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THE EFFECT OF UNSTIRRED LAYERS ON THE MEASUREMENT OF TRANSPORT RATES IN INDIVIDUAL CELLS

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SUMMARY

The effect of unstirred layers on the fluxes produced by saturable cellular transport systems is discussed, and it is shown that diffusion in the external solution should not be limiting provided the half saturation concentration of the system (K, in mM) is very much larger than 10^5 times the transport rate (in mmoles·cm^{-2}·sec^{-1}). This criterion was applied to the data obtained previously by the author on monosaccharide transport in human erythrocytes, to show that in this case diffusion is sufficiently rapid as to have no measurable effects. It was pointed out, however, that this applies only to uniformly dispersed cells, so it was necessary first of all to measure the efficiency of the initial mixing produced by the experimental techniques. This was done through the use of cinematography which revealed that mixing was 90 % complete in 0.7 sec, a time of sufficiently short duration as to allow application of the criterion.

INTRODUCTION

Naftalin[1] has shown that the rate of exchange of sugars across the red cell membrane is affected by inadequate stirring and has concluded from this that unstirred layers surrounding each cell are responsible for certain anomalies found by Miller[2] in the measured rates of exchange between different sugars. The kinetics resulting from such a situation have already been considered[3] and found to be inconsistent with the data. However, since the unstirred layer effect is frequently invoked in other systems[4,5], and in view of Naftalin's claim, it seems appropriate to reconsider here the data which were collected by Miller on monosaccharide transport in human erythrocytes[2,6,7] to see if such layers could have a noticeable effect on this system.

THEORY

In the following we shall consider the effect of diffusion limitation on saturable membrane transport systems which obey the expression

$$R = V\left[\frac{X_i}{X_i + K} - \frac{X_0}{X_0 + K}\right] \qquad (1)$$

where V is the maximum rate at which the system can transport a substrate out of the cell, R is the rate at which transport occurs when operating on a substrate whose concentration is X_i at the inner membrane surface and X_0 at the outer membrane surface, and K is the half saturation constant of the substrate for the system. The diffusion effect will be most evident when such a system is operating maximally, as when the internal concentration is high ($X_i \gg K$) and the external concentration is low ($X_0 \ll K$) so that $R = V$. If, however, X_0 is not low relative to K, as for example when diffusion away from the cell is slow, or when the sugar concentration in the outside solution is not zero, Eqn. 1 becomes

$$R = \frac{VK}{X_0 + K} \tag{2}$$

and R will be only a fraction of V. In fact when $X_0 = K$, $R = 0.5\,V$ which is the basis of the method of Sen and Widdas[8] for obtaining the values of the kinetic constants V and K.

The sugar flowing out of the cell must diffuse through the surrounding solution, and as it will encounter a finite resistance in doing so, a concentration (X_0) will build up next to the cell surface which is higher than that in the solution bulk (X_b). Furthermore, a steady state will be rapidly established in which the flux through the solution equals that through the membrane, or according to Fick's law

$$R = D(X_0 - X_b)/\delta \tag{3}$$

where D is the diffusion coefficient ($5 \cdot 10^{-6}$ cm$^2 \cdot$sec^{-1} for sugars in water) and δ the operational thickness of the layer. The question we must now ask ourselves is whether X_0 will, under our experimental conditions, reach a value comparable to, or in excess of, the lowest value of K measured. This may be readily answered by substituting the appropriate values for the various constants into Eqn. 3, and determining X_0 in comparison with K.

In the experiments we are considering here, the lowest value of K was found to be 1.8 ± 0.3 mM when measured by the the method of Sen and Widdas[3] with glucose as substrate. These same experiments led to a maximum flow rate, when $X_b = 0$, or $R = 104 \pm 12$ mmoles$\cdot$min$^{-1}\cdot$(cell unit)$^{-1}$. A cell unit is a quantity of cells containing a litre of solvent water under isotonic conditions and as this water represents 70 % of the cell volume[9], there will be 1400 cm^3 cells per cell unit. The volume of an individual cell is $0.9 \cdot 10^{-10}$ cm^3 and its area is $1.6 \cdot 10^{-6}$ cm^2, so with this information the value of R can be expressed in cgs units as $7 \cdot 10^{-8}$ mmoles$\cdot$cm$^{-2}\cdot$sec^{-1}.

Next we can make an estimate of δ from the fact that a 1 % suspension of cells was used in these experiments and as this represents a cell population of 10^8 cm^{-3} the average distance between cells must be about $2 \cdot 10^{-3}$ cm. Thus no part of the solution is further than about 10^{-3} cm from a cell surface, a distance which must represent an upper limit to the value of δ.

Substituting this information into Eqn. 3 we find that $X_0 = 14$ μM. As this is less than 1/100 the lowest value of K, we may conclude that diffusion in the solution surrounding the cells, even though unstirred, is so much more rapid than transport

through the membrane that it can have only a negligible effect, and that therefore the maximum value of R which was obtained is indeed equal to V.

If the presence of an unstirred layer cannot account for Naftalin's results, what other explanation is there? To answer this let us consider a second way in which we may be in error in assuming that X_0 is zero. Suppose that, during the course of the experiment, the substrate leaving the cell raises its concentration in the bulk of the solution to a value comparable to that of the affinity constant; then once again, according to equation 2, $R<V$. The experiments of Sen and Widdas[8] were performed by equilibrating the cells, under isotonic conditions, with 130 mM glucose and then suspending them in 100 times their own volume of solution. Assuming as before that the cells are 70 % water, then when the sugar has equilibrated between the cells and the new solution, ΔX_0 (the increase in external concentration due to efflux) will be 0.9 mM. However, measurements were made only until the first 1/3 of the glucose was lost at which point $\Delta X_0 = 0.3$. This is equal to the standard deviation (17 %) for K found by this method.

V was not found by a direct measurement of R when $X_0 = 0$, however, but by determining R for various values of X_0 and extrapolating back to $X_0 = 0$ in accordance with the procedure of Sen and Widdas[8]. ΔX_0 will of course, have less significance at these higher concentrations, being only 3 % of the highest concentration of $X_0 = 10$ mM.

There is, however, a situation where ΔX_0 could have a serious effect on the measurements of V. This would arise in cases of non-uniform cell suspensions, where regions of relatively high cell density result in higher than average values of ΔX_0 and an overall decrease in the measured rate. This could explain why the less efficient stirring employed by Naftalin resulted in lower rates; but it also raises the question as to whether the techniques used by Miller were themselves free from this objection. It is to answer this question, then, that the following experiments were performed to measure the efficiency of mixing in the early stages of Miller's procedure.

EXPERIMENTAL

In this section we will discuss firstly the procedure used in the original work to disperse the cells, and secondly the methods used in the present work to measure the efficiency of mixing during cell dispersal.

Dispersion technique

In the original work, 10 μl red cells were centrifuged into the bottom of a Hopkin's vaccine tube (Fig. 1A), and the supernatant carefully sucked off. Next, 1–2 ml of the medium into which the cells were to be suspended, was slowly added to the upper part of the tube, but did not yet come in contact with the cells, due to the presence of air trapped between them. Sugar movement was initiated when the plunger of a 2-ml Cornwall automatic syringe (available from Becton, Dickinson and Co., Rutherford, N.J.) fitted with a No. 17 three-inch hypodermic needle was manually depressed and the needle rapidly pushed to the bottom of the vaccine tube. As the needle struck the bottom, the plunger was released allowing a spring to force it up against an adjustable stop set at 1 ml. This caused the medium to be

sucked down to the bottom of the vaccine tube and up the needle, sweeping the cells along with it into the syringe where mixing occurred (Fig. 1B).

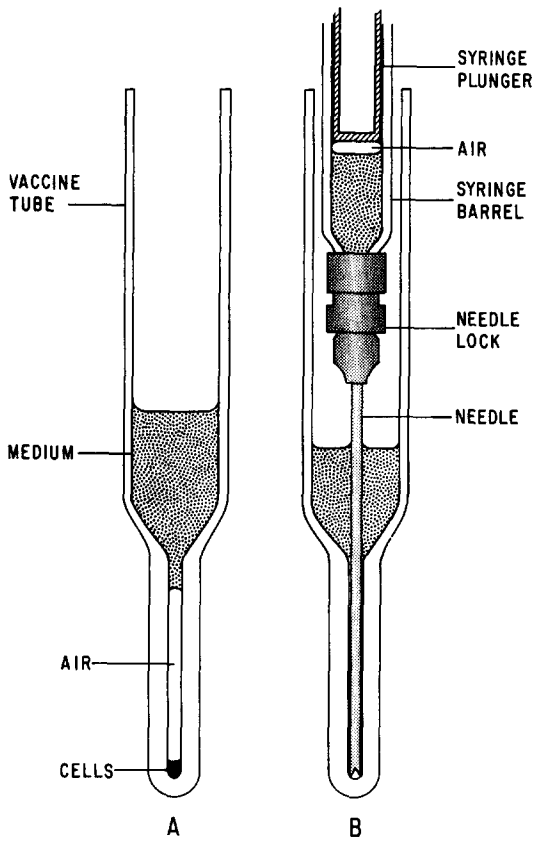

Fig. 1. Apparatus for dispersion of red cells in medium. A, before B, after. See text for details.

Measurement of the rate of mixing

To measure the rate of mixing in the syringe the following procedure was employed. The medium in the tube was replaced by 1–2 ml dilute HCl solution (approx. 0.01 M) containing a small quantity of the indicator phenolphthalein, and the cells were replaced by 10 μl of NaOH solution of sufficient strength to neutralize 0.9 ml of the acid. With the previous technique, 1.0 ml of acid was used to carry the alkali into the syringe where the subsequent changes in color were observed. The initial solution entering the syringe was colored red by the indicator, due to the presence of an excess of alkali, but when 90 % of the acid had entered and mixed with the alkali, the color disappeared. The time of disappearance of the color represented the time at which mixing had progressed to a point where, in no region of the solution was the alkali (or cell) concentration in excess of 10 % of the average. This time was found to be much too short to be determined visually, however, so color cinemotography was employed, using a Bolex H16 camera (Paillard, Ste. Croix, Switzerland) at 64 frames·sec^{-1}, and 16 mm Kodachrome IIA film.

The face of a 10-sec sweep hand stopwatch, mounted beside the syringe, was simultaneously photographed to allow the time of each frame to be determined to about 0.02 sec.

RESULTS

Thirteen sequences were photographed and the films examined. Although there were variations from run to run, the following general observations could be made:

The plunger moved from its lowest to its highest position in less than 0.05 sec and since at the time no liquid had entered the syringe, a partial vacuum was developed. The liquid then rose through the needle and entered the syringe in a jet, which impinged on the bottom of the plunger and ran down the walls of the barrel. The jet was visible for about 0.2 sec, after which streaming into the accumulated liquid resulted in a period of extreme turbulence lasting about 0.3 sec. As the syringe continued to fill, the turbulence decreased, and the pink color present until then could be seen to fade. This usually occurred throughout the liquid, although occasionally a slight wisp or pocket of color remained for a further 0.1 to 0.2 sec. On the average, however, total fading had occurred by 0.7 sec after release of the plunger.

DISCUSSION

The procedure employed by Miller in the measurement of sugar transport is similar to conventional stop-flow experiments, with an initial short period of turbulent mixing followed by a timed interval, with little or no mixing, during which transport took place. The initial mixing period was probably very short, since all the cells are dispersed in several times their own volume of medium in less than 0.1 sec, allowing transport to start during this period. Further dilution of the cells to 100 times their volume occurred over the next 0.6 sec, with agitation sufficient to reduce differences in concentration between all regions of the solution to less than 10 %. Thus since no gross differences in concentration occurred for times which were significant by comparison with the shortest interval of measurement (about 5 sec in this work), and since we have shown that diffusion is so much faster than transport that significant local concentration gradients could not have formed near the cell membrane, we may conclude that Naftalin's objections do not apply to this work.

Naftalin's observations appear to arise from two causes. Firstly, he used a 5 % suspension of cells loaded with sugar at 120 mM, so that ΔX_0 would have reached at least 1–2 mM during his period of measurement. This is small relative to the half saturation value for exchange at room temperature, but would have become more important as the temperature was lowered and the value of K decreased. Secondly his method of mixing (by rotational stirring in a beaker) would have involved essentially streamlined flow with little turbulence, so that if regions of high cell concentration did occur, they would likely have persisted throughout his 15-sec measurement period.

Finally, we may derive a rough guide from Eqn. 3 to determine whether or not diffusion in the solution is likely to affect the rate of a saturable transport system

located in suspended single cells. If R is the flow rate when $X_b = 0$, then, since for most compounds $D = 5 \cdot 10^{-6}$ $cm^2 \cdot sec^{-1}$ and cell populations are generally 10^8 cm^{-3} or greater, we can write that $X_0 = 10^5 R$ mM. Thus if K (in mM) $\gg 10^5 R$ (in $mmoles \cdot cm^{-2} \cdot sec^{-1}$), diffusion in the solution phase should not be limiting. One must realize, however, that this applies to single cells in suspension only, and not to tissues, where much thicker unstirred layers can develop.

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