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PURINE TRANSPORT IN POLYMORPHONUCLEAR LEUKOCYTES

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SUMMARY

The uptake of adenine and xanthine has been studied in rabbit polymorphonuclear neutrophilic leukocytes. Since purines were found to enter neutrophils rapidly a technique was developed for taking initial rate samples as quickly as 10 sec, using cell monolayers on glass coverslips. Adenine was found to enter neutrophils by two saturable transport systems, one with a high affinity and a low maximal velocity, the other with a low affinity and a high maximal velocity. Xanthine enters predominantly by a transport carrier that is distinct from either of the adenine systems. In addition some xanthine entry occurs which cannot be overcome by a competitive inhibitor.

Within the cell adenine was metabolized to adenosine nucleotides and xanthine to uric acid. Both products accumulated within the cell. From the absence of free adenine within the cell it was concluded that at low concentrations the limiting step to adenine incorporation into nucleotides was its entrance into the cell.

INTRODUCTION

Human erythrocytes have been shown to allow equilibration of adenine and hypoxanthine, as well as several nucleosides, within a few minutes[1]. Further studies with uric acid and hypoxanthine in the erythrocyte have indicated that these compounds enter by a saturable transport system[2–4]. On the other hand, the rate of entry of adenine into Ehrlich ascites tumor cells appears to be directly proportional to concentration and has not been distinguished from unmediated diffusion[5]. Uptake of 6-mercaptopurine into a heterogenous group of human leukocytes has been ascribed to simple diffusion[6]. Bone marrow cells have been shown to require preformed purines[7,8]. The capacity for *de novo* synthesis of purines appears to be limited in marrow cells[8,9] and almost non-existent in normal or leukemic circulating leukocytes[10,11]. However, normal and leukemic leukocytes of the blood do take up intact purines[10,12–14]. Because leukocytes utilize preformed purines at a high rate they are suited to a study of purine transport. Rabbit neutrophils, which can be prepared as a homogenous group, were chosen in order to study a single cell type rather than a mixed population. Neutrophils also adhere well to glass, a property which has permitted the development of a rapid sampling technique.

The following study deals primarily with the purines adenine and xanthine. Adenine is actively incorporated into nucleotide in neutrophils while xanthine is an

intermediate breakdown product of purine metabolism. It was found that adenine enters neutrophils by two saturable entry mechanisms and that at low concentrations the entry of adenine is rate limiting to its incorporation into nucleotides. Xanthine enters mainly by a saturable transport mechanism which is distinct from either of the adenine systems.

MATERIALS AND METHODS

Chemicals

Adenosine, cytosine, orotic acid, thymine, uric acid, 2,6-diaminopurine, and 6-mercaptopurine were obtained from Mann Research Laboratories. Adenine, guanine, hypoxanthine and xanthine were bought from Pabst Laboratories as were the nucleotides AMP, ADP and ATP. The free purine base was bought from Aldrich Chemical Company and uracil from Eastman Kodak. The radiochemicals [8-^{14}C]adenine and [8-^{14}C]hypoxanthine were obtained from Schwarz Research, Inc. [8-^{14}C]Guanine for earlier studies was obtained from Nuclear Chicago and later Schwarz. Xanthine was produced from guanine by deamination with nitrous acid and purified to greater than 99% purity using chromatography on Dowex 2 resin. Eosin y and corning glass coverslips were bought from Fisher Scientific Products.

Animals

New Zealand white rabbits of either sex, weighing between 1 and 3 kg, were used for this study.

Preparation of leukocytes

Polymorphonuclear neutrophilic leukocytes were obtained from sterile peritoneal exudates by the method of KAISER AND WOOD[15]. The neutrophils were centrifuged at 200 $\times$ *g* for 5 min at 4° and the ascitic fluid discarded. The cells were then resuspended in modified Hanks solution which consists of the following: 139 mM NaCl, 20 mM K^+, 10 mM phosphate, and 5 mM glucose, adjusted to pH 7.4 $\pm$ 0.05 with NaOH. It has been found previously that modified Hanks solution will maintain neutrophils in a viable condition, as judged by their permeability to eosin y for many hours[16]. Contaminating cells, usually about 3%, were primarily macrophages. Occasionally erythrocytes were seen and preparations containing enough erythrocytes to appear pink were discarded.

Chromatography

Nucleotides were separated from purines by chromatography on the anion-exchange resin Dowex 2 (formate form) in 0.6 cm $\times$ 1 cm columns. The purines were eluted completely in 8 ml of 0.1 M formic acid, while all nucleotides were retained. These were then eluted with 2 ml of 23 M formic acid. For further identification of the purines and their metabolites, ascending paper chromatography was employed using two solvent systems: (1) isobutyric acid–ammonium hydroxide–water (66:1:33, v/v/v)[17], and (2) isobutanol–acetic acid–water (2:1:1, v/v/v)[18]. The latter system was used primarily for the separation of purine bases. The positions of the various compounds on the paper were identified by including sufficient carrier, non-radioactive purine to be detected under ultraviolet light. The amount of radioactivity in a given

area was measured using either a Nuclear Chicago Actigraph II strip counter or, for greater sensitivity, by cutting the chromatographs into sections of uniform size, placing these in vials and counting them by scintillation in toluene solution.

Cell water content

The water content of a cell pellet was determined by drying to a constant weight and correcting for extracellular fluid contamination by dilution of a ^{14}C marker. The residual material was hydrolyzed in 1 M KOH at 38° for 24 h. The solution was adjusted to a known volume and the protein measured by the method of LOWRY *et al.*[19]. From these data it was calculated that there was 5.47 μl of intracellular water per mg protein. In several experiments the protein content per million cells was determined and from these values the water was calculated to be 0.346 μl $\pm$ 0.016 μl ($\pm$ standard error) per million cells. An independent determination gave a value of 0.328 μl cell water per million cells. The latter measurement was obtained by suspending leukocytes in a known volume and subtracting the extracellular fluid as measured by dilution of [^{14}C]inulin.

Determination of purine uptake by neutrophil suspensions

Two methods were used to study purine uptake. The first employed suspensions of cells which were separated from the medium, after incubation, by centrifugation and then washed. This procedure is not suitable for taking samples at times of less than 2 min. Also one can never be sure when the uptake has stopped. For this reason a more rapid technique was develeped (see next section). However, the suspension method is suitable when longer incubations are necessary and was carried out in the following way: Neutrophils in known numbers, usually about 20 million cells per ml, were incubated in modified Hanks solution containing radioactive purines at 38.5°. At regular intervals samples were removed and cells separated from the medium by centrifugation. The cells were then washed twice in 5 ml cold modified Hanks solution. This reduced extracellular contamination to negligible levels as judged by removal of [^{3}H]inulin. The cells were then rapidly suspended in cold water and mixed vigorously for 5–10 sec. Concentrated perchloric acid was added to a final concentration of 0.1 M. The samples were allowed to stand in ice for 30 min for protein precipitation. The cell debris was removed by centrifugation, the solution neutralized with KOH to phenol red, and the potassium perchlorate which formed was allowed to precipitate. This procedure completely disrupted the cells as judged by the agreement between the amount of radioactivity removed from the incubation medium and that recovered from the cells. Samples of the supernatant fluid were then taken for liquid scintillation counting and paper chromatography.

Rapid measurement of purine uptake by neutrophils

Because of the speed of purine entry into neutrophils, a rapid sampling technique, based upon the ability of the neutrophil to adhere to glass, was developed. Cell monolayers were made by pipetting 0.5 ml of a neutrophil suspension containing 4 million cells per ml onto a round glass coverslip of 22 mm diameter. The neutrophils were allowed to adhere to the coverslips during a 30-min incubation at 38.5°. (To incubate the coverslips a watertight box was constructed of plastic sides and a brass top. The temperature of the brass plate was maintained at 38.5° by circulating

water through the box with a constant temperature pump. Coverslips were placed on strips of rolled steel 2 mm thick bolted to the brass plate with an overhanging edge, so that they could easily be gripped with forceps. To minimize evaporation a removable plastic top was fitted to the brass plate and wet filter paper placed inside to humidify the chamber). Approx. 90% of the cells adhere to the coverslip, as determined by protein analysis, during this time forming a monolayer occupying about 50% of the available glass surface. No clumping of the cells was observed and the monolayers could be rinsed vigorously without detaching cells. The coverslips were then drained and about 350 μl of modified Hanks solution containing radioactive purines, prewarmed to 38.5°, were placed over the monolayer. Then the coverslips were replaced on the incubation table. After incubation for various periods the coverslips were drained and passed through four beakers containing cold modified Hanks solution. This washing procedure removes all detectable extracellular contamination. The coverslips were broken and placed into counting vials and the cells were hydrolyzed in 0.5 M KOH for 30–60 min at 38.5° and neutralized to phenol red with perchloric acid. (This step is necessary to remove the radioactive label from the coverslip and dissolve it in the scintillation fluid, to obviate self absorption). The vials were filled with Brays solution and counted by liquid scintillation.

The method has proved easy to use and very reproducible. In practice samples were taken in quintuplicate and averaged. Individual monolayers usually fell within 10% of the mean. It is possible to take sample times as short as 10 sec using this technique.

Controls for rapid sampling technique

Before using the rapid sampling technique described in MATERIALS AND METHODS it was necessary to examine two potential sources of error: first, the possibility that diffusion of the purine in the medium to the underlying monolayer might be limiting and second, the possible loss of purines from the cell during rinsing of the adhered neutrophils. Neither of these was found to be a source of significant error.

The largest volume of medium cleared by neutrophil monolayers in any experiment was a little less than 1 μl in 45 sec incubation. This would represent a layer about 2.6 μ deep. The time required for small molecules to diffuse into this layer can be estimated[20]. Using constants measured for glucose at 25° (ref. 21) the time necessary for this zone to come within 95% of equilibrium is approx. 1 sec, a small portion of the reaction time. This estimate represents an upper limit, because of the lower temperature assumed, the larger size of the glucose molecule compared to that of most purines, and to the fact that some mechanical mixing probably occurs.

To examine the possibility that there was inadequate mixing of the medium in contact with the monolayer, uptake was measured in coverslips immersed in a large volume of fluid, which was mechanically stirred. No difference was found between the amounts of purines taken up by these monolayers as compared to those described above.

The possible loss of intracellular label due to diffusion during the rinsing phase was examined since the coverslips had to be rinsed well with cold modified Hanks solution to remove all traces of the incubation medium. This procedure lasted about 20–25 sec, and was carried out by passing the coverslips through 4 20-ml beakers containing cold modified Hanks solution (4°). To determine if significant loss of

intracellular label occurs during the rinsing procedure 5 mM adenine was incubated over neutrophils for 25 min to allow equilibration of the purine between the cells and the medium. (At this concentration more than 88% of the adenine taken up by the cell exists in the free form, which readily effluxes from the cell at 38.5° as shown in Fig. 4.) The coverslips were then leeched in cold modified Hanks solution for periods

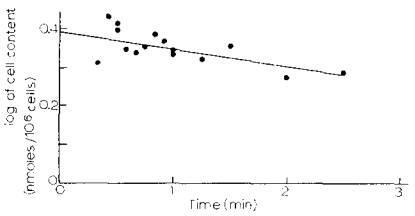

Fig. 1. Adenine release at 4°. Neutrophil monolayers were incubated with 5 mM [^{14}C]adenine in modified Hanks solution for 25 min, then leached in 200 ml of cold modified Hanks solution (4°) for various periods and the radioactivity remaining in the cells counted as described in MATERIALS AND METHODS.

up to 150 sec (Fig. 1). The loss of label from the cells follows first-order kinetics. The portion of adenine lost during the time required for rinsing was 3–4% which may be neglected. A similar experiment utilizing 0.035 mM xanthine showed no detectable loss of label.

RESULTS

Purine uptake by neutrophils

The purines adenine, guanine and hypoxanthine at relatively low concentrations were found to be actively taken up by rabbit neutrophils; xanthine, however, was not (Table I). In these experiments the amount of radioactivity removed from the medium closely approximated that recovered from the cells in the acid-soluble fraction indicating that relatively little, if any, synthesis of nucleic acids occurred.

After 2 h incubation in [^{14}C]adenine the final concentration of label inside the cell, based on 0.346 μl cell water per million cells, is 1.1 mM. This represents a ratio

TABLE I

PURINE UPTAKE BY NEUTROPHILS

Neutrophil suspension, 12.2–19.8 million cells per ml, were incubated in modified Hanks solution containing radioactive purines at the following concentrations: 0.02 mM adenine, 0.016 mM guanine, 0.0285 mM hypoxanthine and 0.01 mM xanthine. At the times listed below, aliquots were removed and the cells extracted, see MATERIALS AND METHODS. Except for xanthine, the uptake figures are the averages of values calculated from cell recovery and medium disappearance of radioactivity. Xanthine values were obtained from cell recovery only since the medium concentration did not change measurably during the experiment. The values given are expressed as pmoles/10^6 cells.

Time (min)	*Adenine*	*Guanine*	*Hypoxanthine*	*Xanthine*
30	92	143	80	6.7
60	209	230	126	7.5
90	313	317	161	8.6
120	386	390	188	—

(intracellular to extracellular concentration of radioactivity) of about 92. More than 94% of the intracellular material was present as nucleotide, as determined by column chromatography on Dowex 2 (see MATERIALS AND METHODS). To examine more closely the intracellular fate of adenine, an experiment was designed using adenine of a higher specific activity and incubation for shorter times. Cell extracts were made after 5, 15, 25 and 35 min, chromatographed with Solvents I and II and the intracellular radioactive compounds were identified (see MATERIALS AND METHODS). In both solvents the predominant amount of radioactivity corresponded to the nucleotide fractions. The relative amounts of ATP, ADP, AMP, adenosine and adenine were determined from chromatograms run in Solvent I (Table II). It is apparent that the free adenine content of the neutrophil is extremely low. Even at 5 min adenine accounts for only about 1% of the total radioactivity, corresponding to a concentration of about 0.0015 mM as compared to the extracellular concentration of approx. 0.02 mM. From this we conclude that adenine added at low concentrations is phosphorylated as rapidly as it enters the cell.

Of the naturally occurring purines tested, only xanthine was not incorporated into nucleotides by neutrophils. At the end of 90 min the concentration of cellular label was 2.48 times the extracellular concentration (Table I). The cell contents were passed over the anion-exchange resin Dowex 2 as described in MATERIALS AND METHODS. All the radioactivity passed through the column with the purine fraction, and none was present in the nucleotide portion. It was shown, however, that xanthine is oxidized to uric acid. After incubation of neutrophils with 0.096 mM xanthine for 60 min at 38.5°, cell extracts were made (see MATERIALS AND METHODS) and both the extracts and medium were chromatographed in Solvents I and II. The extent of conversion to uric acid was found to be 155 pmoles per million cells per h. No degradation of urate to allantoin was detected by paper chromatography.

Of note was the fact that after 60 min, 42% of the uric acid remained inside the cell, yielding a concentration of 0.112 mM, compared to an extracellular concentration of 0.00175 mM. This represents a concentration ratio, cell to medium, of 64 compared

TABLE II

ADENINE UPTAKE AND CONVERSION TO ADENOSINE PHOSPHATES

Neutrophils (17.8 million cells per ml) were suspended in modified Hanks solution containing 0.0203 mM [^{14}C]adenine. After 5, 15, 25, and 35 min of incubation, aliquots were removed and cells extracted, see MATERIALS AND METHODS. The cellular contents were identified and the relative amounts of radioactive material estimated after separation by paper with solvent system I. The uptake values given were determined from cell recovery of radioactivity and the concentration calculated for cell water of 0.346 μl per million cells. ND, not detectable; —, not determined.

Time (min)		0	5	15	25	35
Adenine concentration in medium (mM)		0.0203	—	—	—	0.0162
Cellular concentration of radioactivity (mM)		0	0.176	0.329	0.445	0.527
Percent radiolabel as	ATP	0	87	—	86	88
	ADP	0	8	—	10	10
	AMP	0	2	—	2.4	2.5
	Adenosine	0	2	0	0.9	ND
	Adenine	0	1	—	0.9	ND

to a ratio of 0.78 for xanthine. Thus it appears that uric acid formed inside the cell does not readily efflux.

Adenine entry at low concentrations

The uptake of 0.01 mM adenine by neutrophil monolayers shows a linear rate during the first 16 min of incubation (Fig. 2). As expected the intracellular concentration of free adenine was very low under these conditions. This is shown by the failure of a high concentration of unlabelled adenine to trap significant quantities of label from cells preincubated with a low concentration of [^{14}C]adenine (Fig. 3). In contrast cells preincubated with high adenine concentrations lose at least 90% of the free label during this time (Fig. 4). This is additional evidence that very little free adenine exists inside the neutrophil when exposed to low concentrations and the entrance of adenine into the cell is rate-limiting to its incorporation to nucleotide under these conditions.

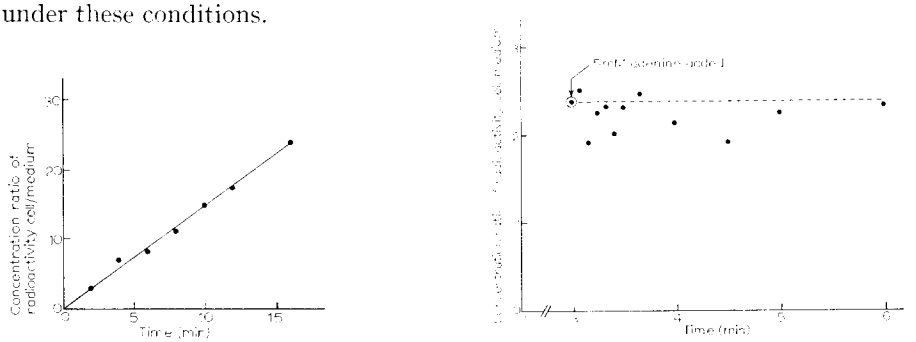

Fig. 2. Uptake of adenine at 0.01 mM. Neutrophil monolayers were incubated with 0.01 mM [^{14}C]adenine for the times indicated and the radiolabel of the cells measured as described in MATERIALS AND METHODS.

Fig. 3. Chase of radioactivity from cells preincubated in 0.032 mM [^{14}C]adenine. Neutrophils were incubated with 0.032 mM [^{14}C]adenine for 3 min, then the medium was removed and the monolayers leeched in 5 mM non-radioactive adenine at 38.5° for the indicated periods. The cellular radioactivity was determined as described in MATERIALS AND METHODS. The circled point represents the concentration ratio before the addition of unlabelled adenine and is an average of 8 determinations. The dotted line is a reference to the original concentration ratio.

Adenine entry at high concentration

As mM concentrations are reached the capacity of the neutrophil to convert adenine to nucleotide is exceeded and free adenine exists inside the cell. The time course of adenine entry into cells at a medium concentration of 2 mM (Fig. 4) shows a rapid initial uptake, followed after 5–7 min, by a slower more linear rate. The second phase is probably due to the continuing incorporation of adenine into nucleotides while the first is the equilibration of free adenine. In order to determine what portion of the cellular radioactivity was free after 25 min, the incubation medium was drained and the cells covered with purine-free modified Hanks solution. Within 5 min 71% of the intracellular radioactivity was lost. The radioactive effusion was identified as adenine by paper chromatography, as described in MATERIALS AND METHODS.

Kinetics of adenine uptake

Initial rates of adenine influx were measured using the rapid sampling technique (see MATERIALS AND METHODS). An incubation time of 45 sec was chosen since during

this period uptake was a linear function for all concentrations tested. Although at low concentrations the intracellular concentration of label rapidly rises to relatively high levels (Fig. 2) the conversion of adenine to nucleotides prevents backflux (Table II, Fig. 3). The kinetics of adenine entry was observed over a concentration range from 0.0125 mM to 73 mM expressed in a double reciprocal plot (Fig. 5). This curve is interpreted as two additive entry mechanisms, one operating more effectively at low concentrations with a K_m of 0.007 mM and a $V_{max.}$ of 5.7 pmoles per million cells per 45 sec, the other system predominant at high concentrations, with a K_m of 100 mM and a $V_{max.}$ of 13700 pmoles per million cells per 45 sec. To display the kinetics of the second system it is necessary to look at concentrations of adenine greater than 5 mM (Fig. 6). Thus the uptake of adenine at relatively high concentrations follows typical saturation kinetics.

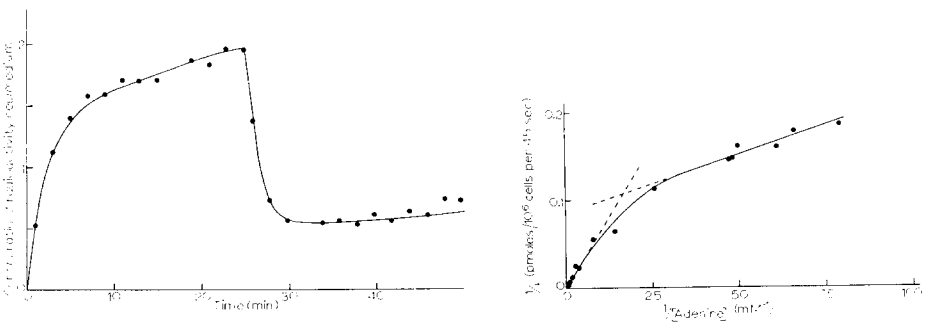

Fig. 4. Efflux of radioactivity from cells preincubated in a high concentration of [^{14}C]adenine. Neutrophil monolayers were incubated in the presence of 2 mM [^{14}C]adenine. After 25 min the medium was replaced with purine-free modified Hanks solution. At various times the cellular radioactivity was measured (see MATERIALS AND METHODS).

Fig. 5. Kinetics of adenine uptake. Cell monolayers were incubated in the presence of [^{14}C]adenine in concentrations from 0.0125 to 73 mM for 45 sec. The monolayers were rinsed and counted as described in MATERIALS AND METHODS. All points plotted were averages of five determinations. The straight portions of the line were drawn through the experimental points with the aid of the method of least squares, while the curving portion was fitted by eye. All solutions over 10 mM were adjusted to isotonicity by omission of an appropriate amount of NaCl.

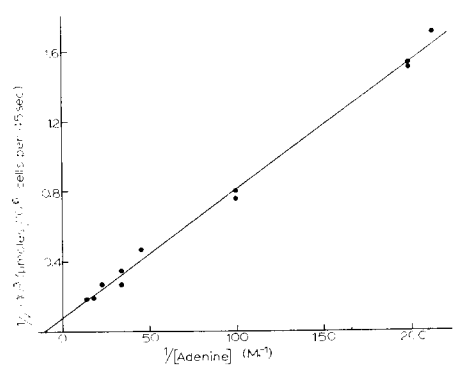

Fig. 6. Kinetics of adenine uptake at high concentrations. This figure is an expansion of Fig. 5 at concentrations between 5 and 73 mM. Since more than 99% of adenine uptake is mediated by the high concentration transport system in this concentration range, the kinetic constants can be derived directly from the experimental values.

(The kinetic constants were determined in the following way: a preliminary estimate of the $V_{max.}$ for the low K_m carrier was made directly from Fig. 5 by extrapolating the straight line at low concentrations. From this value it was determined that at concentrations above 5 mM the contribution to total entry of the low K_m system was less than 2%. Therefore the kinetics for the high K_m carrier could be taken directly from data accumulated using concentrations greater than 5 mM adenine (Fig. 6). Once the kinetic constants were known for the high K_m system it was possible to go back and subtract its contribution from the data gathered at low concentrations. From the resultant data the constants were derived.)

In experiments where adenine was above 10 mM, isotonicity was adjusted to 310 mosmoles/l by omission of an appropriate amount of NaCl. In order to test the possibility that NaCl influenced adenine entry NaCl was completely replaced by sucrose and uptake examined using 5 mM adenine. Entry in the absence of NaCl was identical to entry in its presence.

Inhibition of the low K_m system for adenine

Inhibition of adenine uptake by neutrophil monolayers was examined by incubating the test compound in the presence of a low concentration of adenine and comparing the rate of entry to a control without inhibitor. It was recognized that adenine was entering by both the high and low K_m transport systems and that the high K_m system was unaffected by the inhibitors tested in the concentrations used (see below). In order to obtain the net effect of the inhibitor on the low K_m system, an estimate of the rate of adenine entrance *via* the high K_m system was made on the basis of the previously measured kinetic constants and substracted from both the control and test samples before comparison. The validity of this computation was examined by overwhelming the low K_m system with 100 mM purine which has no significant effect upon the high K_m system. The entry rates obtained in the presence of 100 mM purine agreed well with those predicted from the kinetic constants for the high K_m system. The mechanism of inhibition shown by adenine analogues (Tabe III)

TABLE III

INHIBITION OF THE LOW CONCENTRATION SYSTEM FOR ADENINE TRANSPORT

Neutrophil monolayers were incubated with adenine at various concentrations in the presence of the inhibitor candidate. The amount of inhibition was determined by comparison with a control lacking inhibitor. The uptake of adenine was measured as described in Fig. 1. A correction for the effect of entrance *via* the uninhibited high concentration system was made as described in the text. Each value represents the average of 3–5 determinations. −, inhibition; +, stimulation; *, statistically insignificant at the 5% level.

Compound	*Concentration of inhibitor (mM)*	*Concentration of adenine (mM)*	*Inhibition or stimulation (%)*
Hypoxanthine	1.0	0.0097	−42
Xanthine	5.0	0.021	−41
Purine	1.0	0.0097	−23
Guanine	0.125	0.021	−19
Adenosine	1.0	0.0097	−5*
Uric acid	5.0	0.021	0
Cytosine	1.0	0.0097	+5*
Adenosine monophosphate	1.0	0.0097	+8*

was not examined. Both guanine and purine are good inhibitors of AMP pyrophosphorylase (unpublished observations) at 1 mM concentrations and it is possible that at low adenine concentrations, where entry is rapid, guanine and purine inhibited AMP pyrophosphorylase, thus decreasing the trapping of adenine in the form of the nucleotide. This could result in an increase in the efflux of label from the cell. Such an effect would appear as a depression in the rate of adenine entry. However, a different mechanism is necessary for those compounds which do not inhibit nucleotide formation but do inhibit adenine uptake (hypoxanthine and xanthine).

Inhibition of the high K_m system of adenine transport

The following compounds were tested for their ability to inhibit the high K_m system: 30 mM hypoxanthine, 100 mM purine, 30 mM adenosine, 30 mM AMP, and 2 mg/ml RNA (yeast). This was done by incubating the possible inhibitor in the presence of 5 mM adenine and comparing the rate of entry to that of a control without inhibitor. No correction for entry due to the low K_m system is necessary at this level since more than 99% of adenine entry is mediated by the system in question. The inhibitions achieved were minor and in no case statistically significant at the 5% level.

Temperature coefficient of the high K_m system for adenine transport

To further characterize the high K_m system for adenine the temperature coefficient of the initial rate of entry was measured at a concentration of 5 mM between 24.2° and 38.5°. The values obtained were adjusted to a 10° difference by the equation $\log Q_{10} = 10/(T_2 - T_1) \cdot \log (k_2/k_1)$. A Q_{10} of 2.26 was obtained in this manner, which is approximately equal to the value of 2.21 found for the saturable uric acid transport system in the erythrocyte[2] and is compatible with a carrier mediated process.

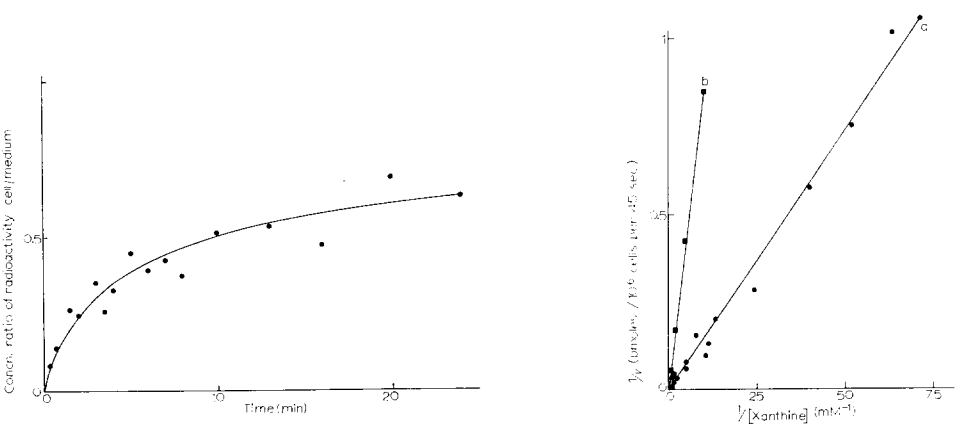

Fig. 7. Xanthine uptake by neutrophils. Neutrophil monolayers were incubated with 1 mM [^{14}C]xanthine. At the times indicated the monolayers were rinsed and counted as described in MATERIALS AND METHODS.

Fig. 8. Kinetics of xanthine uptake. Cell monolayers were incubated in the presence of [^{14}C]xanthine in concentrations ranging from 0.0132 to 5 mM, for 45 sec (Curve a). Curve b contains in addition to [^{14}C]xanthine, 2.5 mM non-radioactive adenine. The monolayers were rinsed and counted as described in MATERIALS AND METHODS. Each point is the average of five determinations and lines were drawn with the aid of the method of least squares.

Xanthine uptake

The transport of xanthine into neutrophils was studied and found to be slower than adenine (Fig. 7). After 25 min incubation in 1 mM xanthine the intracellular concentration appears to be close to a steady state at about 0.62 mM. It is difficult to derive a theoretical distribution ratio for comparison since both the intracellular pH and electrochemical potential are unknown but affect the distribution of xanthine which has a pK of 7.44 (ref. 22). In addition xanthine is slowly oxidized to uric acid which accumulates within the cell.

Kinetics of xanthine uptake

The entrance of xanthine into neutrophils was measured after 45 sec when uptake is linear with time. Xanthine entry at concentrations ranging from 0.0132 mM to 5 mM can be described as a simple system obeying saturation kinetics (Fig. 8). There is no obvious deviation from linearity suggestive of two systems such as that seen for adenine uptake. However, it was considered that some xanthine entry may occur by a second route. To test this possibility, xanthine entry was examined in the presence of an overwhelming concentration of a competitive inhibitor, either 2.5 mM adenine or 100 mM purine (Fig. 8). Both compounds at these high concentrations

TABLE IV

INHIBITION OF XANTHINE TRANSPORT

Inhibitors were incubated for 45 sec over neutrophil monolayers as described in MATERIALS AND METHODS, with 0.023 to 0.026 mM [^{14}C]xanthine, concentrations that are well below the K_m. The rate of entry in the presence of the test compound was compared to a control containing only xanthine. The values reported are the averages of five determinations except that for cytosine which is a single observation. No correction was made for the non-inhibitable route of entry.

Inhibitor	*Concentration of inhibitor (mM)*	*Inhibition (%)*
Adenine	0.02	44
	0.25	62
	1.0	82
	2.5	82
Allopurinol	1.0	48
Guanine	0.25	40
	0.5	67
Hypoxanthine	0.25	40
	1.0	74
Purine	1.0	68
	100.0	81
Uric acid	1.0	48
2,6-Diamino purine	1.0	84
6-Mercaptopurine	1.0	84
8-Aza-adenine	1.0	66
Adenosine	1.0	65
Adenosine monophosphate	1.0	42
Adenosine diphosphate	1.0	38
Adenosine triphosphate	1.0	35
Cytosine	1.0	27
Orotic acid	1.0	28
Thymine	1.0	23
Uracil	1.0	36
4-Hydroxypyrimidine	1.0	39

should inhibit xanthine uptake by more than 99% as judged by inhibition data (see below). It can be seen that 2.5 mM adenine did not inhibit xanthine uptake completely, nor did 100 mM purine which showed identical effects. The inhibitor-insensitive route accounts for about 19% of the entry at very low xanthine concentrations and about 42% at 5 mM xanthine. A K_m of 2.3 and a $V_{max.}$ of 128 pmoles per million cells per 45 sec were determined for the xanthine transport mechanism after correction of the data for the non-inhibitable portion.

Thus xanthine entry occurs predominantly by a saturable transport system although some entry appears to occur *via* another mechanism.

Inhibition of xanthine transport

Inhibition of xanthine entry was examined with the rapid sampling technique by incubating the potential inhibitor with a concentration of xanthine well below the K_m and comparing the rate of entry to a control containing no inhibitor (Table IV). In contrast to adenine at low concentrations, measurement of the initial rate of xanthine uptake does not depend upon its intracellular metabolism to a non-diffusable form since entry is much slower and the final intracellular concentration is less than 20% of the extracellular. Thus the effects, if any, of the various compounds upon xanthine oxidation (the only metabolism of xanthine which occurs) need not be considered and the inhibitions observed must occur upon the entry mechanism.

The kinetics of adenine inhibition of xanthine entry were examined and expressed in a double reciprocal plot (Fig. 9). The results are explicable by competitive inhibition as would be anticipated from the chemical similarity of these purines. The extrapolated intercepts deviate in the direction of the origin since in the presence of adenine the non-inhibitable mechanism accounts for a greater portion of entry. Thus the $V_{max.}$ is shifted closer to that of the non-inhibitable component which presumably has a high or infinite $V_{max.}$

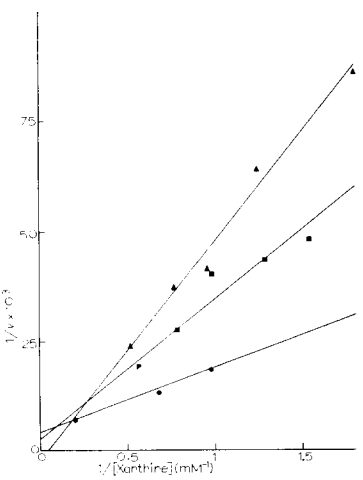

Fig. 9. Inhibition of xanthine entry by adenine. Cell monolayers were incubated in the presence of [^{14}C]xanthine and non-radioactive adenine in concentrations of 0.02 mM (■—■) and 0.15 mM (▲—▲). Curve ●—● describes xanthine entry in the absence of adenine and was obtained from Fig. 8. The monolayers were rinsed and counted as described in MATERIALS AND METHODS. Each point is the average of five determinations and lines were drawn with the aid of the method of least squares.

DISCUSSION

Entry of adenine at high concentrations is consistent with mediation by a transport system having a relatively high K_m and V_{max}. A Q_{10} of 2.26 is also consonant with this concept. The possibility of a third component, unmediated diffusion, is difficult to examine because of the absence of an effective inhibitor of the high K_m system and because adenine is insoluble at concentrations greater than the K_m. The absence of good inhibitors for the high K_m system can best be explained on the basis of a poor affinity of the transport mediator for the compounds tested. Purine secretion in the hamster intestine, an active process, similarly takes place by a mechanism having a high K_m and V_{max}. (ref. 23). Inhibition of this system also could not be shown.

The contribution to adenine entry into neutrophils mediated by the high K_m system becomes of less importance at low concentrations. Physiological concentrations of adenine have thus far escaped detection in contrast to hypoxanthine which exists in concentrations ranging from 0.003 mM to 0.02 mM (refs. 24, 25). The low K_m system is much better suited to facilitate entry of adenine in and below this range. It has been shown that when added at low concentrations almost all the adenine that enters these cells is converted to adenosine nucleotides, predominantly ATP, the limiting step to adenine incorporation occurring at the membrane.

Xanthine enters neutrophils predominantly by a saturable carrier that appears to be distinct from either of the adenine transport systems. The evidence for this is that the maximal rate of xanthine entry is 22 times that of the adenine low K_m system and 1/100th that of the high K_m system. Also xanthine entry could be inhibited significantly by adenosine, adenine nucleotides, uric acid and pyrimidines, while these compounds had no effect on either of the adenine mechanisms of entry. These data taken as a whole strongly indicate that adenine and xanthine enter neutrophils by separate transport systems. Interestingly, although adenine appears to enter *via* another mechanism it is a very potent inhibitor of xanthine entry.

It was found impossible to block more than 82% of xanthine entry using overwhelming amounts of either purine or adenine. This suggests that xanthine also enters by two mechanisms. It is possible that some xanthine entry is mediated by the high K_m system for adenine but that xanthine has a much poorer affinity than adenine for this carrier.

The best inhibitors of xanthine transport were found to be purines. The presence of an amino group in the 2 or 6 positions enhances inhibition while alterations or additions to the imidazole portions, such as those occurring in 8-aza-adenine, allopurinol or adenosine, significantly reduce the inhibitory effect. It is of interest that compounds which do not readily enter the cell, such as the nucleotides and possibly uric acid, also inhibit xanthine entry markedly.

It is difficult to assign a physiological role for the adenine high K_m system since entry *via* this mechanism does not predominate until unphysiologically high concentrations are reached. Xanthine is not incorporated into nucleotides in neutrophils, but is oxidized to uric acid to which these cells are relatively impermeable. This again raises a difficulty in establishing a function for the xanthine transport system *in vivo*. However, the significance of these transport systems may derive from the fact that neutrophils are phagocytic cells which ingest and digest large amounts of organic

material, including nucleic acids. Since the cells probably do not incorporate such large quantities of material, it would be advantageous to allow the products of digestion to leak from the cell before they reach possibly toxic or osmotically injurious levels. In essence, this would act as a safety mechanism. If this is true, one might expect to find transport systems for other digestive products that allow efflux at relatively high concentrations.

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