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Purine and pyrimidine transport and permeation in human erythrocytes

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Time courses of the uptake of radiolabeled hypoxanthine, adenine and uracil were measured by rapid kinetic techniques over substrate ranges from 0.02 to 5000 μM in suspensions of human erythrocytes at 25 or 30 °C. At concentrations above 25 μM, the rate of intracellular phosphoribosylation of hypoxanthine and adenine was insignificant relative to their rates of entry into the cell and time courses of transmembrane equilibration of the substrates could be measured and analyzed by integrated rate analysis. Hypoxanthine and uracil are transported by simple facilitated carriers with directional symmetry, high capacity and Michaelis-Menten constants of about 0.2 and 5 mM, respectively. Adenine is probably transported by a carrier with similar properties but no saturability was detectable up to a concentration of 5 mM. Cytosine entered the cells much more slowly than the other three nucleobases, and its entry seems not to be mediated by a carrier. The hypoxanthine transporter resembles that of one group of mammalian cell lines, which does not exhibit any overlap with the nucleoside transporter and is resistant to inhibitors of nucleoside transport. Results from studies on the effects of the nucleobases on the influx and countertransport of each other were complex and did not allow unequivocal conclusions as to the number of independent carriers involved. At concentrations below 5 μM, radiolabel from adenine and hypoxanthine accumulated intracellularly to higher than equilibrium levels. Part of this accumulation reflected metabolic trapping, especially when the medium contained 50 mM phosphate. But part was due to an apparent concentrative accumulation of free adenine and hypoxanthine up to 3-fold at medium concentrations $\ll 1$ μM and when cells were incubated in phosphate-free medium. This concentrative accumulation could be due to the functioning of additional high-affinity, low-capacity, active transport systems for adenine and hypoxanthine, but other factors could be responsible, such as saturable binding to intracellular components.

Introduction

Mammalian cells almost universally possess a single non-concentrative nucleoside transport system that exhibits very broad nucleoside specificity and similar kinetic properties [1–3], but which may differ in sensitivity to inhibition by nitrobenzylthioinosine and other transport inhibitors in different types of cells [4–6]. Nucleoside transport is well described by the simple carrier model [1,7] and exhibits directional symmetry [1,8,9]. However, whereas the mobility of the carrier of various cell lines is the same whether or not loaded with any nucleoside, the carrier of human and pig erythrocytes moves considerably faster when uridine-loaded than when empty [8–10].

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The nucleoside carrier fails to transport nucleobases, with the possible exception of hypoxanthine, which may be transported by the same carrier as nucleosides in some, but not other, cell lines [1,11–13]. Most cultured cell lines investigated express non-concentrative, low-affinity transportsystems that are specific for hypoxanthine (K_m = 0.3 to 3 mM), uracil (K_m = 5–15 mM) and adenine (K_m = 2–5 mM), but lack a transporter for cytosine [11–16]. In addition high-affinity uptake systems (K_m < 50 μM) have been described for adenine and hypoxanthine in various types of cells [17–20] (see also Ref. 1), but it seems that most of the observed saturation of uptake was mediated at the level of intracellular phosphoribosylation rather than at a transport step [1,11–13] (see Discussion). Transport of hypoxanthine, uracil and adenine in cell cultures is also well described by the simple carrier model. The transporters exhibit directional symmetry and equal mobility when empty and substrate-loaded [12–15]. Transport of these nucleobases is probably mediated by distinct carriers, but their exact numbers and relationship to each other have not been elucidated. To gain further information on this point we turned to human erythrocytes, which have been used widely in the study of nucleoside transport, but whose nucleobase transport has previously not been examined in such detail. This information is also of interest, because these cells, in view of their relatively simple membrane protein composition, have been the system of choice for the isolation of the sugar [21–23] as well as the nucleoside transporter [24,25]. Both have been identified as band 4.5 proteins and there is the likelihood that non-concentrative transporters for other substrates may have similar molecular properties as the sugar and nucleoside carriers and may be present in the same membrane protein fraction.

Experimental procedures

Human erythrocytes. Erythrocytes from freshly drawn blood were kindly supplied by Dr. J. Kersey (University of Minnesota) as a byproduct of lymphocyte isolation. The cells were thrice washed in cold saline containing 5 mM Tris-HCl, pH 7.4 (Tris-saline) and suspended in Tris-saline to (6–8) $\cdot 10^8$ cells/ml as enumerated with a Coulter counter.

Measurement of uptake of hypoxanthine and adenine at low concentrations. 'Uptake' denotes the accumulation of radioactivity, derived from exogenous, labeled substrate, within the cell, regardless of its metabolism [1,26]. Time courses of uptake of radiolabeled hypoxanthine and adenine in the 0.02 to 50 μM range were measured at 30 °C by rapid kinetic techniques (15 time points per time course) as described previously [1,12,27]. The method consists of mixing cell suspension with a solution of radiolabeled substrate in short intervals with a dual syringe apparatus (5 ml and 1 ml syringes) in a ratio of 7.3 : 1. The cells are separated from the medium by centrifugation through oil and analyzed for radioactivity (radioactivity in total cell material). Radioactivity per cell pellet was corrected for that trapped in the extracellular space of cell pellets, which was estimated by the use of [^{14}C]inulin [27] in each experiment. Corrected radioactivity values were converted, where indicated, to pmol/μl cell water on the basis of the intracellular water space determined in each experiment by the use of ^3H_2O [27]. For the purpose of fractionating the acid-soluble pool of cells, the cells from duplicate samples of cells were centrifuged through an oil layer directly into a solution composed of sucrose and trichloroacetic acid, which ensures rapid quenching of metabolism [28]. The acid layer was further processed and analyzed by ascending paper chromatography with a solvent composed of 3 volumes of 1 M ammonium acetate, pH 5, and 7 volumes of 95% ethanol (solvent 28) as described previously [29].

Measurements of nucleobase transport. 'Transport' denotes solely the transfer of unmodified substrate across the membrane as mediated by a saturable, selective carrier [1]. Transport of adenine and hypoxanthine was measured at concentrations above 20 μM because at these concentrations the conversion of radiolabeled purines to intracellular nucleotides during 1–2 min of incubation was insignificant in relation to the amount of purine accumulated by the cells and the transmembrane accumulation of the purines in unmodified form could be measured (see later). Zero-*trans* accumulation and inward equilibrium exchanges of the

purines and of uracil was measured at 25 °C by the rapid kinetic techniques used in uptake measurements, except that the higher substrate concentrations were employed. For equilibrium exchange measurements, the cells were preincubated with appropriate concentrations of unlabeled purines at 37 °C for 1 h. Data were evaluated by fitting appropriate integrated rate equations based on the simple carrier model to the time courses of transmembrane equilibration of the radiolabeled substrates [1]. For estimating the Michaelis-Menten parameters for hypoxanthine and uracil transport, 6 or 7 substrate concentrations were employed and the kinetic parameters extracted by least-squares regression analyses of the pooled data. In the case of zero-*trans* influx, the integrated rate analysis assumed directional symmetry of the carrier and equal mobility when empty or substrate-loaded (see later). The slopes of these fits at $t = 0$ were taken as initial velocities [1]. For estimating initial transport velocities from single time courses of transmembrane equilibration of the substrate, the integrated rate equation was fitted with the Michaelis-Menten constant (K) fixed at 200 μM for hypoxanthine and 5 mM for uracil (see later). In the case of adenine, a first order rate equation, $S_{2,t} = S_1(1 - e^{-kt})$, was fitted to the time courses of transmembrane equilibration, where k = first-order rate constant and S_1 and S_2 = extracellular and intracellular substrate concentrations, respectively. This equation adequately describes the zero-*trans* transmembrane equilibration of a substrate at concentrations well below the Michaelis-Menten constant for transport [1,27].

For measuring the efflux of radiolabeled adenine, samples of suspension of about $5 \cdot 10^9$ cells/ml were equilibrated with specified concentrations of radiolabeled adenine. Then, the suspension of preloaded cells was mixed at timed intervals in a ratio of 1 : 7.3 (opposite to that in influx measurements) with Tris-saline (exit) or with Tris-saline containing unlabeled adenine at the same concentration as labeled adenine used in preloading the cells (outward equilibrium exchange). Exit measurements with this protocol are not strictly zero-*trans,* since the extracellular concentration of substrate at zero time is not nil, but rather 12% of that inside the cells. Exit velocities were estimated by fits of a first-order rate equation, $S_{2,t} = (S_{2,0} - S_{2,\infty})\ e^{-kt} + S_{2,\infty}$, to the data, where $S_{2,0}$ and $S_{2,\infty}$ are the intracellular concentrations of substrate at 0 time and at infinity, respectively.

Measurement of uptake of radiolabeled cytosine, L*-glucose and 8-azaguanine.* Suspensions of red cells were supplemented with radiolabeled substrate and sampled manually at appropriate time intervals of incubation. The cells were collected by centrifugation through oil and analyzed for radioactivity as described for transport assays. Initial velocities of uptake were estimated by fitting a first-order rate equation to the time courses of substrate transmembrane equilibration as already described for adenine influx.

Materials. [2-^{3}H]Hypoxanthine, [8-^{14}C]hypoxanthine, [2-^{3}H]adenine, [8-^{14}C]adenine, [5-^{3}H]uracil and [5-^{3}H]cytosine were purchased from Moravek Biochemicals (Brea, CA) and L-[^{3}H]glucose from New England Nuclear (Boston, MA). Unlabeled nucleosides, purines, pyrimidines and L-glucose were obtained from Sigma Chemical Company (St. Louis, MO). Dipyridamole and dilazep were gifts from Geigy Pharmaceuticals (Yonkers, NY) and Asta Werke (Frankfurt, F.R.G.), respectively.

Results

Hypoxanthine uptake

As reported by other investigators [30,31] we have previously found that the phosphoribosylation of hypoxanthine and adenine by human erythrocytes is very inefficient when incubated in media without phosphate or containing a physiological concentration of phosphate (1 mM), but that it is strongly stimulated by phosphate concentrations in the 5 to 80 mM range [32]. In the present study, we have used rapid kinetic techniques to determine detailed initial time courses of uptake by these cells of radiolabeled hypoxanthine in the 0.02 to 50 μM range in the absence (Fig. 1A and presence of 50 mM phosphate (Fig. 1B). The concentration of [^{3}H]hypoxanthine was kept constant while its specific radioactivity was decreased by addition of unlabeled hypoxanthine. The acid-soluble pools were extracted after 80 s of incubation and analyzed chromatographically for the proportion of radioactivity in IMP and hypoxanthine (Table I). At low concentrations of

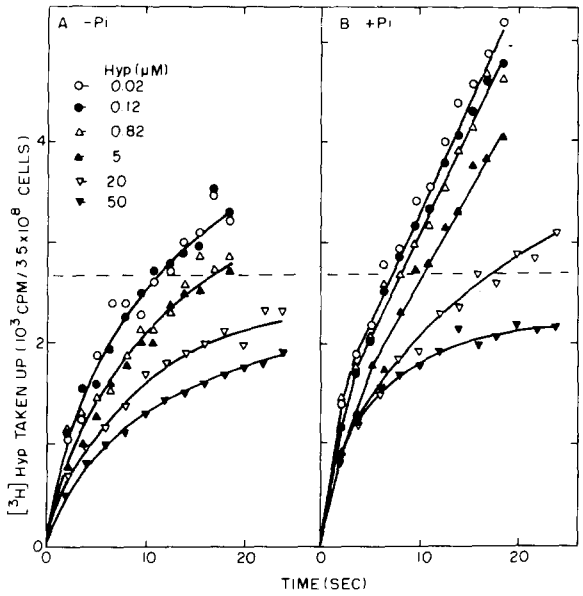

Fig. 1. Time courses of uptake of various concentrations of hypoxanthine by human erythrocytes at 30 °C in the absence (A) and presence of 50 mM phosphate (B). The uptake of the indicated concentrations of [^{3}H]hypoxanthine (106 cpm/μl, irrespective of concentration) by samples of a suspension of $7 \cdot 10^8$ red cells/ml of Tris-saline was measured by rapid kinetic techniques as described under Experimental procedures. The portion of the suspension used in (B) was supplemented with 50 mM phosphate (P_i) 10 min prior to the beginning of uptake measurements. The acid-soluble pools were extracted from samples of cells at 80 s of incubation and chromatographed with solvent 28 (see Table I). The broken lines indicate the intracellular concentration of radioactivity equivalent to that in the medium.

[^{3}H]hypoxanthine (< 5 μM), radioactivity was taken up rapidly and accumulated above equilibrium levels, but in the absence of phosphate, uptake slowed down within 5–10 s (Fig. 1A). In cells incubated in the presence of phosphate, considerable amounts of radiolabeled IMP accumulated, while the intracellular concentration of unmodified hypoxanthine at 80 s of incubation was $< 50\%$ of that in the medium (Table I). As expected (see Ref. 33), IMP was the only nucleotide formed. The amounts of radioactivity accumulated by the cells (Fig. 1B) and the proportion converted to IMP (Table I) decreased progressively with increase in absolute hypoxanthine concentration, obviously due to saturation of the hypoxanthine phosphoribosyltransferase ($K_m \approx 10$ μM, Ref. 26). At 20 or 50 μM [^{3}H]hypoxanthine,

TABLE I

INTRACELLULAR CONCENTRATIONS OF RADIOLABELED IMP AND HYPOXANTHINE IN ERYTHROCYTES INCUBATED WITH VARYING CONCENTRATIONS OF [^{3}H]HYPOXANTHINE FOR 80 s IN THE ABSENCE AND PRESENCE OF 50 mM PHOSPHATE AT 30 °C

Details of the experiment are described in the legend to Fig. 1. The acid-soluble pools were prepared from samples of cells after 80 s of incubation with [^{3}H]hypoxanthine (Hyp) and chromatographed with solvent 28. All values are corrected for [^{3}H]hypoxanthine trapped in extracellular space in the acid extracts. The intracellular concentration of radioactivity equal to that in the medium was 2680 cpm/$3.5 \cdot 10^8$ cells (see Fig. 1).

Hypoxanthine (μM)	(cpm/$3.5 \cdot 10^8$ cells)					
	without P_i			with 50 mM P_i		
	IMP	Hyp [a]	Total	IMP	Hyp [a]	Total
0.02	350	4280	4630	11640	1010	13000
0.12	340	4130	4470	10500	820	11320
0.82	150	3640	3810	8740	1080	9820
5	50	2920	2970	6070	1780	7850
20	20	2620	2640	1700	2250	3950
50	0	2140	2140	460	1750	2210

[a] Fraction contains any inosine that might have been formed.

relatively little of the radioactivity became converted to IMP in 80 s of incubation and the concentrations of unmodified [^{3}H]hypoxanthine accumulated intracellularly approached that in the external medium (Table I).

When the red cells were incubated in phosphate-free medium, on the other hand, little of the intracellular radioactivity was associated with IMP, even at [^{3}H]hypoxanthine concentrations < 1 μM (Table I). At the latter concentrations, unmodified [^{3}H]hypoxanthine was found intracellularly at levels about 60% higher than that in the medium. We have observed this apparent concentrative accumulation of hypoxanthine when present in the medium at low concentrations in several experiments as well as with low concentrations of adenine (see later). At concentrations of 5 μM hypoxanthine or higher, on the other hand, unmodified [^{3}H]hypoxanthine accumulated only to about equilibrium levels (Fig. 1A, Table I).

Hypoxanthine transport

The finding that little of the hypoxanthine taken up at concentrations above 20 μM became phos-

phoribosylated in 1 min of incubation allowed measurements of its transmembrane equilibration at these concentrations. Fig. 2 illustrates representative time courses of hypoxanthine equilibration across the membrane, which indicate saturation of hypoxanthine entry. Fitting an integrated rate equation based on the simple carrier model [1,7] to the time courses of transmembrane equilibration of six concentrations of hypoxanthine ranging rom 30 to 960 μM yielded a Michaelis-Menten constant (K) for hypoxanthine transport of about 200 μM in three independent experiments (Table II). The fit was based on a simple carrier with directional symmetry and equal mobility of empty and hypoxanthine-loaded carrier [1]. This model seems to describe adequately hypoxanthine transport in human erythrocytes. This is indicated first by the excellent fit of the appropriate integrated rate equation to the experimentally determined time courses of hypoxanthine uptake (see Fig. 2 and S.E. of estimate of the fitted kinetic parameters in Table II). Second, the velocities of equilibrium exchange (v^{ee}) and of zero-*trans* entry (v_{12}^{zt}) at a hypoxanthine concentration well above the K_m, which thus approached maximum velocities, were about the same (Fig. 3A). Equality of maximum velocity of equilibrium exchange (V^{ee}) and zero-*trans* entry (V_{12}^{zt}) is only observed with a completely symmetrical system [1], since by definition $V^{zt}/K^{zt} = V^{ee}/K^{ee}$ for the simple carrier [1,7]. The time courses of transmembrane equilibration of [^{3}H]hypoxanthine at a concentration of 1 mM under zero-*trans* and equilibrium exchange conditions clearly differed (Fig. 3A), but this difference was entirely consistent with the simple symmetric carrier (for computer simulations see Ref. 12) and the kinetic parameters determined experimentally for the hypoxanthine transporter (Table II). In addition, directional symmetry was confirmed by direct experimental analysis of the zero-*trans* influx and efflux of 2.5 mM hypoxanthine (data not shown, for protocol see Fig. 6).

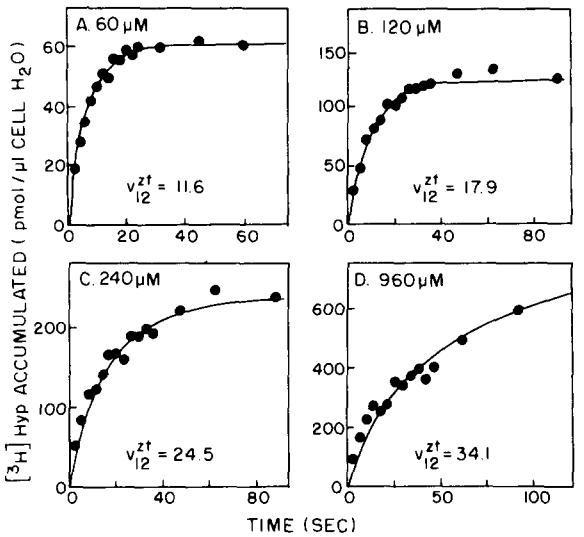

Fig. 2. Zero-*trans* influx of hypoxanthine in human erythrocytes at 25 °C. Time courses of transmembrane equilibration of radioactivity from 30, 60, 120, 240, 480 and 960 μM [^{14}C]hypoxanthine (35 cpm/μl, irrespective of concentration) were determined by rapid kinetic techniques as described in Experimental procedures. Radioactivity values/cell pellet were converted to pmol/μl cell water on the basis of an experimentally determined cell water space. The integrated zero-*trans* rate equation for a simple carrier with directional symmetry and equal mobility when empty and loaded was fitted to the time courses pooled for all six concentrations. The best fitting kinetic parameters are stated in Table II (first line). Representative time courses are illustrated in panels A–D. v_{12}^{zt} values are expressed in pmol/μl cell water per second.

TABLE II

KINETIC PARAMETERS FOR THE TRANSPORT OF HYPOXANTHINE, URACIL AND ADENINE IN HUMAN ERYTHROCYTES AT 25 °C

The kinetic parameters in the first line were estimated from the data in Fig. 2. The other experiments were conducted in the same manner. K and V are the Michaelis-Menten constant and maximum velocity, respectively, for a carrier with directional symmetry and equal mobility when empty and substrate-loaded [1] and k = first-order rate constant. Adenine transmembrane equilibration was measured in the same way at 100, 200, 400, 800, 1600, 3200 and 5000 μM, but k was estimated by fitting the integrated first-order rate equation to each time course as described in Experimental procedures. The value presented is the mean of the estimates for the seven adenine concentrations.

Substrate	Range tested (μM)	K (μM)	V (μM/s)	$V/K = k$ (s^{-1})
Hypoxanthine	30– 960	143 ± 10	39.2 ± 1.0	0.27
	30– 960	284 ± 18	34.6 ± 0.8	0.12
	30– 960	190 ± 13	45.5 ± 1.1	0.24
Uracil	24–3840	5543 ± 512	190 ± 10	0.034
Adenine	100–5000	no saturation		0.055

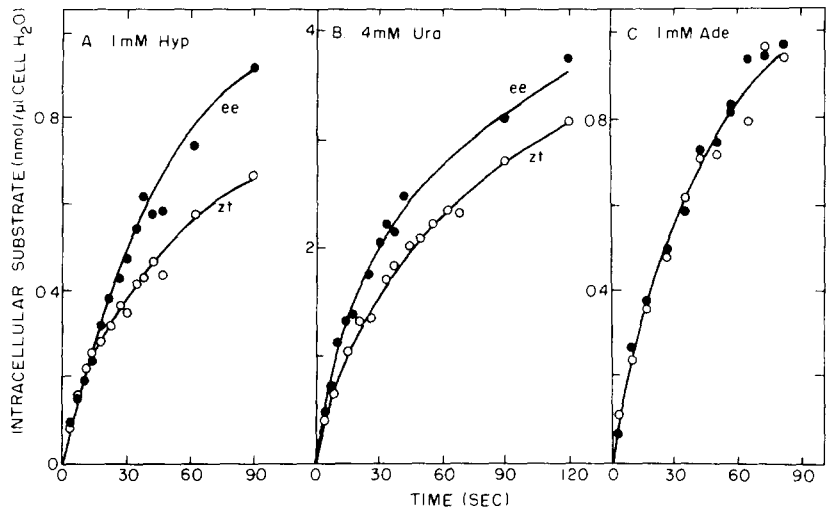

Fig. 3. Comparison of time courses of transmembrane equilibration of [^{14}C]hypoxanthine (A), [^{3}H]uracil (B) and [^{14}C]adenine by human erythrocytes in zero-*trans* (zt) and equilibrium exchange (ee) modes at 25 °C. Samples of suspensions of about $6 \cdot 10^8$ erythrocytes/ml of Tris-saline were preincubated with 1 mM unlabeled hypoxanthine (Hyp), 4 mM uracil (Ura) or 1 mM adenine (Ade) at 37 °C for about 1 h. After cooling to 25 °C, the uptake of 1 mM [^{14}C]hypoxanthine (31 cpm/nmol), 4 mM [^{3}H]uracil (130 cpm/nmol) or 1 mM [^{14}C]adenine (70 cpm/nmol) was measured by rapid kinetic techniques in the preloaded cells (ee) and non-preloaded cells (zt). Radioactivity values for cell pellets were converted to nmol of nucleobase/µl cell water on the basis of experimentally determined water spaces.

Uracil transport and permeation of cytosine

Uracil and cytosine are not metabolized by human red cells, which simplifies measurement of their transmembrane equilibration in unmodified form. Uracil entry into the red cells was found to be saturable with a Michaelis-Menten constant of about 5.5 mM (Table II). The value is only a rough estimate, since it is based on velocities obtained in a concentration range of 24 to 3840 µM uracil. Because of solubility limitations, higher concentrations could not be tested. The same arguments already presented for hypoxanthine indicate that uracil transport is mediated by a simple carrier with directional symmetry and equal mobility when empty and uracil-loaded (Fig. 3B). However, the transport efficiency of uracil transport as quantitated by the first-order rate constant ($k = V/K$) was considerably lower than that of hypoxanthine transport (Table II).

Cytosine transmembrane equilibration was much slower than that observed for uracil and hypoxanthine and thus required measurements over time periods of minutes rather than seconds (Fig. 4). Entry into the cells was probably non-mediated. This conclusion is indicated by the slowness of uptake and the finding that the rate of cytosine permeation in relation to its lipid solubility (measured by its solubility in octanol relative to that in water) was similar to that of 8-azaguanine and L-glucose (Fig. 4 and Table III), whose entry into cells is also thought not to be mediated by a carrier [16,34]. In agreement with this conclusion is the finding that the uptake of 400 µM [^{3}H]cytosine by the red cells was not significantly affected by the presence of 2 mM uracil, hypoxanthine or adenine, that cytosine uptake did not show any sign of saturation up to a concentration of 4 mM (data not shown) and that cytosine had no effect on the transport of hypoxanthine, uracil and adenine (see later).

Adenine uptake and transport

The time courses of uptake of [^{3}H]adenine in the absolute concentration range of 0.02 to 50 µM in the absence (Fig. 5A) and presence of phosphate (Fig. 5B) were comparable to those observed with [^{3}H]hypoxanthine. Adenine entered the cells rapidly and, at the lower concentrations, radioactivity accumulated intracellularly to concentrations well above that in the medium. Much of the intracellular radioactivity was associated with adenine nucleotides (Table IV). Much higher con-

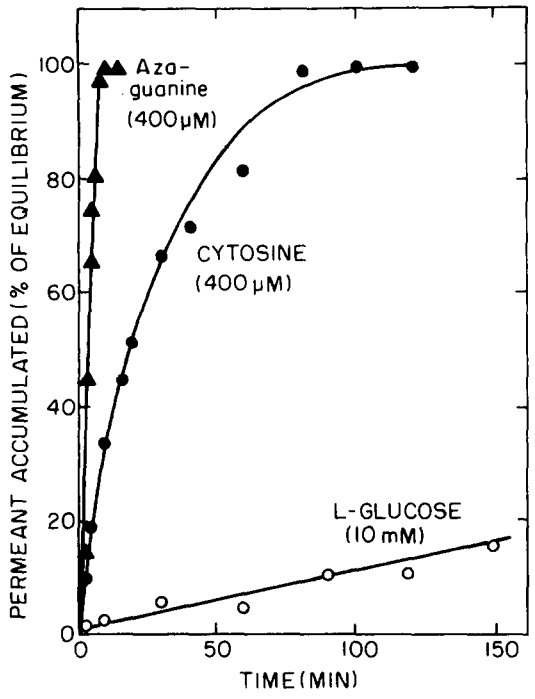

Fig. 4. Time courses of uptake of 8-azaguanine, cytosine and L-glucose by human erythrocytes at 25 °C. Samples of a suspension of $8 \cdot 10^8$ red cells/ml of Tris-saline were supplemented with 400 μM [^{14}C]azaguanine (80 cpm/μl), 400 μM [^{3}H]cytosine (210 cpm/μl) or 10 mM L-[^{3}H]glucose (350 cpm/μl). The suspensions were sampled manually at intervals and the cells separated from the medium by centrifugation through oil and analyzed for radioactivity. Intracellular radioactivity values are averages of duplicate 0.5-ml samples of cell suspension and are expressed as percent of intracellular-extracellular equilibrium of permeant, which was calculated on the basis of an experimentally determined cell water space. A first-order rate equation was fitted to the curves (see Experimental procedures) and the best-fitting first-order rate constants (k) are included in the means presented in Table III.

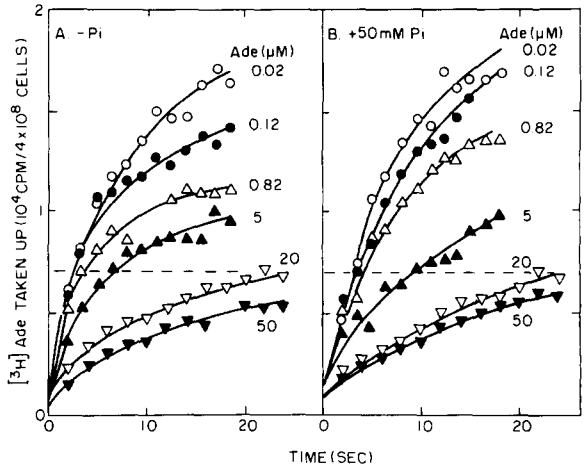

Fig. 5. Time courses of uptake of various concentrations of adenine in the absence (A) and presence (B) of 50 mM phosphate by human erythrocytes at 30 °C. Suspensions of $8 \cdot 10^8$ red cells/ml of Tris-saline or Tris-saline supplemented with 50 mM phosphate (P_i) were incubated at 37 °C for 15 min. After cooling to 30 °C, the uptake of [^{3}H]adenine (220 cpm/μl, irrespective of concentration) was measured by rapid kinetic techniques as described under Experimental procedures. The acid-soluble pools were extracted from samples of cells at 80 s of incubation and chromatographed with solvent 28 (see Table IV). The broken lines indicate the intracellular concentrations of radioactivity equivalent to those in the medium.

centrations of labeled adenine nucleotides accumulated in cells incubated in the presence than in the absence of phosphate. Uptake of radioactivity decreased with increase in absolute adenine concentration whether or not phosphate was present and this apparent saturation of uptake, as in the case of hypoxanthine, correlated mainly with a decrease in the incorporation of radioactivity into nucleotides. However, in the absence of phosphate there was also a decrease in absolute adenine concentration in the medium. Results similar to those shown in Fig. 5 and Table IV were obtained in three independent experiments. In all experiments, the intracellular concentrations of free adenine accumulating in the cells exceeded that in medium, especially in cells incubated with low concentrations of adenine in the absence of phosphate (Table IV).

In most experiments dealing with [^{3}H]adenine uptake, the uptake curves did not seem to extrapolate to zero (see Fig. 5), but the apparent intercept in relation to the equilibrium level varied in independent experiments with different populations of erythrocytes. The reasons for this finding are not clear; it could reflect variable binding of adenine to cell surface components as we have observed with some cultured mammalian cells [15]. In other experiments, we found the time courses of uptake of radiolabeled adenine and of its conversion to nucleotides by rabbit erythrocytes to be comparable to those observed with human erythrocytes (data not shown, see Fig. 5).

In extension of the data in Fig. 5, we measured the transmembrane equilibration of adenine at concentrations ranging from 100 to 5000 μM, the

TABLE III

OCTANOL PARTITION COEFFICIENTS AND FIRST-ORDER RATE CONSTANTS FOR NON-MEDIATED PERMEATION AND FACILITATED TRANSPORT OF VARIOUS NUCLEOBASES IN HUMAN ERYTHROCYTES AT 25 °C

Z is the partition coefficient: concentration of substance in octanol/concentration in aqueous buffer solution determined previously [18]. k, apparent first-order rate constant for transport or permeation; calculated as V/K for hypoxanthine and uracil transport from the data in Table II. The value for adenine is a mean of values obtained in a single experiment with adenine concentrations ranging from 100 to 5000 μM. A similar value has been obtained in three other experiments with varying adenine concentrations. The values for cytosine, 8-azaguanine and L-glucose were estimated from the data in Fig. 4 and results from other experiments conducted in a similar manner.

Permeant	Z (mean ± S.E.)	$10^3 \times k$ (s^{-1}) (mean ± S.E.)	k/Z (s^{-1})
Hypoxanthine	0.115 ± 0.009	210 ± 46 ($n = 3$)	1.83
Adenine	0.105 ± 0.015	55 ± 3.6 ($n = 7$)	0.52
Uracil	0.0778 ± 0.004	34 ($n = 1$)	0.44
Azaguanine	0.173 ± 0.064	5.7 ($n = 1$)	0.033
Cytosine	0.0352 ± 0.0007	0.55 ± 0.42 ($n = 3$)	0.016
L-Glucose	0.00158 ± 0.00003	0.034 ± 0.007 ($n = 3$)	0.022

highest concentration of adenine that could be tested because of solubility limitations. Initial entry velocities were estimated by integrated rate analysis as the slopes of the equilibration curves at zero time. As demonstrated already (Table IV), at these concentrations and in the absence of phosphate, < 5% of the entering adenine became phosphoribosylated during the time period of the transport assay. There was no indication of any saturation of entry up to 5 mM; all first-order rate constants fell within 0.0525 and 0.0576/s. This result was confirmed in two other independent experiments. Combined, our results support the view that adenine is transported by an adenine-specific transporter with relatively low affinity for its substrate, but this conclusion would not be proved unequivocally. The main evidence for carrier mediated entry of adenine is its rapidity. The first-order rate consant for adenine entry was of the same order of magnitude as those for hypoxantine and uracil transport (Table II) as well as for adenine transport in various cultured mammalian cell lines, which exhibit Michaelis constants for transport of 2–5 mM at 25 °C [1,15]. The same applied to the ratio of the first-order rate constant of entry over the octanol partition

TABLE IV

INTRACELLULAR CONCENTRATIONS OF RADIOLABELED ADENINE NUCLEOTIDES AND ADENINE IN ERYTHROCYTES INCUBATED WITH VARYING CONCENTRATIONS OF [^{3}H]ADENINE FOR 80 s IN THE ABSENCE AND PRESENCE OF 50 mM PHOSPHATE AT 30 °C

The details of the experiment are described in the legend to Fig. 5. The acid-soluble pools were prepared from samples of cells after 80 s of incubation with [^{3}H]adenine (Ade) and chromatographed with solvent 28. All values are corrected for [^{3}H]adenine trapped in acid extracts via the extracellular water space of cells. The intracellular concentration of radioactivity equal to that in the medium was 7000 cpm/$4 \cdot 10^8$ cells. AXP = > 85% ATP, the remainder ADP and AMP.

Adenine (μM)	cpm/$4 \cdot 10^8$ cells					
	without P_i			with 50 mM P_i		
	AXP	Ade	Total	AXP	Ade	Total
0.02	18000	26000	54000	88800	8400	97200
0.12	6800	21000	27800	87000	8000	95000
0.82	1500	15600	17100	32000	12900	44900
5	500	13300	13800	5100	11900	17000
20	300	12500	12800	1100	11700	12800
50	200	9500	9700	600	10200	10800

coefficient (k/Z), which was much higher than those observed for the non-mediated permeation of cytosine, 8-azaguanine and L-glucose (Table III). Zero-*trans* influx and efflux of adenine at a concentration of 5 mM were about equal as was inward and outward equilibrium exchange (Fig. 6), which indicates that the adenine transporter exhibits directional symmetry. The time courses of [^{3}H]adenine transmembrane equilibration under zero-*trans* and equilibrium exchange conditions were identical (Figs. 3C and 6) as expected on the basis of the simple carrier model for substrate concentrations in the first order range ($\ll K_m$). However, because of the lack of information on the Michaelis-Menten constant for adenine transport, this test does not allow any conclusions as to the relative mobility of empty and adenine-loaded carrier. The zero-*trans* influx of 500 μM hypoxanthine, adenine and uracil was not significantly affected by pH between 6.5 and 7.5 (data not shown).

Effects of nucleobases on the transport of each other

In order to obtain further information on the number of base transport systems in human erythrocytes and their specificity, we have determined the effects of hypoxanthine, uracil and adenine on the zero-*trans* entry and countertransport of each other and at the same time have examined the effects on their transport of various substances that act as inhibitors of nucleoside transport [1–3]. The results were complex and are not readily interpretable according to presently available concepts. First, hypoxanthine influx was relatively strongly inhibited by adenine (Table V). This result might suggest that hypoxanthine and adenine are transported by a single carrier, and this suggestion is supported by the finding that hypoxanthine effected a similar 'countertransport' of [^{3}H]adenine as of itself (Fig. 7A and B). Countertransport refers to the transient concentrative accumulation of a substrate on the *trans* side of the membrane, which is caused by the presence of an excess concentration of the same or an alternate substrate on the *trans* side. The mechanism(s) involved have not been entirely elucidated, but may involve several different factors [1,7]. In our study, we assayed for countertransport by measur-

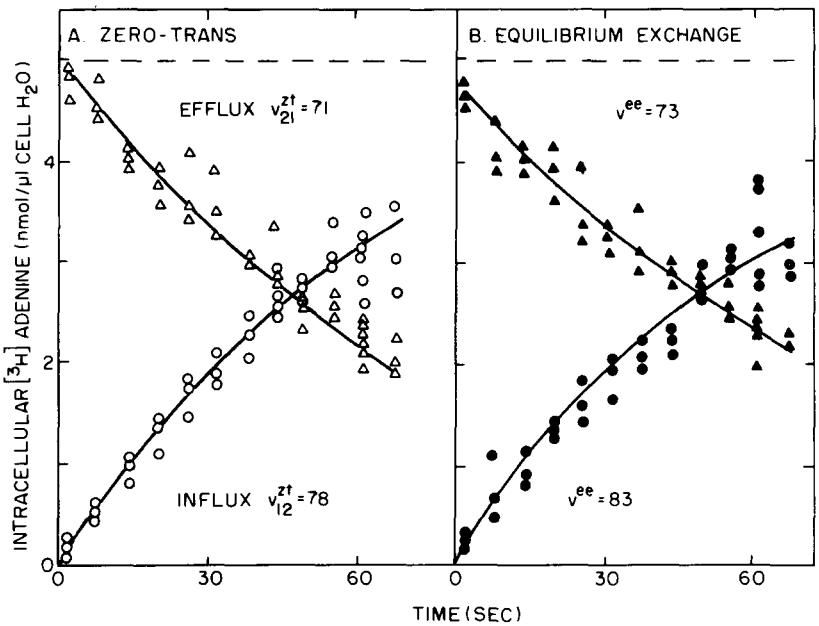

Fig. 6. Comparison of efflux and influx (A) and inward and outward equilibrium exchange (B) of 5 mM adenine in human erythrocytes at 25 °C. Transmembrane equilibration of 5 mM [^{14}C]adenine was measured in triplicate in the four experimental protocols in the same population of erythrocytes (final density = $6 \cdot 10^8$ cells/ml of Tris-saline) by rapid kinetic techniques as described under Experimental procedures. The appropriate first-order rate equations were fitted to the time courses and the initial velocities (in pmol/μl cell water) were calculated from the fitted first-order rate constant, $v = Sk$. The broken lines indicate the intracellular concentration of adenine equivalent to that in the medium.

TABLE V

EFFECTS OF DIPYRIDAMOLE, DILAZEP AND URIDINE ON THE ZERO-*TRANS* INFLUX OF NUCLEOBASES IN HUMAN ERYTHROCYTES AND OF THE NUCLEOBASES ON THE TRANSPORT OF EACH OTHER

The zero-*trans* influx of the radiolabeled nucleobases was measured by rapid kinetic techniques at 25 °C in suspensions of $(6.5–8)\cdot10^8$ red cells/ml as described under Experimental procedures. The substances tested for inhibition were mixed with cells simultaneously with the substrate to the final concentrations shown. Ade, adenine; Hyp, hypoxanthine; Ura, uracil; Cyt, cytosine; Urd, uridine; DIP, dipyridamole; DLZ, dilazep. The results are compiled from several experiments. In the main experiments, the initial velocities (v_{12}^{zt}) for control suspensions were 28.4 ± 1.7; 21.7 ± 3.3, and 14.3 ± 0.5 pmol/μl cell water per second for hypoxanthine, adenine and uracil influx, respectively. % *I*, percentage inhibition.

Substrate					
500 μM hypoxanthine		500 μM adenine		500 μM uracil	
Addition	% *I*	Addition	% *I*	Addition	% *I*
0.1 mM Ade	64	0.5 mM Hyp	30	0.5 mM Hyp	33
0.3 mM Ade	85	1 mM Hyp	40	2 mM Hyp	47
1 mM Ura	16	3 mM Ura	2	0.5 mM Ade	30
2 mM Ura	28			2 mM Ade	42
3 mM Cyt	0	3 mM Cyt	0	3 mM Cyt	0
1 mM Urd	14	1 mM Urd	18	3 mM Urd	42
20 μM DIP	38	20 μM DIP	20	40 μM DIP	30
100 μM DLZ	25	20 μM DLZ	16	100 μM DLZ	22

ing the uptake of radiolabeled hypoxanthine, adenine or uracil, all at 50 μM, into cells that had been preloaded with 5 mM hypoxanthine, adenine or uracil (Fig. 7).

The view that adenine and hypoxanthine are transported by the same carrier, however, is contradicted by the finding that hypoxanthine had far less affect on adenine influx than adenine had on

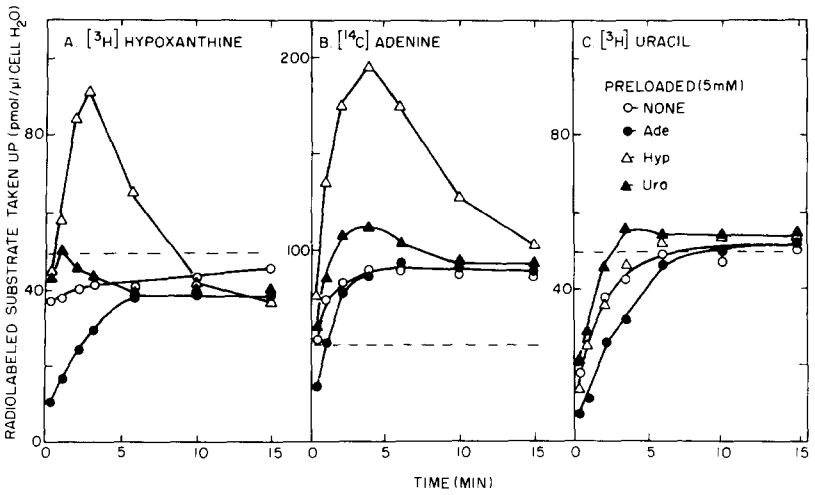

Fig. 7. 'Countertransport' of hypoxanthine (A), adenine (B) and uracil (C) by each other in human erythrocytes. Samples of a suspension of $7.7\cdot10^8$ red cells/ml of Tris-saline were supplemented with 5 mM adenine (Ade), 5 mM hypoxanthine (Hyp) or 5 mM uracil (Ura) or remained without addition (control). The suspensions were incubated at 37 °C for 1 h. The cells were collected by centrifugation at 4 °C and washed once by suspension and centrifugation in ice cold Tris-saline and then suspended to the original density in Tris-saline containing 50 μM [^{3}H]hypoxanthine (143 cpm/μl), 50 μM [^{14}C]adenine (77 cpm/μl) or 50 μM [^{3}H]uracil (64 cpm/μl) at 35 °C. After various times of incubation at 25 °C, the cells from 0.5-ml of suspension were collected by centrifugation through oil and analyzed for radioactivity. All values are averages of duplicate samples and were corrected for radioactivity trapped in the extracellular space of cell pellets and converted to pmol/μl cell water on the basis of an experimentally determined cell water space. The broken lines indicate the intracellular concentrations of substrate equal to those in the medium.

hypoxanthine influx (Table V), even though the K_m for its transport is much lower than that for adenine transport. Furthermore, preloading the cells with 5 mM adenine inhibited the uptake of [^{3}H]hypoxanthine rather than effecting its countertransport (Fig. 7A). In addition, it slightly inhibited the uptake of 50 μM [^{14}C]adenine (Fig. 7B). Results similar to those shown in Fig. 7 were obtained in three independent experiments. Furthermore, in each, radiolabeled adenine accumulated intracellularly above equilibrium levels (see Fig. 7B), even though conversion to intracellular nucleotides was negligible (data not shown).

Uracil, at a concentration of 5 mM, effected only a slight countertransport of itself, as expected from the high K_m for its transport. However, it effected a similar countertransport of hypoxanthine and adenine (Fig. 7) even though it inhibited the influx of hypoxanthine and adenine only slightly or not at all, respectively (Table V). Its own influx was similarly inhibited by hypoxanthine and adenine, but only at relatively high purine concentrations. Preloading the cells with hypoxanthine had no effect on uracil influx. In contrast, preloading with adenine inhibited uracil influx just as it inhibited the influx of [^{3}H]hypoxanthine and of itself (Fig. 7). Cytosine had no effect on the zero-*trans* influx of adenine, hypoxanthine and uracil (Table V) and did not countertransport with the three substrates (data not shown).

Uridine, a substrate for the nucleoside transporter, had little effect on hypoxanthine and adenine transport (Table V). It had a slightly greater effect on uracil influx, but the inhibition was still low considering the high concentration of uridine that was tested (3 mM, i.e., about 10-times the K_m for uridine influx Ref. 8).

Dipyridamole and dilazep, two strong inhibitors of the equilibrium exchange of 500 μM uridine in human red cells ($IC_{50} \approx 30$ nM for both; Refs. 35 and 36) had relatively little effect on adenine, uracil and hypoxanthine transport (Table V).

Discussion

The kinetic analyses clearly indicate that human erythrocytes possess transport systems for hypoxanthine and uracil (Table II). The hypoxanthine transporter is not significantly inhibited by uridine (Table V) and nucleoside transport in these cells is not significantly inhibited by hypoxanthine or adenine [8]. In these properties and its kinetic parameters hypoxanthine transport in human red cells resembles that of one group of mammalian cell lines, which includes mouse P388, L1210 and L929 cells [13]. Hypoxanthine transport in the latter cells is also highly resistant to inhibition by dipyridamole [13] another property shared with the hypoxanthine transporter of human red cells (Table V). In another group of cell lines that includes Novikoff and HTC rat hepatoma cells and Chinese hamster ovary cells, on the other hand, there exists a clear overlap of nucleoside and hypoxanthine transport and hypoxanthine transport is inhibited by dipyridamole with an IC_{50} of about 0.3 μM [13].

Uracil transport in human erythrocytes is comparable in its kinetic parameters and efficiency (Table II) with that observed in a few types of cultured mammalian cells that have been examined ($k = 0.06–0.028/s$; Ref. 14). Also, both uracil and hypoxanthine transport in the red cells exhibit directional symmetry and equal mobility of loaded and empty carrier, and the cells lack a cytosine transporter just as observed in cultured cells.

The rapidity of entry of adenine into the erythrocytes as compared to the rate of non-mediated permeation of similar hydrophilic substances suggests that it is facilitated by a transporter (Tables II and III) but further evidence in support of this conclusion is desirable. The adenine transporter must exhibit a very low affinity for adenine, since no saturation of influx was apparent up to a concentration of 5 mM (Table III). However, the affinity of the adenine transporter of cultured cells for its substrate is also relatively low ($K = 2–5$ mM; Refs. 1, 15).

Overall the results with red cells were similar to those with cultured cells and have not yielded definitive conclusions as to the number of independent nucleobase transporters that are present. Nevertheless, we favor the view that hypoxanthine, uracil and adenine are transported by independent carriers since hypoxanthine has little effect on adenine and uracil transport (Table V), even though the Michaelis-Menten constant for its

transport is much lower than those for the other two nucleobases. The reverse inhibition of hypoxanthine transport by adenine might be explained by binding of adenine to the hypoxanthine transporter without being transported itself. Precedents for such effects have been reported [7,37]. Mainly unexplained are the inhibitions of the influx of the radiolabeled nucleobases into red cells that had been preloaded with 5 mM adenine in countertransport experiments, the countertransport of adenine by hypoxanthine, and the slight but similar countertransport of all three nucleobases by uracil (Fig. 7). Resolution of these observations requires information on the molecular nature of these transporters. Unfortunately, their identification at the molecular level is impeded by the lack of high-affinity probes for them.

We also have no unequivocal explanation for the apparent slight concentrative accumulation of hypoxanthine and adenine when present at low concentrations (Tables I and IV). It could reflect operation of a high-affinity active transport system, but other factors might be responsible, since the initial rates of uptake of the nucleobases at low concentrations are commensurate within experimental errors with the rates predicted on the basis of first-order rate constants for their nonconcentrative, low-affinity transport. One alternate explanation is the saturable binding of the nucleobases to intracellular components. Another is that the concentrative accumulation of hypoxanthine and adenine, especially in the absence of P_i, reflects the initial trapping of the entering nucleobases by phosphoribosylation, thereby consuming the limited amounts of *P*-Rib-*PP* available in the cells, coupled with rapid turnover of the nucleotides. These questions can only be resolved by measuring nucleobase uptake in cells, in which all phosphoribosylation is completely blocked either genetically or chemically and which can be readily depleted of ATP.

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References

1 Plagemann, P.G.W. and Wohlhueter, R.M. (1980) Curr. Top. Membranes Transp. 14, 225–330
2 Paterson, A.R.P., Jakobs, E.S., Harley, E.R., Cass, C.E .and Robbins, M.M. (1983) in Development of Target-Oriented Anticancer Drugs (Cheung, Y.-C., Goz, B. and Minkoff, M., eds.), pp. 41–56, Raven Press, New York
3 Young, J.D. and Jarvis, S.M. (1983) Biosci. Rep. 3, 309–322
4 Belt, J.A. (1983) Mol. Pharmacol. 24, 479–484
5 Plagemann, P.G.W. and Wohlhueter, R.M. (1984) Biochim. Biophys. Acta 773, 39–52
6 Plagemann, P.G.W. and Wohlhueter, R.M. (1985) Biochim. Biophys. Acta 816, 387–395
7 Stein, W.D. (1986) Transport and Diffusion Across Cell Membranes, pp. 231–251, Academic Press, Orlando, FL
8 Plagemann, P.G.W., Wohlhueter, R.M. and Erbe, J. (1982) J. Biol. Chem. 257, 12069–12074
9 Jarvis, S.M., Hammond, J.R., Paterson, A.R.P. and Clanachan, A.S. (1983) Biochem. J. 210, 457–461
10 Plagemann, P.G.W. and Woffendin, C. (1987) Biochim. Biophys. Acta 903, 18–30
11 Marz, R., Wohlhueter, R.M. and Plagemann, P.G.W. (1979) J. Biol. Chem. 254, 2329–2338
12 Plagemann, P.G.W. and Wohlhueter, R.M. (1982) Biochim. Biophys. Acta 688, 505–514
13 Plagemann, P.G.W. and Wohlhueter, R.M. (1984) J. Membrane Biol. 81, 255–262
14 Wohlhueter, R.M., McIvor, R.S. and Plagemann, P.G.W. (1980) J. Cell. Physiol. 104, 309–319
15 Puziss, M.B., Wohlhueter, R.M. and Plagemann, P.G.W. (1983) Mol. Cell. Biol. 3, 82–90
16 Graff, J.C., Wohlhueter, R.M. and Plagemann, P.G.W. (1977) J. Biol. Chem. 252, 4185–4190
17 Müller, M.M., Kraupp, M., Falkner, G. and De Bruyn, C.H.M.M. (1978) Monogr. Hum. Genet. 10, 116–121
18 Templeton, B.A. and Chilson, O.P. (1981) J. Biol. Chem. 256, 285–290
19 Aranow, B., Toll, D., Patrick, J., Hollingsworth, P., McCartan, K. and Ullman, B. (1986) Mol. Cell. Biol. 6, 2957–2962
20 Ullman, B., Patrick, J. and McCartan, K. (1987) Mol. Cell. Biol. 7, 97–103
21 Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390
22 Baldwin, S.A., Baldwin, J.M., Gorga, F.R. and Lienhard, D.G.E. (1979) Biochim. Biophys. Acta 552, 183–188
23 Mueckler, M., Caruso, C., Baldwin, S.A., Panico, M., Blench, I., Morris, H.R., Allard, W.J., Lienhard, G.E. and Lodish, H.F. (1985) Science 229, 941–945
24 Wu, J.-S.R., Jarvis, S.M. and Young, J.D. (1983) Biochem. J. 24, 995–997
25 Tse, C.M., Belt, J.A., Jarvis, S.M., Paterson, A.R.P., Wu, J.-S. and Young, J.D. (1985) J. Biol. Chem. 260, 3506–3511
26 Wohlhueter, R.M. and Plagemann, P.G.W. (1980) Int. Rev. Cytol. 64, 171–240
27 Wohlhueter, R.M., Marz, R., Graff, J.C. and Plagemann, P.G.W. (1978) Meth. Cell. Biol. 20, 211–236

28 Plagemann, P.G.W. and Wohlhueter, R.M. (1983) J. Cell. Physiol. 116, 236–246
29 Plagemann, P.G.W. (1971) J. Cell. Physiol. 77, 213–240
30 Hershko, A., Razin, A., Shoshani, T. and Mager, J. (1967) Biochim. Biophys. Acta 149, 59–73
31 Gutensohn, W. (1975) Physiol. Chem. 256, 1105–1112
32 Plagemann, P.G.W. (1986) J. Cell. Physiol. 126, 491–500
33 Bartlett, G.R. (1985) Progr. Clin. Biol. Res. 195, 335–345
34 Plagemann, P.G.W., Marz, R., Wohlhueter, R.M., Graff, J.C. and Zylka, J.M. (1981) Biochim. Biophys. Acta 647, 49–62
35 Plagemann, P.G.W. and Kraupp, M. (1986) Biochem. Pharmacol. 35, 2559–2567
36 Woffendin, C. and Plagemann, P.G.W. (1987) J. Membrane Biol. 98, 89–100
37 Krupka, R.M. and Deves, R. (1983) Int. Rev. Cytol. 84, 303–352