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HYPOXANTHINE TRANSPORT IN HUMAN ERYTHROCYTES

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SUMMARY

Using rapid sampling by filtration, it has been possible to follow and describe the transport of hypoxanthine across the human red blood cell membrane.

The dependence of the transport rate upon the concentration of hypoxanthine is complex, and suggests a two-component mechanism. The first is a "saturable carrier system" with a K_m of 0.4 mM, a value comparable to the K_i value for hypoxanthine as inhibitor of uric acid transport. This is taken as evidence for uric acid and hypoxanthine, at least in part, using the same transport system across the red-cell membrane. The second part of the transport system seems non-saturable.

Experiments at different temperatures and concentrations did not reveal further details of the mechanism.

With hypoxanthine as substrate for the purine-transporting system, it was of interest to demonstrate "uphill transport induced by counterflow"[1,2] to disclose whether the transport system was of the "mobile type". Experiments on this point did not give evidence for counterflow of labelled hypoxanthine against a net influx of non-labelled hypoxanthine. As discussed, this might be due in part to restrictions in the practicable experimental conditions.

INTRODUCTION

It is well established that hypoxanthine is able to penetrate the human erythrocyte membrane[1-3]. WHITTAM[1] reported that the fractional uptake of hypoxanthine from the suspending medium was independent of hypoxanthine concentrations in the range 0.2 to 6.8 mM. The method used by WHITTAM did not, however, allow an estimation of the velocity of uptake until the attainment of steady state. It has earlier been shown[3] that there is mutual inhibition between hypoxanthine and adenine influxes into human erythrocytes. Furthermore, hypoxanthine has been shown to inhibit uric acid transport across the red-cell membrane[4,5]. Thus it is reasonable to assume the presence of a specific transport system, common to several purine derivatives[3].

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The transport of hypoxanthine across the red-cell membrane is rapid[1,3]. At 37° and with low molar concentrations, complete equilibrium for hypoxanthine is reached in less than 1 min. The conventional separation of cells from the incubating medium by centrifugation is not satisfactory, as the time of separation is poorly defined. For this reason a previously described sample-taking device[6] has been used in the present study. The principle of this apparatus is a pressure filtration to obtain rapid sampling of the supernatant from the incubation mixture. The method is convenient when using low cell fractional volumes in the incubation mixtures. With cell fractional volumes higher than approx. 15%, the filters tend to clog, thus causing hemolysis. Hypoxanthine in the concentrations used is not actively accumulated in the cells. Addition of labelled hypoxanthine to a cell suspension with low hematocrit only causes a minor decrease in extracellular radioactivity during the penetration. Thus the determination of influx of radioactivity is rather inaccurate, and fluxes were measured by following loss of labelled hypoxanthine from preloaded erythrocytes. The results show that the transport system for hypoxanthine is complex, presumably having two components.

METHODS

The experiments were performed on heparinized human blood (5 I.U. of heparin per ml) not more than 30 h after withdrawal. The blood was stored at 6° until use.

Red cells were suspended in Krebs–Ringer bicarbonate solutions[7] with sufficient bicarbonate added to give a pH of 7.38–7.42 after 1 h equilibration at 37° with 4.7–5.3% CO_2 in O_2. In the experiments where different temperatures were employed, it proved difficult to maintain a constant pH with bicarbonate media. For this reason, a medium with the following composition was used in these experiments: instead of the $NaHCO_3$ solution, 10 parts of 0.11 M sodium phosphate buffer (pH 7.35) were added; $CaCl_2$ was omitted to avoid precipitation. The rest of the ingredients were as for the usual "Krebs–Ringer" solution. Hypoxanthine solutions of various concentrations were prepared by adding hypoxanthine to the 0.154 M NaCl with heating to approx. 80° and stirring. After cooling and readjustment of the volume this NaCl solution was used to make up the Krebs–Ringer buffer. The media contained 5 mM glucose and 1 g bovine albumin per l solution.

Blood cells were prepared for the experiment in the following way: heparinized blood was centrifuged for 30 min at approx. 2000 × *g* and 10° in a refrigerated centrifuge. The plasma and buffy coat were sucked off, and the erythrocyte concentrate was resuspended in approx. 2 volumes of a Krebs–Ringer solution having a predetermined hypoxanthine concentration and a constant concentration of [8-^{14}C]hypoxanthine (approx. 0.1 μC/ml) with a specific activity of 8.4 μC/μmole (Calbiochem, Los Angeles). This suspension was then incubated for 30 min at 37° under constant aeration (the above CO_2–O_2 mixture) and shaking in a Dubnoff metabolic shaker. The incubate was then centrifuged for 30 min in the cold; the supernatant was carefully sucked off and the cells (3–4 ml) were drawn into a syringe. This syringe was reheated to 37° (unless otherwise stated) and fitted on the rapid sampling device together with another syringe containing approx. 20 ml of Krebs–Ringer solution with the same "equilibrium concentration" of hypoxanthine as the first incubate (see below). The experiment was then started by simultaneous injection of the two syringes into the mixing tube of the sample-taking device. A total of 8 samples was usually taken within 80 sec from the

mixing of cells and suspending medium. 25–50-μl aliquots of the cell-free filtered samples were counted in a conventional liquid-scintillation spectrometer system (Packard Model-314 EX) using toluene–water-free methanol (70:30, by vol.) as the solvent. To obtain samples for the evaluation of extracellular counting rate at equilibrium, centrifuged aliquots of the incubate were taken in the interval 5 – 20 min after start of the experiment. Further experimental details are reported in a previous publication[6].

In experiments at or below the ambient temperature the incubate and sampling machine were kept at constant temperature by circulation from a refrigerated thermostat.

Cell fractions of the different incubates were measured in quadruplicate by a microhematocrit method*. To check the purity of the commercial hypoxanthine used (Calbiochem), a crude milk xanthine oxidase (EC 1.2.3.2) preparation (Worthington) was used, applying the rise in absorbance at 292 nm as a measure of the formed uric acid[8]. Both radioactive and non-radioactive hypoxanthine were checked for purity by paper chromatography on Whatman No. 1 paper with water-saturated butanol–concentrated NH_4OH (100:1, by vol.) as the solvent (descending). Both preparations were free of detectable impurities, especially other purines. The paper strips from the runs of radioactive hypoxanthine were measured in a Vanguard Autoscanner 880. By this method, a radiopurity of more than 99% was found.

When loading the cells with radioactive hypoxanthine, a cell concentrate was added to a medium containing the labelled material at different specific activities. For the calculation of transport velocities and for preparing the medium in which efflux of radioactivity was followed, it was necessary to know the amount of hypoxanthine taken up by the cells at equilibrium. From the extracellular counting rates before addition of the cell concentrate and at equilibrium after the addition, and from the hematocrits, the ratio of hypoxanthine concentrations in intracellular to extracellular water was 1.40 ± 0.07 (14 determinations) taking the intracellular water to be 65% of the cell volume. This ratio was independent of hypoxanthine concentrations in the range 0.2 to 12 mM. With the procedure used for the initial centrifugation of the blood, the cell concentrate to be suspended in the radioactive medium had a hematocrit of approx. 90%. The extracellular equilibrium concentration of the incubate in which the cells were loaded could therefore be approximated as:

$$[Hx]_{eq} = [Hx]_{init} \frac{\text{volume of medium}}{\text{volume of medium} + \text{volume of "cells"}}$$

where $[Hx]_{eq}$ is the equilibrium concentration and $[Hx]_{init}$ the hypoxanthine concentration of the medium before addition of the cell concentrate. This expression was checked by direct analyses, and was found to agree within 7% (maximal deviation in 11 determinations). For calculation of transport rates, the following expressions were used[9]:

$$\left(1 + \frac{a}{b}\right)^{-1} \ln\left[z_0 + \frac{a}{b}x_0 - x\left(1 + \frac{a}{b}\right)\right] = -k_1 t + N_1$$

$$z_0 = x_{eq}\left(1 + \frac{a}{b}\right) - x_0 \frac{a}{b}$$

$$k_1 = k_2 \frac{b}{a}$$

* B. VESTERGAARD-BOGIND, personal communication.

where a and b are the apparent volumes of distribution for hypoxanthine in extra- and intracellular compartments, x and z are the concentrations of radioactivity in the extra- and intracellular compartments, the subscript "o" referring to time zero (start of the experiment) and "eq" meaning at equilibrium. k_1 and k_2 are the steady-state rate constants for turnover of extracellular and intracellular hypoxanthine. N_1 and N_2 (below) are constants.

The above equations can be reduced to:

$$\ln (x_{eq} - x) = -k_2\left(1 + \frac{b}{a}\right)t + N_2$$

The experimental results are treated according to this expression, and the k_2 value found is used to calculate the transport velocity:

$$v_{efflux} = 0.91 \cdot k_2[Hx]_{eq}$$

The unit of velocity is mmoles hypoxanthine (l cells·min)$^{-1}$. The factor 0.91 is the water fraction of the cells (0.65) times the ratio of intracellular to extracellular hypoxanthine concentration in the water phases (1.4).

RESULTS

Fig. 1 illustrates experiments with determinations of efflux of labelled hypoxanthine at six different hypoxanthine concentrations from 0.67 mM to 6.67 mM. The results are plotted as extracellular counting rate in per cent of the equilibrium value found in later samples separated by centrifugation. The filtered and centrifuged

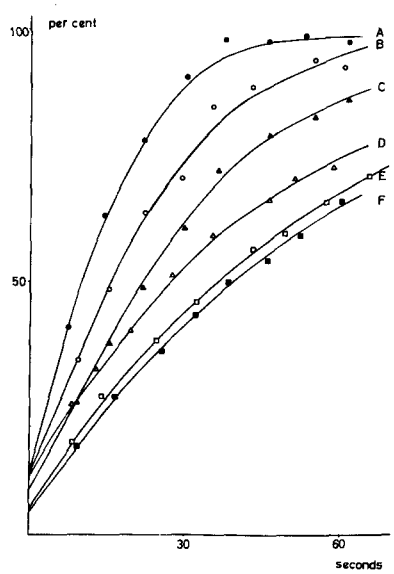

Fig. 1. Efflux of radioactive hypoxanthine from preloaded erythrocytes at different concentrations of hypoxanthine. Abscissa: time in sec; ordinate: extracellular counting rate in per cent of counting rate at equilibrium. Samples were obtained by filtration of the incubation mixtures. Concentrations of hypoxanthine: A, 0.67 mM; B, 1.35 mM; C, 2.00 mM; D, 3.33 mM; E, 4.73 mM; F, 6.67 mM.

samples are directly comparable*. The figure shows that increasing concentrations of hypoxanthine diminish the apparent rate constant of exit of labelled hypoxanthine from the cells. This behaviour is different from that of a simple diffusion in an ideal system, where the molar concentration should not influence the tracer movement.

In Fig. 2 the concentration dependence of the hypoxanthine transport is measured over a wider concentration range. The ordinate depicts the transport velocity calculated as indicated in METHODS. The experiments were performed on batches of blood from different donors. The experiments show the same general tendency: at low concentrations, rising concentration gives a rapid rise in transport velocity. At higher concentrations (above 1–2 mM), there is also a good correlation between concentration and transport velocity, but the slope of the curve, which approaches a straight line, is considerably smaller. This overall type of concentration dependence is neither in accordance with saturation of a simple, enzymatic-type sys tem, nor is it, as indicated above, a behaviour expected from a diffusion-type system. However, from immediate inspection, the shape of the curve resembles a "diffusion-type system" superimposed on a saturable system. To see whether this would give a satisfactory description of the transport mechanism, the plot in Fig. 3 was calculated from the data in Fig. 2. Here "V" is the result of subtracting from each transport velocity in Fig. 2 the corresponding values of the line $y = 0.63\ x$. This corresponds to subtracting the non-saturable part of the transport ("diffusion part") from the total transport. The remaining figures are plotted according to LINEWEAVER AND BURK[10]. It may be noted that the experimental points now fit a straight line reasonably well. From the intercept of the line with the horizontal axis, K_m was found to be 0.4 mM.

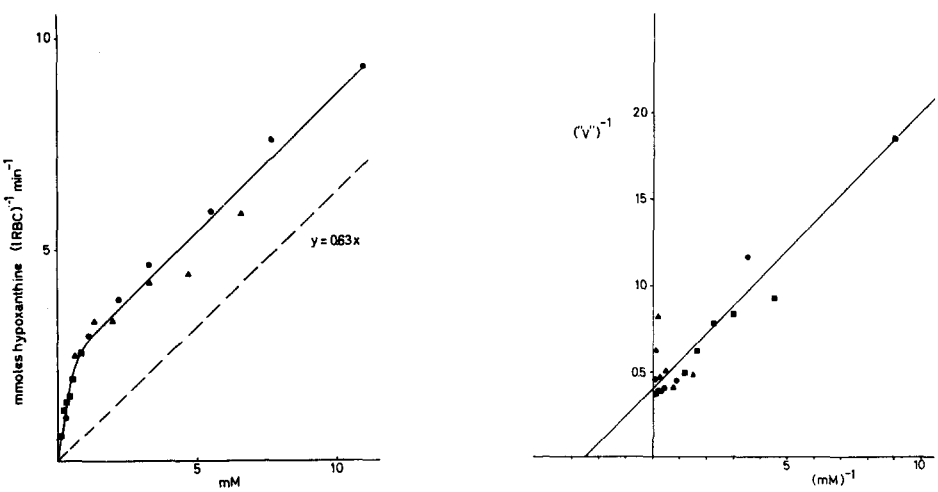

Fig. 2. Transport of hypoxanthine across the red-cell membrane as a function of hypoxanthine concentration. Abscissa: hypoxanthine concentration in mM; ordinate: transport velocity in mmoles/l red-blood cells (lRBC) per min. The different symbols refer to experiments on cells from different batches of blood. Triangles represent values from experiments in Fig. 1. The broken line is drawn parallel to the velocity *vs.* concentration curve at high concentrations.

Fig. 3. Double-reciprocal plot of "saturable part of the transport". Abscissa: reciprocal concentration of hypoxanthine; ordinate: reciprocal of transport velocity ***minus*** the corresponding values on the broken line in Fig. 2. The plot is discussed further in the text.

* U. V. LASSEN, unpublished results.

However, the implications and validity of such a treatment need further discussion, as given below.

If the transport of hypoxanthine across the red-cell membrane were in part due to a saturable "carrier" system and partly to diffusion, the effect of different temperature on transport at high and at low concentrations of hypoxanthine might be markedly different. At low concentrations, transport would be mainly *via* the saturable "carrier" system, whereas at the high concentration of hypoxanthine "diffusion" should prevail. To test this hypothesis, experiments as shown in Fig. 4 were performed. The rate constants for efflux of radioactive hypoxanthine were measured at two concentrations ($[Hx]_{eq} = 0.5$ mM or 5 mM) and four different temperatures. The logarithms of the resulting rate constants (k_2 values) are then plotted against the reciprocal of the absolute temperature to give an Arrhenius-type plot. It may readily be seen from the figure that the lines through the points at the two concentrations are parallel.

WILBRANDT AND ROSENBERG[11] suggested that the transport system for purines in the red-cell membrane might possibly be of a "movable" type similar to the sugar transport system in these cells. Attempts to demonstrate a "counterflow" of uric acid from preloaded cells did not give any indication on the mobility of the carrier system*. However, as pointed out by ROSENBERG, this might have been due to (1) low relative

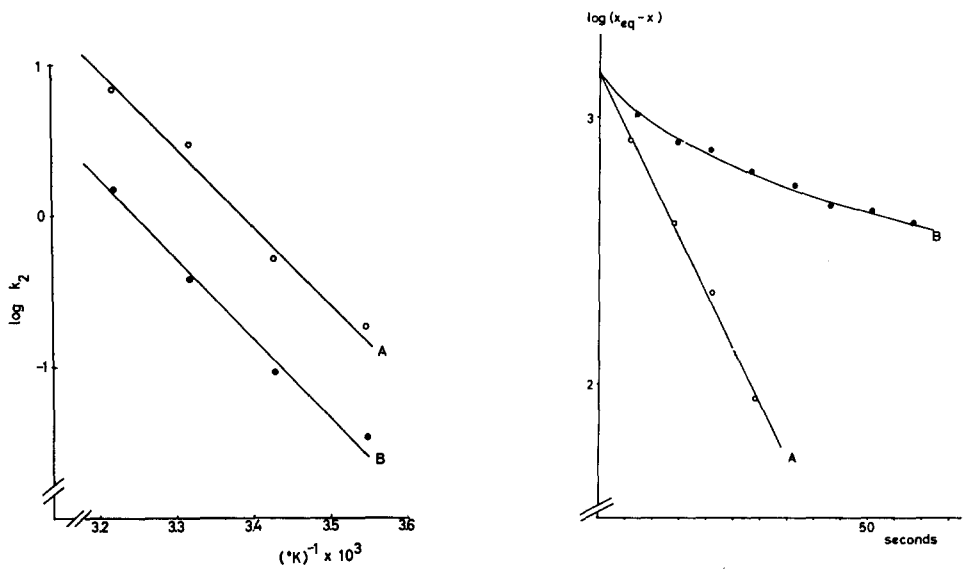

Fig. 4. Rate constants for exit of radioactive hypoxanthine from red cells as a function of temperature. Curve A is at a hypoxanthine concentration of 0.5 mM and B at a concentration of 5 mM. Ordinate: logarithm of the rate constants (see under METHODS); abscissa: reciprocal of absolute temperature in degrees Kelvin. The experiments were performed at 9, 18, 29, 39°.

Fig. 5. Exit of radioactive hypoxanthine from red cells. The cells were preloaded at a hypoxanthine concentration of 0.14 mM. In Expt. A, the preloaded cells were mixed in the sampling device with medium without hypoxanthine; in Expt. B, the medium contained 10 mM hypoxanthine. Ordinate: logarithm of difference between final counting rate and actual counting rate in the extracellular phases. Abscissa: time in sec.

* U. V. LASSEN, unpublished results.

concentrations of uric acid (about equal to the K_s for the transport system (3 mM)), and (2) the fact that in cases where counterflow[12] of uric acid was to occur against a large net-influx of hypoxanthine, the much higher affinity of hypoxanthine for the transport system (as indicated by a ratio of the Michaelis constants of approx. 10) would rapidly lead to a block of the uric acid efflux. For these reasons, the case where an efflux of labelled hypoxanthine was measured with or without a large net-influx of non-labelled hypoxanthine should have a better chance of revealing any possible counterflow. These experimental conditions were sought in the experiment represented in Fig. 5. Cells preloaded at a total hypoxanthine concentration of 0.14 mM ($[Hx]_{eq}$) were injected into the chamber of the sample-taking device together with media with or without 10 mM non-labelled hypoxanthine. The counting rates of the filtered samples are plotted logarithmically as indicated. The points from the experiment with no hypoxanthine in the suspending fluid fit a straight line (up to about 90% of equilibrium). Contrary to this, the points from the experiment with 10 mM hypoxanthine are non-linear, although apparently they have the same origin as the former. It is to be noted that there is no sign of an initial velocity in the "high-hypoxanthine experiment" being higher than in the case with no hypoxanthine. The curve of log $(x_{eq}—x)$ *vs.* time not being a straight line in one of the above experiments is different from the other experiments in this work, which all yielded straight lines when treated similarly. The validity of this experiment for disclosing possible "mobile carriers" might be questioned as discussed below.

DISCUSSION

In the present study, the transport of radioactive hypoxanthine has been studied under steady-state conditions (apart from the experiments in Fig. 5). It has been inferred that the kinetics of transport of labelled hypoxanthine gives information on the total transport of hypoxanthine. For this reason it is important to know whether hypoxanthine is metabolized to any great extent in human red cells. BISHOP, RANKINE AND TALBOTT[13] found no inosine monophosphate (IMP) in human red cells. However, LOWY, WILLIAMS AND LONDON[14] isolated small amounts of IMP from human erythrocytes, and showed that labelled hypoxanthine is readily incorporated into it. No compounds other than IMP were found to be extensively labelled by the [^{14}C]-hypoxanthine. In fresh heparinized blood, the fractional uptake into IMP of labelled hypoxanthine in concentrations above 0.1 mM is negligible (less than 5%)*. This means that the molar concentration of bound, labelled hypoxanthine need not to be taken into consideration for the present calculations of hypoxanthine "space" in the cells.

Another factor might influence the transport of hypoxanthine. During incubation of erythrocytes in the absence of glucose, breakdown of adenosine nucleotides leads to formation of hypoxanthine[15]. This was avoided in the present study by the presence of glucose. The cellular content of ATP did not change significantly during the incubation*.

It was found in earlier work[2], and in the present study, that hypoxanthine is present in erythrocytes in higher concentration than would be expected merely from

* U. V. LASSEN, unpublished results.

knowledge of the water content. This fact had been taken as being suggestive of the active transport of this compound[2], but another explanation seems more relevant. It is generally assumed that Cl^- is passively distributed across the red-cell membrane. Making several critical assumptions, it is possible to calculate membrane potentials of these cells in the order of 9–12 mV, inside negative (see ref. 16). A potential of this magnitude has been confirmed by direct measurements**. Using the Nernst equation, the passive distribution for a freely permeable cation (as hypoxanthine at physiological pH) is calculated to have a ratio between concentrations in the intra- and extracellular water phases of approx. 1.3–1.4, *i.e.*, approximately the same as for hypoxanthine. Thus the trans-membrane potential may well account for the uneven distribution of hypoxanthine.

The results shown in Fig. 2 suggest that we are dealing with a two-component system, one of the components being saturable. This hypothesis was used in Fig. 3 for a conventional double-reciprocal plot. It should be noted that the basis of the double-reciprocal plot is the Michaelis–Menten equation derived for effective end-product concentrations of zero. Thus the derivation only applies to initial net velocities. In the present study we are almost exclusively following transport at equilibrium for hypoxanthine. Thus if influx is coupled to efflux, for example by having the same carrier, the plot has to be considered with some reservation. The reason for showing Fig. 3 is that it gives a simple method for estimating an apparent K_m for the saturable system.

Hypoxanthine inhibits uric acid transport into human red cells[5], having an apparent inhibitor Michaelis constant of 0.3 mM. This value is well compatible with the K_m of approx. 0.4 mM for hypoxanthine found in Fig. 3. It fits with the idea that the erythrocytes have a transport system common to at least uric acid and hypoxanthine. The complex fashion by which hypoxanthine inhibits uric acid efflux from preloaded cells to give a net depletion of uric acid is not immediately explained by the present data, but is subject to further investigation.

The experiments at different temperatures did not reveal differences in temperature dependence at a high and a low concentration of hypoxanthine. Referring again to a two-component mechanism, it is possible that the saturable transport behaves like an enzymatic system, *i.e.* has a strong temperature dependence. However, this may very well also be the case for the non-saturable part. As pointed out by DANIELLI[17], a strong influence of temperature might be expected in membranes with a high resistance to diffusion. Assuming a diffusion coefficient for hypoxanthine (mol.wt. 136) in the same range as glycine (mol.wt. 75) and arginine (mol.wt. 174)[18], and setting 1–2 μ as a reasonable mean path for a molecule to escape a region of the size of an erythrocyte, the mean time for this escape (free diffusion) would be of the order of a few msec. As seen from the experimental data, this means that the erythrocyte membrane slows the transport relative to free diffusion by a factor of approx. 10^4. It thus seems safe to assume that the membrane imposes a severe restriction on the transport of hypoxanthine. This in turn may explain the equal temperature dependence at the two concentrations of hypoxanthine.

The experiment in Fig. 5 was designed to disclose the possible presence of a "mobile carrier" in the WIDDAS[19] and ROSENBERG AND WILBRANDT[12,20] sense. It is

** O. STEN-KNUDSEN AND U. V. LASSEN, unpublished results.

to be noted that, due to the large initial net influx of hypoxanthine, the intracellular concentration of hypoxanthine must have risen some 6 times within the first 10 sec after the mixing of cells and suspensing medium, thus causing saturation of the transport system on both sides of the membrane. This will tend to conceal any initial acceleration of radioactive efflux occurring before the first sample was taken. For this reason it was desirable to perform experiments with a better time resolution in the first few seconds of the experiments. This could not be done, however, with the present method of sampling. Furthermore, a much higher initial extracellular concentration of hypoxanthine is desirable, but this was not possible due to the low solubility of this compound. A distinction between "stationary" and "mobile carrier systems" for hypoxanthine transport is thus not possible with the available data.

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