellular level of ATP; depletion of the cell's ATP or the addition of 10^{-4} M Ca^{2+} caused a loss in the deformability of the erythrocyte membrane and a sharp rise in the viscosity of the erythrocyte suspension (see refs. 9 and 10). ATP could control membrane deformability by (1) chelating the intracellular Ca^{2+} and thus reducing the amount of Ca^{2+} bound to the cytoplasmic aspect of the cell membrane, or by (2) acting as a substrate for a membrane-

bound and contractile ATPase[11–14]. This paper provides evidence for the first hypothesis. This work also provides a quantitative analysis which predicts that the membrane-bound Ca^{2+} should be high when the erythrocyte is in the arterial blood and should be low when the cell is in the venous blood. Recent direct observations by LACELLE[15] indicate that the membrane deformability is directly proportional to the degree of cell oxygenation.

METHODS

Erythrocyte ghosts were prepared from stored human blood[16–19]. Since all the hemolysing and washing solutions contained 1 mM EDTA, the ghosts were considered free of any residual membrane-bound Ca^{2+} (ref. 20). The method for measuring the binding of Ca^{2+} to erythrocyte ghost membranes has been described[17,18].

The binding of ATP to erythrocyte ghost membranes

[^{3}H]ATP (generally labelled), tetrasodium salt (New England Nuclear Corp., U.S.A.) of specific activity 15.7 C/mmole was used. All dilutions and solutions were made with 15 mM Tris–HCl buffer (pH 7) and readjusted to pH 7 when necessary. The binding of ATP (Sigma Chem. Co.) to erythrocyte ghost membranes was measured in two ways. In the 'Supernatant method', an aliquot of 0.2–0.5 ml of erythrocyte ghosts (1–2 % dry wt.) was weighed into a 10 mm × 75 mm Pyrex test tube, and an aliquot of 0.2 ml of the [^{3}H]ATP stock solution was added. After incubating at 4° for 10 min, the tubes were centrifuged at 36900 × *g* at 4° for 10 min. The supernatant was counted[21]. The amount of membrane-bound ATP was calculated as for Ca^{2+} (refs. 17 and 18), where the free ATP concentration had been corrected for the amount of ATP hydrolysed during the incubation. The amount of ATP broken down to ADP and AMP was ascertained by descending chromatography (isobutyric acid–NH_4OH (28 %)–water (33:1:66, by vol.)). In the 'Millipore-filter method', aliquots of ghost membranes [*carboxyl*-^{14}C]inulin (2.03 mC/g, Mallinckrodt Nuclear, 25 mg/100 ml buffer) and [^{3}H]ATP were weighed into a 10 mm × 75 mm pyrex test tube, everything kept at 4°. Within 15 sec after adding the final aliquot (*i.e.* the [^{3}H]ATP) the sample was weighed and poured onto a Millipore filter disc (0.5-μm diameter pores) or onto a glass fibre filter disc (Grade 934 AH, Reeve Angel-Whatman) and filtered by vacuum. The amount of ATP hydrolyzed was negligible. The disc was put into a vial and BRAY'S[21] solution added. Because of incomplete transfer of the mixture onto the disc, a residual amount remained in the test tube. This residual was weighed, permitting subsequent corrections in calculating the amount of ATP adsorbed to the membranes. The procedure for counting two isotopes simultaneously and making the appropriate separation has been presented elsewhere[17]. The radioactive inulin served to correct for the residual supernatant left on the filter paper and which was harboured between the erythrocytes and within the open erythrocyte membranes. The number of moles of ATP bound to the erythrocyte membranes (M^{ATP}_{memb}) was calculated as follows.

$$M^{ATP}_{memb} = cpm^{ATP}_{memb} \times S \qquad (1)$$

where S is the specific activity (in moles/cpm) of the ATP in the test tube, and where cpm^{ATP}_{memb} is the number of cpm (counts/min) of radioactive ATP adsorbed to the erythrocyte membranes, and is determined according to Eqns. 2–8.

$$\mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{memb}} = \mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{mil,\,total}} - \mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{mil,\,free}} \tag{2}$$

where $\mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{mil,\,total}}$ is the total number of cpm of [^{3}H]ATP found experimentally on the Millipore filter, and where $\mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{mil,\,free}}$ is the number of cpm of free (*i.e.* unbound) [^{3}H]ATP on the Millipore filter. Incorporating the [^{14}C]inulin data, Eqn. 2 can be rewritten as Eqn. 3.

$$\mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{memb}} = \mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{mil,\,total}} - \frac{\mathrm{cpm}^{\mathrm{inulin}}_{\mathrm{mil,\,free}}}{R} \tag{3}$$

where $\mathrm{cpm}^{\mathrm{inulin}}_{\mathrm{mil,\,free}}$ is the number of [^{14}C]inulin cpm found experimentally on the Millipore filter and assumed to be completely free and unadsorbed. The ratio R is defined by Eqn. 4.

$$R = \frac{\mathrm{cpm}^{\mathrm{inulin}}_{\mathrm{mil,\,free}}}{\mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{mil,\,free}}} \quad \text{and} \quad R = \frac{\mathrm{cpm}^{\mathrm{inulin}}_{\mathrm{tube,\,free}}}{\mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{tube,\,free}}} \tag{4}$$

where $\mathrm{cpm}^{\mathrm{inulin}}_{\mathrm{tube,\,free}}$ and $\mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{tube,\,free}}$ are the number of cpm of free (*i.e.* unbound) [^{14}C]inulin and [^{3}H]ATP, respectively, and where it is assumed that the ratio, R, of free inulin to free ATP on the Millipore filter is identical to the ratio, R, of free inulin to free ATP in the test-tube contents. Eqn. 4, therefore, becomes,

$$\mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{memb}} = \mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{mil,\,total}} - (\mathrm{cpm}^{\mathrm{inulin}}_{\mathrm{mil,\,free}})/(\mathrm{cpm}^{\mathrm{inulin}}_{\mathrm{tube,\,free}}/\mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{tube,\,free}}) \tag{5}$$

$$\text{But since } \mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{tube,\,free}} = \mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{tube,\,total}} - \mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{memb}} \tag{6}$$

therefore, Eqn. 5 can be written as Eqn. 7.

$$\mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{memb}} = \mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{mil,\,total}} - \frac{\mathrm{cpm}^{\mathrm{inulin}}_{\mathrm{mil,\,free}}}{\mathrm{cpm}^{\mathrm{inulin}}_{\mathrm{tube,\,free}}} \times (\mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{tube,\,total}} - \mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{memb}}) \tag{7}$$

Finally, Eqn. 7 can be re-arranged to give the desired expression for $\mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{memb}}$,

$$\mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{memb}} = \frac{(\mathrm{cpm}^{\mathrm{inulin}}_{\mathrm{tube,\,free}} \times \mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{mil,\,total}}) - (\mathrm{cpm}^{\mathrm{inulin}}_{\mathrm{mil,\,free}} \times \mathrm{cpm}^{\mathrm{ATP}}_{\mathrm{tube,\,total}})}{\mathrm{cpm}^{\mathrm{inulin}}_{\mathrm{tube,\,free}} - \mathrm{cpm}^{\mathrm{inulin}}_{\mathrm{mil,\,free}}} \tag{8}$$

where all values on the right-hand side of Eqn. 8 are experimentally measured. After calculating the $M^{\mathrm{ATP}}_{\mathrm{memb}}$, therefore, by Eqn. 1–8, the membrane concentration of ATP or $C^{\mathrm{ATP}}_{\mathrm{memb}}$ was derived by Eqn. 9.

$$C^{\mathrm{ATP}}_{\mathrm{memb}} = M^{\mathrm{ATP}}_{\mathrm{memb}}/w_{\mathrm{mil}} \tag{9}$$

where w_{mil} is the dry weight of the membranes on the Millipore filter and is equal to $w_{\mathrm{tube,\,total}} - w_{\mathrm{residual}}$, where w_{residual} is the dry weight of the membranes remaining in the test tube after pouring all the contents onto the Millipore filter.

RESULTS

The effect of ATP and of EDTA on the membrane-bound Ca^{2+}

Fig. 1 depicts the effect of ATP on the membrane-bound level of Ca^{2+}. The membrane-bound Ca^{2+} was reduced to zero at 1.5 mM ATP. The free concentration of Ca^{2+} in these experiments was 0.94 mM. At low levels of ATP the amount of membrane-bound Ca^{2+} was 70 mmoles/kg dry membrane. This compares with a value of 81 mmoles/kg dry membrane when all the Ca^{2+} sites are saturated at high concentrations of free Ca^{2+} (ref. 17). Fig. 2 shows similar effects of EDTA on membrane-bound Ca^{2+}.

The adsorption of ATP to erythrocyte ghost membranes

As will be outlined in DISCUSSION, it is possible to calculate the amount of membrane-bound Ca^{2+} inside the normal erythrocyte using the data of Fig. 1 in conjunction with the data of GARBY *et al.*[22] for the binding of ATP to hemoglobin. In order to derive these values carefully it is important to know whether significant amounts of

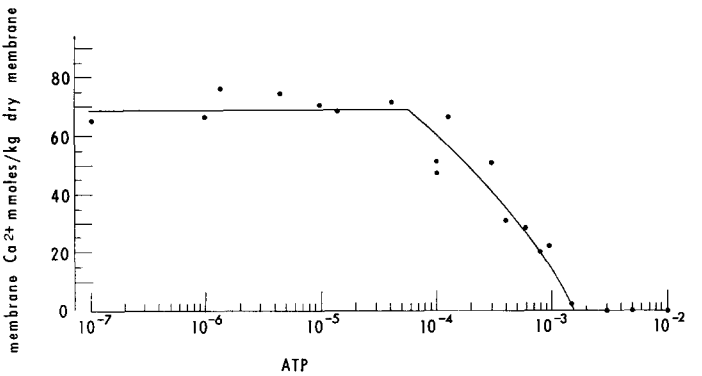

Fig. 1. The effect of ATP on the erythrocyte ghost membrane-bound Ca^{2+} at 21°. The free concentration of Ca^{2+} is 0.94 mM.

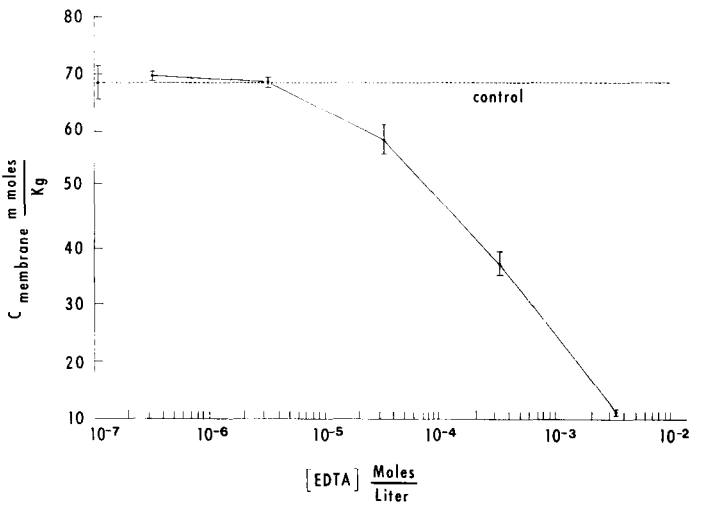

Fig. 2. The effect of EDTA on the erythrocyte membrane-bound Ca^{2+} at 21°.

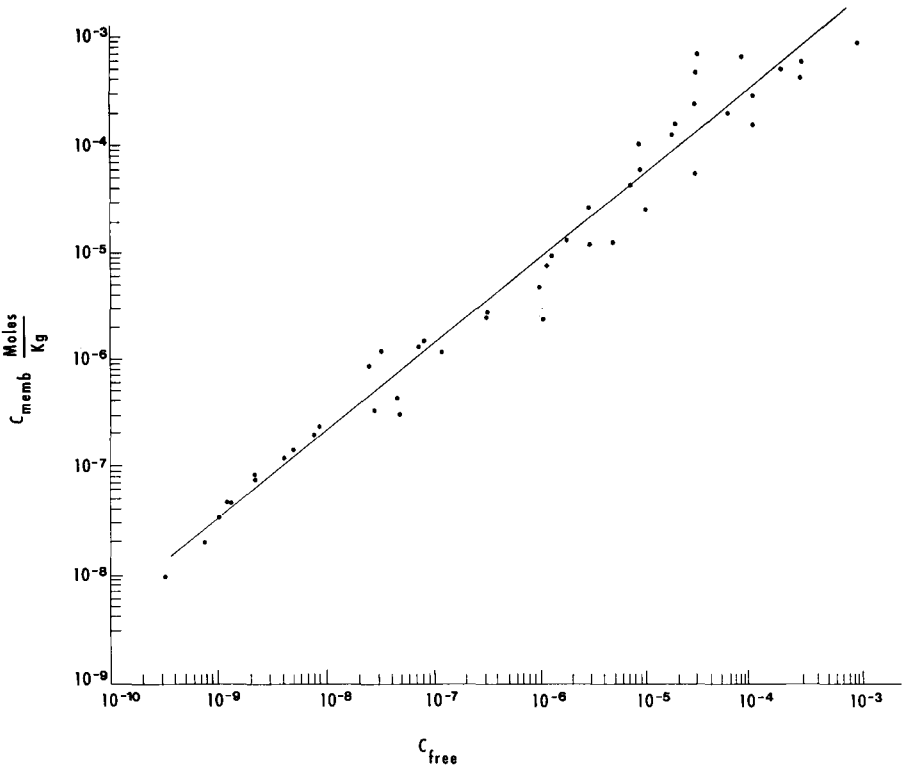

Fig. 3. The adsorption of ATP to erythrocyte ghost membranes at pH 7 and 4°.

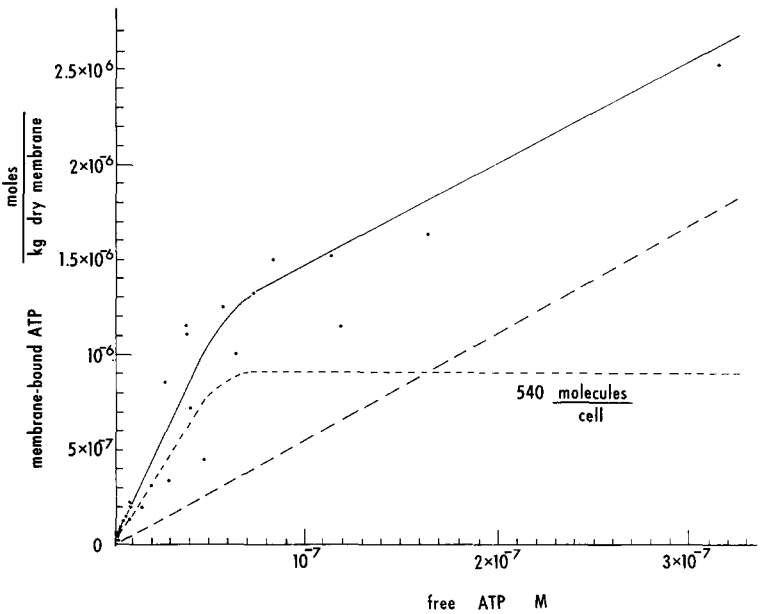

Fig. 4. The adsorption of ATP to erythrocyte ghost membranes at pH 7 and 4°. The data here are taken from Fig. 3 and have been divided into linear and non-linear components of adsorption.

ATP are adsorbed to the erythrocyte membrane. For this reason, the binding of ATP to erythrocyte ghost membranes was studied, and the effect of Mg^{2+}, Ca^{2+} and pH on this ATP binding was also examined.

Fig. 3 shows the binding of ATP to erythrocyte ghost membranes at pH 7 and 4°. Although the majority of the points were obtained by the 'Millipore-filter method', no distinction is made between those values obtained by this method and the 'supernatant method' since the results were indistinguishable; these results represent data from six separate lots of experiments, and each point represents the average of triplicate determinations. The results in Fig. 3 show that at 1 mM ATP the amount of membrane-bound ATP is 1 mmole/kg dry membrane. There is no indication that the membrane was becoming saturated with ATP in the log–log plot of Fig. 3. A linear plot of the data, however, in the low concentration region shows two binding phases, as shown in Fig. 4. A saturable binding phase can be distinguished with a maximal binding capacity of about 0.9 μmole/kg dry membrane (or 540 molecules of ATP per single erythrocyte membrane). 10^{-4} M ouabain generally depressed the amount of ATP bound at all ATP concentrations, without having any selective effect on the saturable phase.

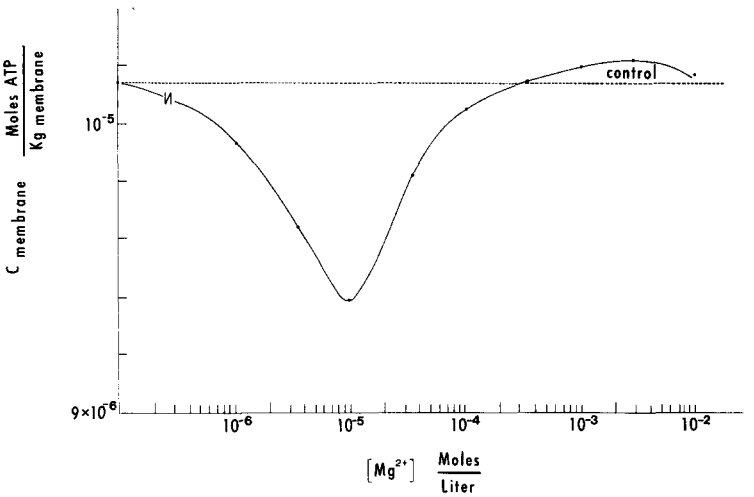

Fig. 5. The effect of Mg^{2+} on the amount of membrane-bound ATP.

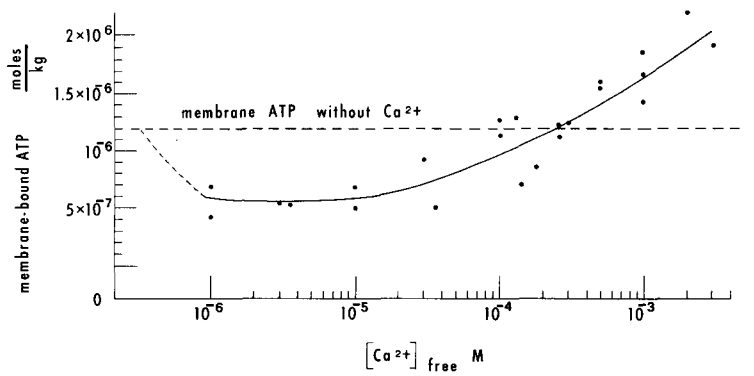

Fig. 6. The effect of Ca^{2+} on the amount of membrane-bound ATP.

The effects of Mg^{2+}, Ca^{2+} and pH on the amount of membrane bound ATP is presented in Figs. 5, 6 and 7, respectively. Both Mg^{2+} and Ca^{2+} in the region of 10^{-5} M depressed the membrane-bound ATP by 25–50 %, but in the region of 1 mM these ions actually increased the membrane-bound ATP by 10–50 %. ATP binding was increased at low pH. At pH 2.7 the binding of ATP was increased by more than 20-fold over the binding at pH 7.0.

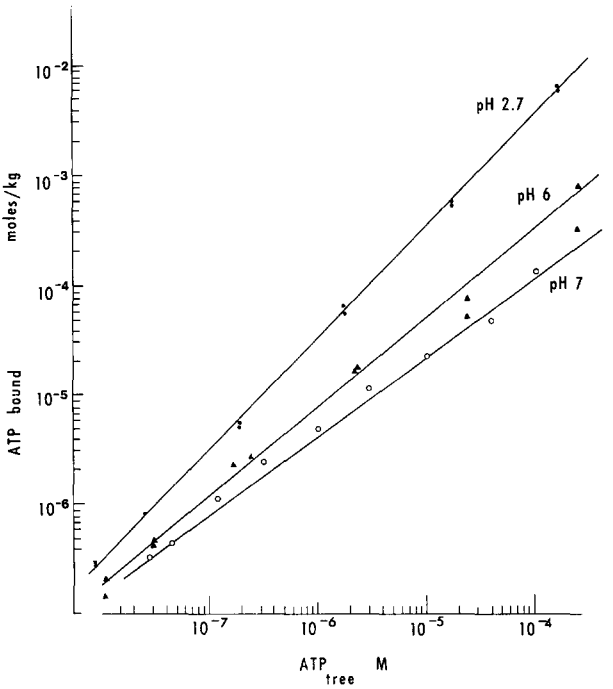

Fig. 7. The effect of pH on the amount of membrane-bound ATP.

DISCUSSION

The first main observation was that a free concentration of 1.5 mM ATP reduced the amount of erythrocyte membrane-bound Ca^{2+} to zero (Fig. 1).

The second main observation was that the amount of ATP bound to the erythrocyte membrane was only of the order of 1 mmole ATP/kg dry membrane when the free ATP concentration was in the physiological range of 1 mM. This means that only approx. 1 % of the intracellular ATP is bound to the erythrocyte membrane. The data in Figs. 5–7 indicate that this value of 1 % ATP binding to the membrane might at most be increased to 2 % under some conditions.

The arterio–venous control of erythrocyte membrane-bound Ca^{2+} by ATP

With these data it is now possible to calculate the expected values for the membrane-bound Ca^{2+} in the erythrocyte.

The oxygenated erythrocyte. The intracellular pH of the oxygenated erythrocyte is 7.2, interpolated from the data of PAYMASTER AND ENGLESSON[23] at the arterial CO_2

partial pressure of 40 mm Hg. The data of GARBY *et al.*[22] indicate that at pH 7.2 the amount of ATP bound to hemoglobin is 0.04 mole ATP/mole hemoglobin when the total concentration of ATP is 1 mmole/l of solution. Since the intracellular concentration of hemoglobin is 5.5 mmoles/l cell, this would amount to 0.22 mmole of ATP bound to the cell hemoglobin in 1 l of cell volume containing 1 mmole of ATP. In other words, at pH 7.2 the oxygenated erythrocyte contains 22 % bound ATP and 78 % free ATP. (According to the results of Figs. 3–7, the amount of membrane-bound ATP is of the order of 1–3 % and may be neglected.)

Since the normal intracellular ATP concentration is 1.2 mmoles ATP/l of cell[24–26] the amount of free ATP will be 0.94 mmole/l of cell. Because, however, only 65 % of the erythrocyte consists of water, the free concentration of ATP is 1.45 mmoles/l cell water. At this free concentration of ATP it can be seen from Fig. 1 that the membrane-bound Ca^{2+} is about 1 mmole Ca^{2+}/kg dry membrane.

The erythrocyte in venous blood. The intracellular pH of the erythrocyte under conditions existing in venous blood (CO_2 partial pressure of 46 mm Hg) is 7.14, as interpolated from the results of PAYMASTER AND ENGLESSON[23]. The data of GARBY *et al.*[22] indicate that the amount of ATP bound to hemoglobin at pH 7.14 is 0.16 mole ATP/mole of deoxygenated hemoglobin when the total concentration of ATP is 1 mmole per l of solution. By the manner outlined in the previous two paragraphs, it can be calculated that 89 % of the ATP in the deoxygenated cell is bound. Since 89 % of the intracellular ATP is bound in a deoxygenated cell, and 22 % is bound in an oxygenated cell, the interpolated value for an erythrocyte in venous blood which is 60 % oxygenated would be approx. 50 % ATP bound.

Hence, with a normal intracellular ATP concentration of 1.2 mmoles ATP/l of

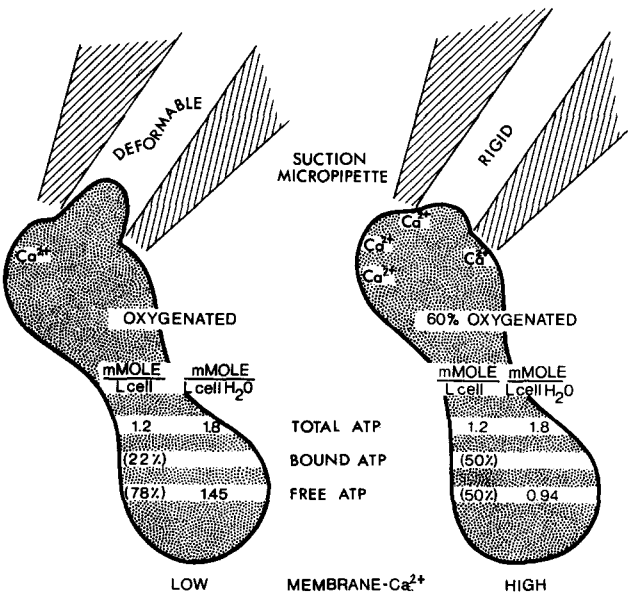

Fig. 8. A summary of the calculations made in the text, indicating that the amount of free ATP in an oxygenated erythrocyte is higher than in an erythrocyte with less oxygen. Accordingly, the level of membrane-bound Ca^{2+} would tend to be lower in the oxygenated cell.

venous erythrocyte, 0.6 mmole would be bound to the hemoglobin and 0.6 mmole would be free. Correcting for the concentration of water in the cell, the free concentration of ATP would then be 0.9 mmole ATP/l of cell water in the venous erythrocyte. This free concentration of ATP would leave about 15 mmoles of Ca^{2+} bound/kg of erythrocyte membrane, as indicated by the data in Fig. 1.

A summary of all these calculations is depicted in Fig. 8, showing that there is less membrane-bound Ca^{2+} in the oxygenated erythrocyte. These calculations point toward the trend in which ATP would alter the membrane-bound Ca^{2+}. Fig. 1 indicates that the maximum amount of membrane-bound Ca^{2+} at low ATP is 69 mmoles/kg dry membrane. In reality, however, the maximum amount of membrane-bound Ca^{2+} cannot exceed 2–6 mmoles Ca^{2+}/kg membrane. (The upper value of 6 is derived from the work of WEED *et al.*[8], who found $5.8 \cdot 10^{-18}$ mole of Ca^{2+} per erythrocyte; the lower value of 2 is from HARRISON AND LONG[20]; GENT *et al.*[27] found saturation values of between 15 and 58 mmoles Ca^{2+}/kg membrane, depending on the final ionic strength of solution in contact with the erythrocyte ghosts). Although these calculations for the amount of membrane-bound Ca^{2+} may be off by as much as a factor of 10, therefore, they emphasize the important role which ATP must play in modulating the deformability of the erythrocyte membrane. A similar conclusion has recently been made by LACELLE[15].

The binding of ATP to erythrocyte ghost membranes

At 1 mM ATP the membranes adsorbed approx. 1 mmole ATP/kg dry membrane. The present results are similar to those of BLAKE *et al.*[28] who reported that approx. 1 mmole ATP was bound per kg erythrocyte membrane at 1 mM ATP. WALZ AND CHAN[29] found that hemoglobin-free ghosts retained about 0.4 mmole [^{14}C]ATP/kg dry membrane after exposing the ghosts to 1 mM ATP and subsequently washing 4 times. ABOOD AND MATSUBARA[30] found that ATP adsorbed to a variety of membranes to the extent of between 10 and 30 mmoles/kg dry membrane. Our present results supplement all these earlier findings by providing a complete ATP adsorption isotherm for the first time.

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