Biochimica et Biophysica Acta, 729 (1983) 97–103
Elsevier Biomedical Press

BBA 71591

UPTAKE OF L-TRYPTOPHAN BY ERYTHROCYTES INFECTED WITH MALARIA PARASITES (*PLASMODIUM FALCIPARUM*) *

H. GINSBURG and M. KRUGLIAK

Department of Biological Chemistry, Institute of Life Sciences, Hebrew University of Jerusalem, 91904 Jerusalem (Israel)

(Received December 1st, 1982)

Key words: Tryptophan; Amino acid transport; Erythrocyte; Malaria; (P. falciparum)

The initial rates of uptake of L-tryptophan into normal human red blood cells and into cells infected by the malarial parasite *Plasmodium falciparum* in vitro, were investigated. We find that transport in non-infected cells, which is mediated by the specific saturable T system and the apparently non-saturable L system (Rosenberg, Young and Ellory (1980) Biochim. Biophys. Acta 598, 375–384) is considerably enhanced by blood preservation and culture conditions. This increase is mostly due to an increase in the maximal velocity of the saturable component and of the rate constant of the linear component. Uptake is further enhanced in non-infected cells by factors released from infected cells into the culture medium and, even more so, in infected cells at the advanced stage of intraerythrocytic parasite development. At these stages the susceptibility of the transport system to the non-specific inhibitor phloretin and to the competitive inhibitor phenylalanine, is virtually lost. The effect of the parasite on L-tryptophan uptake by the host cell membrane is exerted only on the maximal velocity of the T system, which is carrying most of the substrate under physiological conditions. The possible implications of these findings to the life of the intraerythrocytic parasite are briefly discussed.

Introduction

Malarial parasites reproduce asexually inside the red blood cells of their mammalian host. The developing parasite obtains most of the amino acids it requires for protein anabolism by phagocytosis and the subsequent proteolysis of host cell cytoplasm which consists mainly of hemoglobin [1]. However, not only is the amino acid composition of parasite protein significantly different from that of the host cell hemoglobin, but the latter lacks some amino acids which are essential for the synthesis of the proteins of the parasite, i.e., methionine, isoleucine and cysteine [2]. Therefore, the host cell membrane must provide routes for the supply of the necessary amino acids and for the disposal of those acids which are produced in excess during the digestion of host cell cytoplasm. The carrier-mediated transport of amino acids across the membrane of normal erythrocytes is usually relatively slow [3]. Upon infection of *Plasmodium lophurae* into duck erythrocytes, the transport of amino acids into infected cells is significantly increased [4,5]. This increase occurs at a defined stage of parasite development, i.e., the trophozoite stage, and is correlated with vast alterations in structure, composition and function of the host cell membrane [6], which are induced by the invader. In a recent series of studies we have presented evidence for the appearance at the trophozoite stage of narrow, positively charged

* This work was supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

pores in the host cell membrane [7–10]. Since this new permeability pathway excludes large and positively charged amino acids, it is worth investigating whether the transport of these substances across the host cell membrane is also affected by the intraerythrocytic parasite. In the present paper we report our studies on the influx of L-tryptophan in human red blood cells infected with *Plasmodium falciparum* and its susceptibility to some known inhibitors of this transport system. We have chosen to study this system since it is one of the amino acid transport systems that has been thoroughly characterized kinetically [11].

We show that the uptake of L-tryptophan by non-infected red blood cells is increased upon storage and incubation of the cells in culture conditions, and even more so when parasites are present in the culture. The transport is further increased across the membrane of infected cells and its susceptibility to inhibitors is lost.

Materials and Methods

Cultures of Plasmodium falciparum

Blood (Type A^+ or O^+) was obtained from healthy donors and stored in citric acid/citrate/dextrose (ACD) or with EDTA at 4°C for up to 3 weeks. Erythrocytes were obtained by three consecutive washes in sterile phosphate-buffered saline and finally suspended at 2.5% hematocrit in growth medium: RPMI-1640 (Gibco) supplemented with 32 mM $NaHCO_3$, 25 mM Hepes, 1 mM inosine and 10% (v/v) heat inactivated AB^+ plasma. This erythrocyte suspension (15 ml in 75 cm^2 culture flasks (Nunc)) was inoculated with infected cells (Nigerian strain obtained from Dr. W.H.G. Richards) to a final percentage of infected cells, i.e. parasitemia, of 1.5–2%, flushed with a mixture of 5% O_2, 5% CO_2 in nitrogen, and incubated at 37°C. Cultivation proceeded for 4–5 days with daily changes of medium and gas mixture, until parasitemia reached 18–25%, as determined by microscopic inspection of thin smears stained with Giemsa. Cultures were collected, washed once in wash medium (growth medium without plasma), resuspended at 10% hematocrit in wash medium containing 0.7% (w/v) gelatin [12], and left to stand at room temperature for 15–30 min. The upper layer containing erythrocytes harboring trophozoites and schizonts, and the lower layer consisting of uninfected cells and ring forms were collected separately and washed twice in Tris-HCl buffered saline, with 20 min of incubation at room temperature between the washes. (Residues of gelatin were found to interfere with transport of tryptophan.) Control cells either freshly washed from fresh blood (less than 4 days after venipuncture) or from outdated blood or cultivated under culture conditions, were subjected to the same preparation steps. In each experiment we determined number of cells (by means of hemacytometer) and hemoglobin concentration (absorbance at 540 nm after lysis in hemolysing solution: 4 mM CsCl/0.25% (v/v) NH_4OH/50 ppm Triton X-100, as well as parasitemia.

Uptake of tryptophan

All experiments were conducted at 37°C. Uptake experiments were started with the rapid mixing of prewarmed Tris-buffered salt solution containing the desired concentration of ^3H-labeled L-tryptophan (Amersham or New England Nuclear) with a small volume of packed erythrocytes. Inhibitors, when used, were included in the loading solution. At the desired time interval, 8 ml of ice-cold buffer were added and the suspension was quickly centrifuged ($7000 \times g$, 10 s). The supernate was discarded and the pellet washed again with cold buffer. The final pellet was lysed in hemolyzing solution, part of which was used for the determination of hemoglobin concentration. To the other part, trichloroacetic acid was added (5% final concentration) and, after centrifugation, the supernate was taken for scintillation counting (Hydroluma scintillation fluid (Lumac) in a Packard Instruments Prias PL equipped with a dpm calculating module). Zero-time values were obtained by mixing cells with ice-cold loading solution and immediately proceeding with washed as detailed above. All time points were taken in triplicates. It was ascertained that the parasitemia did not change in the course of the above mentioned manipulations. Calculations of the tryptophan content in cells were made according to the following equation:

$$S_t = \frac{D_t \cdot F \cdot A_s}{A_t \cdot N \cdot A}$$

where S_t is the cellular tryptophan content in micromoles per 10^{12} cells. Concentration is expressed per number of cells rather than per cell volume or cell water because the cellular volume of infected cells is larger than that of normal cells and it was assumed that the membrane area per cell does not change upon infection. D_t is the dpm of soluble label associated with the cells at time t; A_t is the absorbance at 540 nm of the lysate of this sample; A_s is the absorbance of the lysate of a known number of cells N. Usually, when working with erythrocytes, it is very convenient to measure hemoglobin content of cell lysates as an adequate indicator of cell number. However, with *Plasmodium*-infected cells this indicator may become rather ambiguous, since the hemoglobin content per cell decreases with parasite development. Since different preparations had different parasitemias, the correlation between cell number and absorbance had to be found for each cell preparation. a is the specific activity of the loading solution and F is a correction factor which accounts for dilutions and orders of magnitude.

The rate of transport in micromoles/10^{12} cells per min is given by:

$$v = \frac{S_t - S_0}{\Delta t}$$

where S_0 is the tryptophan content at time 0 (see above) and Δt is the time elapsed between the initiation of transport and its stopping by the addition of an ice-cold buffer. In preliminary experiments it was found that uptake was linear with time, at least up to 5 min (Fig. 1). Thereafter all experiments were made with 2-min uptake periods, such that true initial rates of uptake were determined. It is assumed that the possibility of countertransport can be excluded because any residual intracellular amino acids should have been reduced to nil during the washing of the cells and the incubation in gelatin.

Analysis of data

The transport of tryptophan into human erythrocytes is mediated by two parallel systems [11], one of them with higher affinity than the other. Thus, the initial rate of uptake (v) vs. substrate concentration (S) was analyzed by the Levenberg-Marquand non-linear least-squares technique, fitting the data to the first order reaction: $v = V_{max} \cdot S/(K_m + S) + P \cdot S$, where V_{max} is the maximal velocity; K_m the half-maximal concentration constant, and P is the rate coefficient of the linear component. At low substrate concentrations, the contribution of the low-affinity system (P) is negligible and the data could be fitted to a simple Michaelis-Menten system, allowing data analysis either by non-linear least squares or by linear regression of one of the linear transformations of the Michaelis-Menten relationship (S/v vs. S or v/S vs. v). The similarity in the values of the parameters derived by the two fitting techniques indicates that the variances of the data are constant and no special weighting of the data is required.

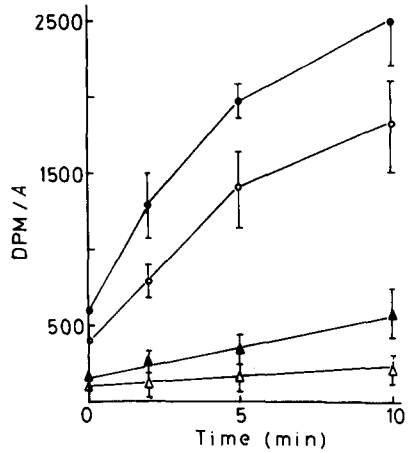

Fig. 1. Time course of uptake of L-tryptophan into normal and malaria infected erythrocytes. Normal erythrocytes were cultivated for 4 days as detailed in Materials and Methods, either in the absence (control) or in the presence of *P. falciparum*. Both cultures were subjected to gelatin floatation, washed, and [^{3}H]tryptophan uptake was measured as a function of time at 0.1 and 10 mM total L-tryptophan concentration. Uptake is expressed in terms of dpm associated with the cell pellet per hemoglobin. △, Control cells, 10 mM; ▲, upper gelatin layer, 40% parasitemia, 10 mM; ○, control cells, 0.1 mM; ●, upper gelatin layer, 40% parasitemia, 0.1 mM.

Results

Transport of tryptophan in normal and P. falciparum infected erythrocytes

A typical experiment of tryptophan uptake into

normal and *P. falciparum* infected cells is depicted in Fig. 2. In agreement with previous reports [13], the uptake of tryptophan into fresh human erythrocytes has been resolved into saturable and linear components (Table I). Long storage in blood bank conditions and, to a greater degree, 4–5 days of cultivation in conditions (which are certainly not optimal for maintenance of the integrity of various erythrocytic functions, but rather an empirical compromise between erythrocyte and parasite requirements) induce further changes in transport properties. Results shown in Table I indicate that while the K_m of the saturable component remains virtually constant, the maximal velocity of the saturable component and, even more, the rate constant of the linear component are substantially increased.

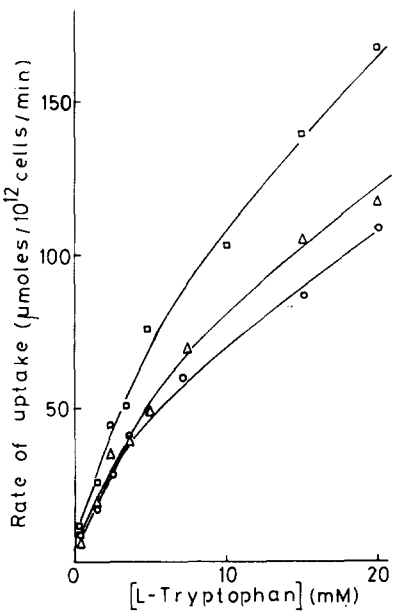

Fig. 2. Uptake of [^{3}H]tryptophan into normal erythrocytes, non-infected and ring-infected erythrocytes from infected cultures, and erythrocytes infected with trophozoites and schizonts as a function of tryptophan concentration (a representative experiment). Control cells were cultivated in parallel with infected cultures. Cultures were floated on gelatin, and upper and lower layers from infected cultures were separated. The lower layer contained 5% cells infected with ring form parasites, and the upper layer contained 40% cells infected with trophozoites and schizonts. Uptake was measured in triplicate for 2 min and results were analyzed by non-linear least squares regression, fitting the data to the first order reaction $v = V_{max} \cdot S/(K_m + S) + P \cdot S$ (see Analysis of Data). Lines were drawn according to the following derived parameters: Control (○), $V_{max} = 57.5$ μmol/10^{12} cells per min; $K_m = 3.9$ mM; $P = 3.03 \cdot 10^{-3}$ min^{-1}. Lower layer (△), $V_{max} = 98.7$; $K_m = 7.88$; $P = 2.6$. Upper layer (□), $V_{max} = 128.3$; $K_m = 6.95$; $P = 3.4$. Standard deviations of the derived parameters were less than 10% of the values.

TABLE I

KINETIC PARAMETERS OF L-TRYPTOPHAN UPTAKE IN HUMAN ERYTHROCYTES (RBC)

Erythrocytes were prepared as detailed in Materials and Methods. Uptake of L-tryptophan was measured in the range of 0.1–10 mM at 37°C. The initial rates of uptake were calculated and the kinetic parameters were obtained by non-linear least-squares analysis fitting the relationship $v = V_{max} \cdot S/(K_m + S) + P \cdot S$.

Preparation	K_m (mM)	V_{max} (μmol/10^{12} cells per min)	P (min^{-1}) ($\times 10^3$)
Fresh RBC	3.2 ± 0.9	12.3 ± 3.8	1.03 ± 0.57
Outdated RBC	3.8 ± 0.1	20.4 ± 0.5	5.40 ± 0.03
Cultivated RBC	5.1 ± 1.3	58.5 ± 12.5	3.03 ± 0.18

The results of tryptophan transport into cells in infected cultures are summarized in Table II. We see that the K_m increased in infected cultures by about 60% and that the maximal velocities are elevated by about 150%. The increase in K_m and in V_{max} is also observed in the lower layer which consists mostly of non-infected cells and a low percentage (4–7.5%) of erythrocytes harboring ring forms.

The rate constant of the linear component was not altered by the presence of parasites, i.e., the rate constants (in 10^{-3} min^{-1}) for control, lower layer and upper layer were 3.03 ± 0.18, 2.09 ± 1.21 and 3.71 ± 1.37, respectively.

Inhibition of tryptophan uptake by various competitors

Phloretin is an effective inhibitor of various transport systems of the human red blood cell membrane [14–16] including that for tryptophan [11]. In the present study we also observed (Table III) a considerable inhibition of tryptophan uptake, though less than that observed by Rosenberg [11]. The degree of inhibition is reduced as the substrate concentration is raised, and in the upper

TABLE II

KINETIC PARAMETERS OF THE SATURABLE COMPONENT OF L-TRYPTOPHAN TRANSPORT INTO NORMAL AND *P. FALCIPARUM* INFECTED ERYTHROCYTES

Initial rates of uptake were measured in the range of 0.1–10 mM L-tryptophan in the following preparations: non-infected erythrocytes maintained in culture conditions; lower gelatin layer and upper gelatin layer (see Materials and Methods). In the left column are shown the parasitemias (lower layer/upper layer). Results were analyzed as detailed in the legend to Fig. 1. V_{max} is given in μmol/10^{12} cells per min; K_m is given in mM and V_{max}/K_m is given in min^{-1} ($\times 10^3$). See text for the meaning of V_{max}/K_m for trophozoites and schizonts.

Expt. No.	Cultivated control cells			Lower layer			Upper layer			Trophozoites and Schizonts
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m	V_{max}/K_m
1. (4/60)	46.5	4.95	9.4	66.0	6.96	9.5	132.0	7.19	18.4	24.3
2. (5/40)	57.5	3.90	14.7	98.7	7.88	12.5	128.3	6.95	18.5	27.4
3. (7.5/45)	71.5	6.55	10.9	109.6	9.40	11.7	160.7	10.20	15.8	20.8
Mean	58.5	5.1	11.7	91.4	8.1	11.2	140.3	8.1	17.5	24.2
±S.D.	12.5	1.3	2.8	22.7	1.2	0.9	17.7	1.8	1.5	1.9

TABLE III

INHIBITION OF L-TRYPTOPHAN UPTAKE IN NORMAL AND *P. FALCIPARUM* INFECTED RED BLOOD CELLS BY PHLORETIN PHENYLALANINE AND CHLOROQUINE

Initial rates of uptake of L-tryptophan were measured at 0.1 and 1.0 mM L-tryptophan in the presence and the absence of the indicated inhibitors. The percentage of inhibition was calculated for each of three individual experiments and means ± S.D. were computed. The (−) sign represents acceleration of transport. The mean parasitemia of the upper layer was 60%.

Inhibitor [Trp] (mM)	Phloretin (0.25 mM)		Phenylalanine (10 mM)		Chloroquine (0.1 mM)	
	0.1	1.0	0.1	1.0	0.1	1.0
Preparation:						
Cultivated RBC	35.2± 9.6	28.1± 6.6	29.0±14.0	8.9± 1.2	15.4±12.8	10.0±15.6
Lower layer	38.9±21.2	31.6±35.4	25.0±20.7	−24.0±68.6	25.8± 9.4	−13.6±41.6
Upper layer	31.4±35.4	−6.5±35.2	11.5±23.5	−5.2±40.7	18.6±19.7	−7.2±27.7

layer there is no inhibition whatsoever.

Phenylalanine is a competitive inhibitor which interferes with both components of tryptophan uptake [13]. It is similarly effective in the present experiment (Table III) but at 1.0 mM tryptophan its inhibition of transport is completely lost in infected cells or in non-infected ones which were isolated from an infected culture.

The inhibitory effect of the widely used antimalarial chloroquine was also tested, since previous observations indicated that tryptophan inhibited the uptake of chloroquinine by red blood cells (Krugliak and Ginsburg, unpublished observations). As can be seen from Table III, chloroquine is the least potent inhibitor of the three compounds tested. Here, again, the transport in the upper layer was the least affected and the inhibitory effect was abolished by increasing substrate concentration.

Discussion

Amino acids translocate across the erythrocyte membrane by means of at least five different transport systems. These are: the ASC system observed for L-alanine and L-cysteine [3]; the Ly

system for the dibasic amino acids [3,17]; the glycine system [18]; the L system observed for L-leucine, L-phenylalanine, L-methionine, L-tryptophan and L-valine [3,19]; and the T system for L-tryptophan [11,13]. Accordingly, tryptophan uptake is characterized by a saturable component identified with the T system and an apparently linear component mediated by the L system [13]. From the present study we see that storage of red cells in blood bank conditions increases the linear component of tryptophan transport and cultivation of the cells under conditions required for the propagation of malarial parasites also increases considerably the maximal velocity of the saturable component. Culture conditions also reduce the susceptibility of those transport systems to inhibition by the non-specific inhibitor phloretin, while it leaves the effect of the competitive inhibitor phenylalanine virtually unaltered (not shown). This point is emphasized because, when alterations of transport functions are investigated, one must be aware of those changes which occur due to the mere exposure of the cells to culture conditions. The kinetic parameters of tryptophan are further altered in the presence of the malarial parasite *P. falciparum*. The effect of the parasite is discernible as well in non-infected cells, inasmuch as both V_{max} and K_m of the saturable component increased by 50–60% in the lower layer consisting mostly (> 92.5%) of non-infected cells. The observed changes could not have been caused by the small number of ring forms that have no detectable effect on the host cell membrane [6]. It seems as though the parasite releases into the culture medium a factor which affects the T system. Such factor(s) has been alluded to by Dunn [20] to explain the increase in Na^+ leak and the decrease in active Na^+ extrusion in Rhesus red cells exposed to the serum of a *P. knowlesi* infected animal. Sherman and Tanigoshi [5] also showed that changes in the transport of amino acids could be caused by an extract of parasitized cells. Extensive non-specific membrane damage which could have caused the observed alterations in tryptophan transport, can be excluded since other transport systems tested, such as that of uridine (Klein and Ginsburg, unpublished observations) are not affected by the parasite. Furthermore, the fact that the non-saturable component is not affected by the parasite and that the other transport system retains its saturable characteristics upon infection, speaks against any significant non-specific leak.

As the intraerythrocytic parasite matures, its effect on V_{max} is apparently increased 1.5-fold (Table II, upper layer). If we assume that all this increase results from alterations in the membranes of infected cells only, it is possible to evaluate the full effect of this increase specifically in infected cells, by the following calculations: The ratio of V_{max}/K_m ($\equiv R$), the limiting permeability [21] of a mixed population of cells (R_t) is the sum of the R values of the individual sub-populations multiplied by their relative number f in the pupulation. Thus, $R_t = f_n \cdot R_n + f_i \cdot R_i$ and $R_i = (R_t - f_n \cdot R_n)/f_i$, where the subscripts n and i stand for non-infected and infected cells respectively. When this correction is made, taking the values of the lower layer for R_n and the parasitemia of the upper layer for f_i and $f_n = 1 - f_i$, we see that the limiting permeability is further increased (Table II, last column). Since K_m of the lower layer is identical to that of the upper layer, we can conclude that the difference in the limiting permeability is due to an increase in V_{max}. Since the T system behaves as a simple symmetric carrier [11], such an increase in V_{max} could be due to an increase in the rate constant of carrier-substrate dissociation or in the rate of translocation of the unloaded carrier, or in both. Similar changes probably do not occur in the L system inasmuch as L-tryptophan transport mediated by this system (the linear component) is not affected by the parasite. As the saturable T system is the relevant transport agency at physiological concentrations of L-tryptophan [22], i.e., it carries 85% of the total transfer at 1 μM, a 2-fold increase in the basic permeability may be of crucial importance for the life of the intraerythrocytic parasite. Elucidation of this supposition must await further experimentation. The physiological role of the T system in normal erythrocytes is not yet known, let alone its importance for the intraerythrocytic parasite. Neverthesless, the inhibition by chloroquine (Table III) might be of some significance to its chemotherapeutic effect at the normal level of free L-tryptophan in the serum, which is in the micromolar range [22].

In conclusion, the dramatic effect that the intracellularly growing parasite exerts on the com-

position, structure and function of its host cell membrane is only marginally reflected in the L-tryptophan transport system in terms of affinity (K_m) and transport capacity (V_{max}). However, the total loss of susceptibility of the transport system to the competitive inhibitor phenylalanine and to phloretin, imply considerable alterations at the binding site of the transport system. Furthermore, it seems that the new permeability pathways, induced in the host cell membrane at the trophozoite stage [9] and which are demonstrably permeable to amino acids whose transport is mediated by the L system [10], do not accommodate tryptophan as well as other essential amino acids [10], inasmuch as the transport of the latter in infected cells is not inhibited by phloretin which is a potent inhibitor of this channel [8,9]. Hence, if the parasite requires for its metabolic advantages a higher permeability of amino acids at the host membrane level, it achieves this need by affecting the membrane in an indiscriminate way by inducing narrow aqueous pores which accommodate various solutes of small molecular size and by non-specific changes in the membrane matrix, where transport agencies are embedded and with which they intimately interact.

References

1 Homewood, C.A. and Neame, K.D. (1980) in Malaria (Seed, T.M. and Kreier, J.P., eds.), Vol. 1, pp. 345–405, Academic Press, New York

2 Sherman, I.W. (1977) Bull. WHO 55, 265–276

3 Young, J.D., Jones, S.E.M. and Ellory, J.C. (1980) Proc. Roy. Soc. London B. 209, 355–375

4 Sherman, I.W. and Tanigoshi, L. (1972) Proc. Helminthol. Soc. Wash. 39, 250–260

5 Sherman, I.W. and Tanigoshi, L. (1974) Exp. Parasitol. 35, 369–373

6 Howard, R.J. (1982) Immunol. Rev. 61, 67–107

7 Kutner, S., Baruch, D., Ginsburg, H. and Cabantchik, Z.I. (1982) Biochim. Biophys. Acta 687, 82–86

8 Kutner, S., Ginsburg, H. and Cabantchik, Z.I. (1982) J. Cell. Physiol. 114

9 Ginsburg, H., Krugliak, M., Edelman, O. and Cabantchik, Z.I. (1983) Mol. Biochem. Parasitol., in the press

10 Ginsburg, H., Krugliak, M., Kutner, S. and Cabantchik, Z.I. (1983) submitted for publication

11 Rosenberg, R. (1981) Biochim. Biophys. Acta 649, 262–268

12 Jensen, J.B. (1978) Am. J. Trop. Med. Hyg. 27, 1274–1276

13 Rosenberg, R., Young, J.D. and Ellory, J.C. (1980) Biochim. Biophys. Acta 598, 375–384

14 Cousin, J.L. and Motais, R. (1978) Biochim. Biophys. Acta 507, 531–538

15 Macy, R.I. and Farmer, R.E.L. (1970) Biochim. Biophys. Acta 211, 104–106

16 LeFevre, P.G. (1948) J. Gen. Physiol. 31, 505–527

17 Gardner, J.D. and Levy, A.G. (1972) Metabolism 21, 413–481

18 Ellory, J.C., Jones, S.E.M. and Young, J.D. (1980) J. Physiol. (London) 310, 22p

19 Winter, C.G. and Christensen, H.N. (1964) J. Biol. Chem. 239, 872–878

20 Dunn, M.J. (1969) J. Clin. Invest. 48, 674–686

21 Stein, W.D. and Lieb, W.R. (1973) Isr. J. Chem. 11, 325–339

22 Daniel, P.M., Love, E.R., Moorhouse, S.R. and Pratt, O.E. (1979) J. Physiol. 289, 87–88