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INFINITE *cis* INFLUX OF CYCLIC AMP INTO HUMAN ERYTHROCYTE GHOSTS

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Summary

Infinite *cis* uptake of cyclic AMP into red blood cell ghosts has been measured. The $K_{\mathrm{ic}}^{\mathrm{oi}}$ is calculated from two different integrated rate equations that are applicable when the substrate concentration is unsufficient to cause volume changes. Values of 0.69 mM and 0.66 mM are obtained for the infinite *cis* K_{m} at 30°C using these procedures. These values are only slightly higher than that predicted from zero *trans* net flux experiments.

Lowering the temperature reduces $K_{\mathrm{ic}}^{\mathrm{oi}}$ from 0.69 mM at 30°C to 0.478 mM at 20°C, 0.108 mM at 10°C and 0.072 mM at 4°C (Q_{10} = 2.4). The Q_{10} for activation of influx permeability of 10^{-5} M cyclic AMP is 1.55.

Recently the transport of cyclic AMP in human erythrocyte ghosts has been measured and characteristics consistent with a mediated diffusion process were evident. Saturation kinetics were obtained for net entry and exit, the latter process showing a lower K_{m} [1]. To characterise further cyclic AMP transport the infinite *cis* K_{m} has been determined using the valuable kinetic analysis of the asymmetric transport system by Stein and Lieb [2]. This analysis has been of use in analysing the infinite *cis* K_{m} values for entry of D-glucose, D-galactose and uridine into human erythrocytes [3,4,5]. The infinite *cis* K_{m} for entry ($K_{\mathrm{ic}}^{\mathrm{oi}}$) is the internal saturation constant. It is determined from the extent of backflux in entry experiments unlike the zero *trans* exit K_{m} which is determined by studying efflux directly. In the studies on sugar entry the integrated rate equation approach has been used as this provides a means of following internal saturation when the internal transported substrate concentration rises with time. The advantage of such an approach is that because of the small internal volume of the red cell linear estimates of rate at fixed internal substrate concentrations are difficult to achieve. This approach has also been adopted here.

Cyclic [8-^{3}H]AMP and D-[^{3}H]glucose were obtained from the Radiochemical Centre, Amersham. Cyclic AMP was obtained from Sigma and phloretin was obtained from K and K Chemicals. Other chemicals (reagent grade) were obtained from B.D.H.

Pink erythrocyte ghosts were obtained by the method of Dodge et al. [6] as previously described [1]. These were resealed at 30°C for 1 h and were then separated from the resealing buffer by centrifugation. A phosphate saline buffer was used for the uptake experiments (154 mM sodium chloride, 12.5 mM sodium phosphate, pH 7.4). 10 μl of packed ghosts were added to 50 μl of a solution containing 10^{-5} M or 10 mM cyclic [^{3}H]AMP. To terminate the incubations 1 ml of ice-cold phosphate saline buffer containing 0.5% ethanol (stopping buffer) was added and this solution was carefully pipetted into the centre of a glass fibre filter (Whatman GF/F) in a Millipore filter chamber. This was followed by washing with 15 ml of stopping buffer. The filter edges were immediately removed with a cork borer. The filter centres (with trapped ghosts) were added to scintillation vials and the transported cyclic [^{3}H]AMP was extracted into 1% trichloroacetic acid before liquid scintillation counting. In all cases zero time and equilibrium isotope levels for 10^{-5} M cyclic AMP were estimated. At equilibrium the cyclic AMP concentration inside is equal to the external concentration. The internal water space was estimated with D-[^{3}H]glucose. When D-glucose equilibrium isotope levels were measured 0.1 mM phloretin was added to the stopping buffer. The initial influx rate constant for 10^{-5} M cyclic AMP was determined from samples taken at 2 min at the indicated temperatures.

The uptake of 10 mM cyclic AMP was measured at 30, 20, 10 and at 4°C (Fig. 1a). To obtain the infinite *cis* K_m from this data Eqn. 1 has been used. This equation was derived by Ginsburg and Stein [4].

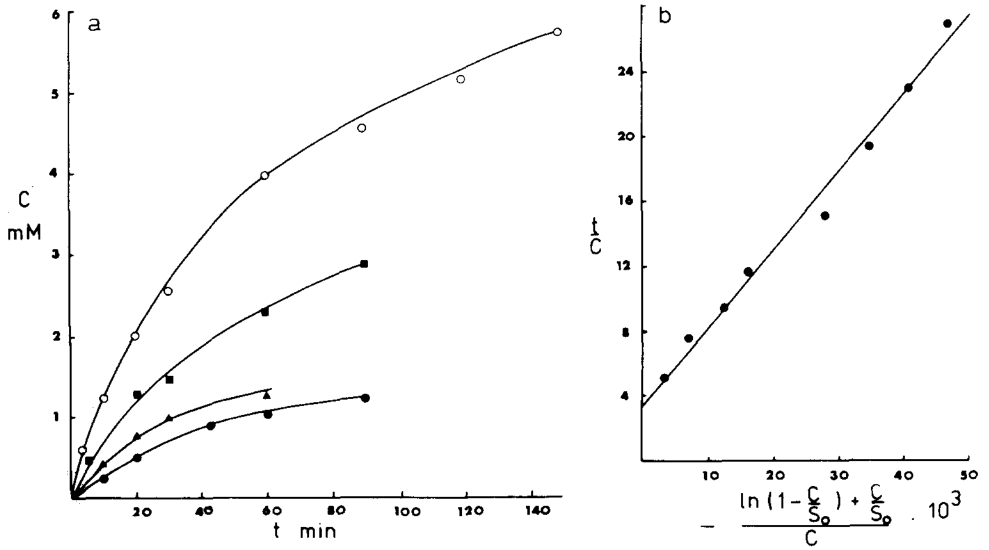


Fig. 1.(a). A time course for the uptake of 10 mM cyclic AMP at 30° C (○), 20° C (■), 10° C (▲) and 4° C (●). (b). Analysis of infinite *cis* entry data at 30° C taking account of volume corrections. The line is derived from median estimates (see text for details).

$$(A + BS_{\mathrm{o}})\left[\frac{\ln\left(1 - \dfrac{C}{S_{\mathrm{o}}}\right) + \dfrac{C}{S_{\mathrm{o}}}}{C}\right] - \frac{A}{S_{\mathrm{o}}} = -\frac{t}{C} \tag{1}$$

where $A = KR_{\mathrm{oo}} + R_{21}S_{\mathrm{o}}$

$$B = \frac{KR_{\mathrm{oo}}}{\pi} + R_{12} + \left(\frac{R_{12}}{\pi} + \frac{R_{21}}{\pi} + \frac{R_{\mathrm{ee}}}{K}\right)S_{\mathrm{o}} + \frac{R_{\mathrm{ee}}S_{\mathrm{o}}^{2}}{K\pi}$$

$$R_{12} + R_{21} = R_{\mathrm{oo}} + R_{\mathrm{ee}} \tag{2}$$

π is the concentration of osmotically active but impermeant material. S_{o} is the external substrate concentration, C is the internal substrate concentration, R_{ee} is the reciprocal of the maximum velocity per unit carrier for the equilibrium exchange experiment, R_{12} is the reciprocal of the maximum velocity per unit carrier for the zero *trans* efflux experiment and R_{21} is the corresponding quantity measured in the influx direction. K is the half saturation constant.

To determine the infinite *cis* K_{m} $(KR_{21}/R_{\mathrm{ee}})$,

$$-\left[\frac{\ln\left(1 - \dfrac{C}{S_{\mathrm{o}}}\right) + \dfrac{C}{S_{\mathrm{o}}}}{C}\right] \text{ (x-axis) is plotted against } \frac{t}{C} \text{ (y-axis)}$$

The slope is $A + BS_{\mathrm{o}}$ and the intercept on the y-axis is A/S_{o}. The intercept on the x-axis is

$$X^{-}\text{ intercept} = \frac{A}{S_{\mathrm{o}}(A + BS_{\mathrm{o}})} \tag{3}$$

When S_{o} is very large Ginsburg and Stein showed that

$$K_{ic}^{\mathrm{oi}} = X^{-}\text{ intercept}\cdot\frac{S_{\mathrm{o}}^{3}}{\pi} \tag{4}$$

Thus this equation is rather limited for cases where $S_{\mathrm{o}} < \pi$. However, if one substitutes for A and B

$$S_{\mathrm{o}}^{2}X^{-}\text{ cept} = \frac{S_{\mathrm{o}}(KR_{\mathrm{oo}} + R_{21}S_{\mathrm{o}})}{KR_{\mathrm{oo}} + R_{21}S_{\mathrm{o}} + \dfrac{KR_{\mathrm{oo}}S_{\mathrm{o}}}{\pi} + R_{12}S_{\mathrm{o}} + \left(\dfrac{R_{12}}{\pi} + \dfrac{R_{21}}{\pi} + \dfrac{R_{\mathrm{ee}}}{K}\right)S_{\mathrm{o}}^{2} + \dfrac{R_{\mathrm{ee}}S_{\mathrm{o}}^{3}}{K\pi}}$$

Substituting for $R_{12} + R_{21}$ in the denominator using Eqn. 2 gives

$$S_{\mathrm{o}}^{2}X^{-}\text{ cept} = \frac{S_{\mathrm{o}}\left(\dfrac{KR_{\mathrm{oo}}}{S_{\mathrm{o}}} + R_{21}\right)K}{\left(\dfrac{KR_{\mathrm{oo}}}{S_{\mathrm{o}}} + R_{\mathrm{ee}}\right)\left(1 + \dfrac{S_{\mathrm{o}}}{\pi}\right)(S_{\mathrm{o}} + K)} \tag{5}$$

Since $\frac{KR_{oo}}{S_o}$ is a small term and $S_o > K$ Eqn. 5 becomes

$$K^{oi}_{ic} = S_o^2\, X^{-}\,\text{cept}\left(1 + \frac{S_o}{\pi}\right) \tag{6}$$

It can be shown that the ratio of the true infinite *cis* K_m over the K_m calculated from Eqn. 4 is very high at low values of S_o and falls as S_o is increased whereas Eqn. 6 is valid even when S_o is low relative to π. Since in the present study the flux of 10 mM cyclic AMP has been studied, Eqn. 6 has been used to estimate the infinite *cis* K_m (Fig. 1b). For an accurate estimate of K_m statistical analysis of the plot is required. Since both axes are dependent on C linear regression is inappropriate. A solution to a problem of this sort is to use a nonparametric approach similar to that used by Eisenthal and Cornish-Bowden [7]. These authors have shown that the Michaelis-Menten equation can be arranged as pairs of simultaneous equations. An equation of the type $y = mx + c$ (as is Eqn. 1, $m = (A + BS_o)$, $c = A/S_o$ can give $n(n-1)/2$ equations for m and c

$$m_{ij} = \frac{y_i - y_j}{x_i - x_j} \qquad c_{ij} = y_i - m_{ij}x_i \tag{7}$$

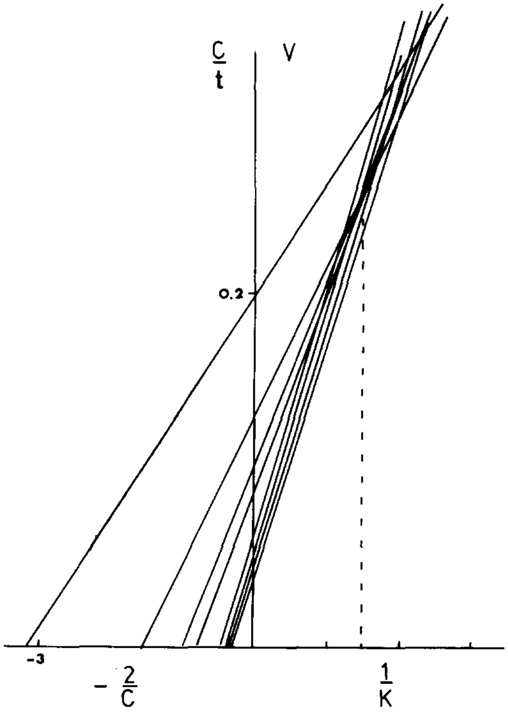
The values of m and c so obtained are then ranked and median estimates are determined. The median m and median c are then used to calculate X^{-} cept $\left(-\frac{c}{m}\right)$. The statistical advantages of this type of approach have been discussed [7]. It is of interest to note that when $S_o \ll \pi$ the Sen/Widdas equation [9] can also provide estimates of the infinite *cis* K_m

$$\frac{dC}{dt} = \frac{VK}{K + C}$$

integrating leads to $V = \frac{C}{t} + \frac{C^2}{2Kt}$

Thus a direct linear plot [10] of V against $1/K$ has an intercept C/t, on the y axis and $-2/C$ as the X^{-} cept. Such a plot for cyclic AMP infinite *cis* influx is shown in Fig. 2.

Immediate inspection of such a plot can give K and V estimates from the intersections but one can also substitute in Eqn. 7 $y = C/t$, $x = C^2/2t$, $m = -1/K$ and $c = V$. From Fig. 1b (which takes account of volume changes) $K^{oi}_{ic} = 0.69$ mM (median estimate). From the direct linear plot $K^{oi}_{ic} = 0.66$ mM (median estimate). Since V_{max} for net and exchange entry of cyclic AMP are equal the predicted value for the infinite *cis* K_m is that which is also obtained in the zero *trans* exit experiment (0.475 mM) [1]. The experimentally determined values for the infinite *cis* K_m are therefore only slightly higher than predicted despite the fact that for most of the data $C > K_m$. No evidence for a high K_m similar to that found in net influx experiments was obtained but would have been detectable if present.



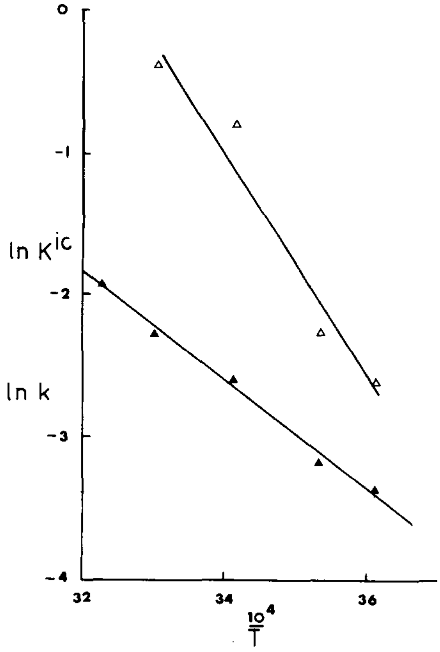


Fig. 2. A direct linear plot for infinite *cis* entry at 30° C. The intersections give $1/K$ estimates. The calculated median estimate is shown as a broken line.

Fig. 3. The effect of temperature on the infinite *cis* K_m (△) (the line is derived by linear regression) and the effect of temperature on the initial uptake rate constant (k) (min^{-1}) of 10^{-5} M cyclic AMP (▲) (the line is derived by linear regression).

Since $K_{ic}^{oi} = KR_{21}/R_{ee}$ and $R_{21}/R_{ee} = 1$ for cyclic AMP transport [1] it can be seen that K_{ic}^{oi} provides a convenient estimate of the dissociation constant for interaction of cyclic AMP and its transport binding sites. Consequently, the effect of temperature on this parameter and the influx rate constant (k) for 10^{-5} M cyclic AMP was determined (Fig. 3). Using Eqn. 6 and the data in Fig. 1a showed that the infinite *cis* K_m values at 30, 20, 10 and 4°C are 0.69 mM, 0.47 mM, 0.1078 mM and 0.072 mM, respectively. From this $\Delta H = 16$ kcal·mol^{-1}; $Q_{10} = 2.4$. The apparent activation energy for transport of 10^{-5} M cyclic AMP is 8 kcal·mol^{-1}; $Q_{10} = 1.55$. Apparent enthalpy terms were calculated as the slopes × R, where R is the gas constant. Since k can be regarded as $1/KR_{oo}$, $1/R_{oo}$ must also show a large apparent activation energy. Unfortunately this data alone cannot give a reliable indication as to whether cyclic AMP transport is mediated by a predominately hydrophobic or hydrophilic pathway. No evidence for a phase transition around the melting temperature of the membrane lipid was obtained.

In conclusion, the equations used here for the estimation of the infinite *cis* K_m for cyclic AMP transport may be of general use for rapidly transported substrates whose concentration relative to K_m is high when their concentration is low relative to π. In the case of cyclic AMP transport the results are of interest since the calculated infinite *cis* K_m is only slightly higher than the value that can be predicted from the zero *trans* and exchange transport experiments that have been previously reported [1]. The glucose transport system in intact red cells has a lower than predicted value for the infinite *cis* K_m (Hankin et al. [3]), and this can be confirmed using Eqn. 6. Hence, compared with the glucose transport system of whole cells the cyclic AMP transport system of red cell ghosts is kinetically a simple system.

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