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AMINO ACID TRANSPORT IN PIG LYMPHOCYTES

ENHANCED ACTIVITY OF TRANSPORT SYSTEM ASC FOLLOWING MITOGENIC STIMULATION

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Changes in neutral amino acid transport activity caused by addition of phytohaemagglutinin-P to quiescent peripheral pig lymphocytes have been evaluated by measurements of ^{14}C-labelled neutral and analogue amino acids under conditions approaching initial entry rates. Utilizing methylaminoisobutyric acid, the best model substrate of System A, we confirmed our previous report (Borghetti, A.F., Kay, J.E. and Wheeler, K.P. (1979) Biochem. J. 182, 27–32) on the absence of this transport system in quiescent cells and its emergence following stimulation. Furthermore, we demonstrated the presence in quiescent cells of an Na^+-dependent transport system for neutral amino acids that has been characterized as System ASC by several criteria including intolerance to methylaminoisobutyric acid, strict Na^+-dependence, the property of transtimulation and specificity for pertinent substrates such as alanine, serine, cysteine and threonine. Analysis of the relationship between influx and substrate concentration revealed that two independent saturable components contribute to entry of alanine in quiescent cells: a low affinity (K_m = ≈4 mM) and a high affinity (K_m = ≈0.2 mM) component. The high affinity component could be inhibited in a competitive way by serine, cysteine and threonine, but methylaminoisobutyric acid did not change appreciably its constants. The enhanced activity of alanine transport through the ASC system observed in activated cells resulted from a large increase in the capacity (V) of the high affinity component without any substantial change in the apparent affinity constant (K_m).

Introduction

Mitogenic stimulation of lymphocytes is associated with changes in the flux across the plasma membrane of many different metabolites [1,2] including synthetic and natural amino acids taken up by an Na^+-dependent pathway [3–6]. Although the increased amino acid uptake may be not essential for subsequent events in activation, the close correlation with mitogenesis [7] suggests that it is a normal component of the pleiotypic proliferative program, and it may be linked in a causal relationship with the triggering of cells into the S phase of the cell cycle. We have previously shown that as an early consequence of the addition of mitogenic lectins to cultured pig lymphocytes the transport activity for some natural amino acids was increased [6]. The activity of a system resembling the A system of other cells was low or absent in rat splenic lymphocytes [8] as well as in pig peripheral blood lymphocytes and increased most dramatically after activation [6]. Indirect evidence has also suggested that a second Na^+-dependent transport system for neutral amino acids (ASC system) is present in quiescent lymphocytes; its activity was also accelerated after incubation with phytohaemagglutinin-P [6].

In this communication we report further studies that compare uptake of pertinent substrates of the

ASC system in quiescent and activated pig lymphocytes. Normal identification of ASC transport system activity has been accomplished by several criteria including inability to transport *N*-methylated amino acids, pH- and cation-dependence, kinetic analysis and properties of trans-effects. Competitive and non-competitive analysis using inhibiting amino acids on the kinetic constants of alanine transport was used as a procedure to ascribe transport of a specific amino acid to the ASC transport system in quiescent and stimulated lymphocytes.

Experimental

Materials

L-[U-^{14}C]Alanine, L-[U-^{14}C]serine and 2-amino-[1-^{14}C]isobutyric acid were obtained from the Radiochemical Centre, Amersham, Bucks., U.K., and 2-[1-^{14}C]methylaminoisobutyric acid came from New England Nuclear Chemicals, Dreieichenain, F.R.G. Unlabelled amino acids were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., except for 2-methylaminoisobutyric acid, which was a gift from Professor Raffello Fusco (Department of Chemistry, University of Milan, Milan, Italy). Phytohaemoagglutinin-P was purchased from DIFCO Laboratories, Detroit, MI, U.S.A.

Preparation and purification of lymphocytes

Lymphocytes were purified from defibrinated pig blood, collected at a local abattoir, as described by Kay et al. [9]. The cells at densities between $1 \cdot 10^6$ and $1.5 \cdot 10^6$ cells/ml were incubated in Dulbecco's modified Eagle's medium containing 100 units of penicillin per ml, 100 μg streptomycin per ml and supplemented with 15% autologous serum for 20 h, except when otherwise stated, with and without addition of 10 μg phytohaemagglutinin-P/ml in an atmosphere of 5% CO_2 in air at 37°C. When lymphocytes appeared contaminated by red blood cells, they were further purified by resuspending in 0.167 ammonium chloride, which was buffered to pH 7.4 with sodium hydroxide, for 10 min for the removal (by lysis) of contaminating cells [8].

Intracellular amino acid pool depletion

Lymphocytes, after incubation with or without mitogen, were collected by centrifugation and resuspended in Earle's Balanced Salt Solution supplemented with 0.1% glucose at 37°C. A preliminary incubation in amino acid-free medium to deplete the intracellular amino acid pool is an obligatory step before transport measurements to avoid trans-effects [10]. After 15 min of preincubation, the transport activity of proline or alanine reached a plateau in activated cells, suggesting that these cells have been completely released from trans-effects. On the contrary, quiescent cells showed a slow but continuous increase in amino acid uptake even after 60 min of depletion. We therefore chose a 30 min preincubation step for further experiments with both cell types: adequate time to maximize alanine transport in activated cells even if not enough for the unstimulated; after 30 min depletion, however most of the trans-effects have been eliminated and the preincubation time is short enough to avoid superimposed adaptive regulation of transport System A [11].

Measurement of amino acid transport and calculation

The initial rate of amino acid uptake was determined essentially as described previously with slight modifications [6]. After depletion, cells collected by centrifugation were resuspended at 10^8 cells/ml in standard buffer (150 mM NaCl/5 mM KCl/1.8 mM $CaCl_2$/1 mM $MgSO_4$/1 mM KH_2PO_4/20 mM Tris-HCl, pH 7.5, at 37°C). Choline chloride was used to balance the osmolarity when Na^+-free medium was used. $1 \cdot 10^7$ lymphocytes were then incubated at 37°C in standard buffer containing the radioactive amino acid (1 μCi/ml) and inhibitor, when present, in a final volume of 0.13 ml. The uptake was stopped by chilling the tubes in ice and adding 2 ml of ice-cold standard buffer. Cells were washed twice with 2 ml of ice-cold standard buffer. Washing was by alternate centrifuging (1 000 × *g*, 4 min) and resuspension. Trichloroacetic acid, 0.4 ml of 10% (v/v), was added to the washed cells. The mixture was agitated and centrifuged (2 000 × *g*, 10 min) and 0.2 ml of the supernatant was dispersed into 2 ml of liquid scintillation mixture. All determinations were corrected for the amount of extracellular radioactivity trapped in the cell pellet, determined in each experiment by addition of radioactive isotope to cells maintained at 0°C and then processed as normal. Three or four replicate determinations were made for each value and results, expressed as nmol amino acid taken up

per min per $1 \cdot 10^7$ cells, are shown with the standard deviation of the mean. Based on an estimated average cell diameter of 7.5 μm and a water content of 75% of the cell volume, the calculated intra- to extracellular concentration ratios achieved after 5 min of alanine uptake, measured at 0.1 mM substrate concentration, were 15 to 21 : 1 for phytohaemagglutinin-P-stimulated and 2 to 3 : 1 for quiescent lymphocytes, indicating active amino acid transport in both treated and control cells.

When kinetic contants of amino acid transport were to be measured, high concentrations of amino acid in the medium were used to determine, in each experiment, the rate constant for the non-saturable component of transport by extrapolation to infinite concentrations; net uptake velocity was then corrected for the non-saturable component.

The results relating substrate concentrations to velocity were analyzed by graphical transformation plotting v against $v/[S]$. When curvilinear plots were obtained the assumption was made that two independent Michaelis-Menten components contributed to transport. Data were submitted to computer analysis to obtain the best fit of the values of kinetic parameters. The method for parameter fitting developed by Feldman [12] was used for this analysis: this procedure involves the lowest value of the sum of squares of deviations of experimental points from the theoretical curve.

Results

A discrimination analysis of amino acid transport systems in lymphocytes

We tested the ability of unstimulated and phytohaemagglutinin-P-activated pig lymphocytes to take up natural and analogue neutral amino acid through Na^+-dependent transport systems. The discrimination between A and ASC systems has been approached by measuring the initial entry rates of aminoisobutyric acid, serine and alanine in the presence and in the absence of sodium (Na^+-dependence) and in the absence and in the presence of methylaminoisobutyric acid as characterizing substrate for System A.

TABLE I

TRANSPORT OF METHYLAMINOISOBUTYRIC ACID, AMINOISOBUTYRIC ACID, ALANINE AND SERINE IN QUIESCENT AND 20 h-ACTIVATED PIG LYMPHOCYTES: DISCRIMINATION ANALYSIS

The Na^+-dependent component has been discriminated as methylaminoisobutyric acid-inhibitable (System A) and methylaminoisobutyric acid-non-inhibitable fraction (System ASC) by adding the inhibitor model substrate at 10 mM final concentration. The Na^+-independent component (presumably System L) has been determined in Na^+-free medium, where choline replaced the cation in the sodium salt of the standard buffer. Initial rates of amino acid uptake were measured by incubating the cells for 1 min at the following substrate concentrations: 0.1 mM for methylaminoisobutyric acid and aminoisobutyric acid, and 0.2 mM for alanine and serine. The values shown are the mean of three to four independent determinations. The amino acid uptake by each component, as a percentage of total uptake is shown in parentheses. PHA, phytohaemagglutinin-P.

Substrate	Transport component		Amino acid uptake (nmol/min per 10^7 cells)	
			−PHA	+PHA
Methylaminoisobutyric acid	Na^+-dependent	(A)	0.004 (10)	0.084 (76)
	Na^+-independent		0.036 (90)	0.026 (24)
Aminoisobutyric acid	Na^+-dependent	(A)	0.000 (0)	0.022 (34)
		(ASC)	0.000 (0)	0.022 (34)
	Na^+-independent		0.019 (100)	0.021 (32)
Alanine	Na^+-dependent	(A)	0.000 (0)	0.045 (5)
		(ASC)	0.189 (88)	0.774 (91)
	Na^+-independent		0.025 (12)	0.035 (4)
Serine	Na^+-dependent	(A)	0.012 (6)	0.014 (1)
		(ASC)	0.144 (73)	0.838 (81)
	Na^+-independent		0.041 (21)	0.181 (18)

Table I shows that the Na^+-dependent uptake of serine, alanine and aminoisobutyric acid was significantly greater in cells treated with phytohaemagglutinin-P for 20 h than in quiescent lymphocytes. The activity of the ASC system (evaluated as methylaminoisobutyric acid not-inhibitable portion of amino acid transport) accounted for all the Na^+-dependent uptake of alanine and more than 90% of the Na^+-dependent uptake of serine in the unstimulated cells, its activity being accelerated about 5-fold after mitogen addition. Aminoisobutyric acid transport by unstimulated cells was restricted to a Na^+-independent route but, following activation, substantial Na^+-dependent uptake emerged through both System A and ASC. This observation suggests that aminoisobutyric acid should not be considered an adequate substrate for System A in lymphocytes as recently recommended for other cells [13]. Moreover, one may note that the transport of methylaminoisobutyric acid was almost negligible in quiescent cells, and its uptake became larger in stimulated lymphocytes. Since methylaminoisobutyric acid has been evaluated so far as the best model substrate of System A in several cells [11,13], this result confirms our previous report [6] and adds new evidence on the emergency of System A after mitogenic stimulation, its activity being almost absent in peripheral pig lymphocytes.

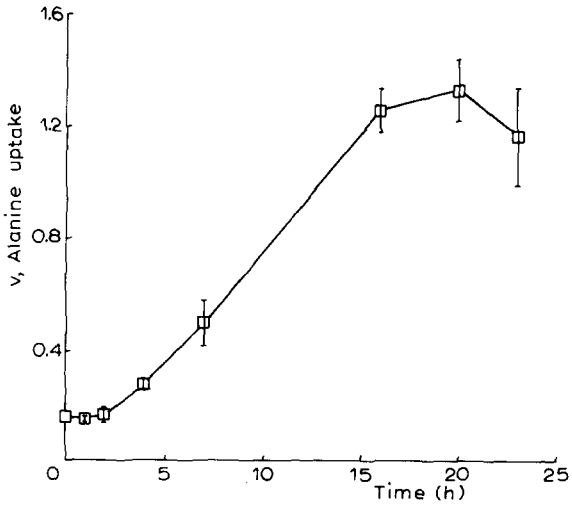

Fig. 1. Time-dependent changes in alanine transport after the addition of phytohaemagglutinin-P of quiescent peripheral pig lymphocytes. Initial rates (1 min assay) of uptake of 0.2 mM alanine were performed at 37°C at the indicated time. The values are shown with the standard deviation of the mean for four independent determinations.

Inability of System ASC to accept Li^+ for Na^+ substitution

It should be also noted that neither System A (as a methylaminoisobutyric acid-inhibitable component of proline uptake), nor System ASC (as a methylaminoisobutyric acid-non-inhibitable component of alanine entry) transport activity of stimulated cells can use Li^+ as a substitute for Na^+ (data not presented here). Our observations are in contrast with previous reports that Li^+ can be substituted for Na^+ for System A in Ehrlich ascites tumour cells [14], and for System ASC [15] and System N [16] in rat hepatocytes, whether freshly isolated or in primary culture. In agreement with our results, however, it has been reported that in pigeon erythrocytes, rabbit reticulocytes [17] and Ehrlich ascites tumour cells [14] little or no tolerance of Li^+ was seen for System ASC.

Time-course of effect of phytohaemagglutinin-P on alanine uptake

When phytohaemagglutinin-P, at the optimum mitogenic concentration, was added to cultures of quiescent pig lymphocytes, the transport of alanine, measured under conditions approaching the initial rate of entry, increased with time of lectin exposure (Fig. 1). The alanine entry increased as early as 2–4 h after addition of mitogen and reached a maximum after 18–24 h. Maximal values remained constantly high thereafter (up to 42 h after addition of lectins). This behaviour is reminiscent of the effect of phytohaemagglutinin-P on the uptake of proline (pertinent substrate of System A) in these cells [6].

Lack of inhibition of alanine uptake by methylaminoisobutyric acid

The rate of transport of a substrate across a membrane may be altered by the presence of other solutes. The use of inhibition analysis by other amino acids on the uptake of a specific substrate may reveal the existence of distinct transport systems.

Figs. 2a and 2b show the effect of increasing concentrations of methylaminoisobutyric acid on the uptake of alanine in quiescent and activated cells. A 2 mM concentration of the inhibitor does not reduce

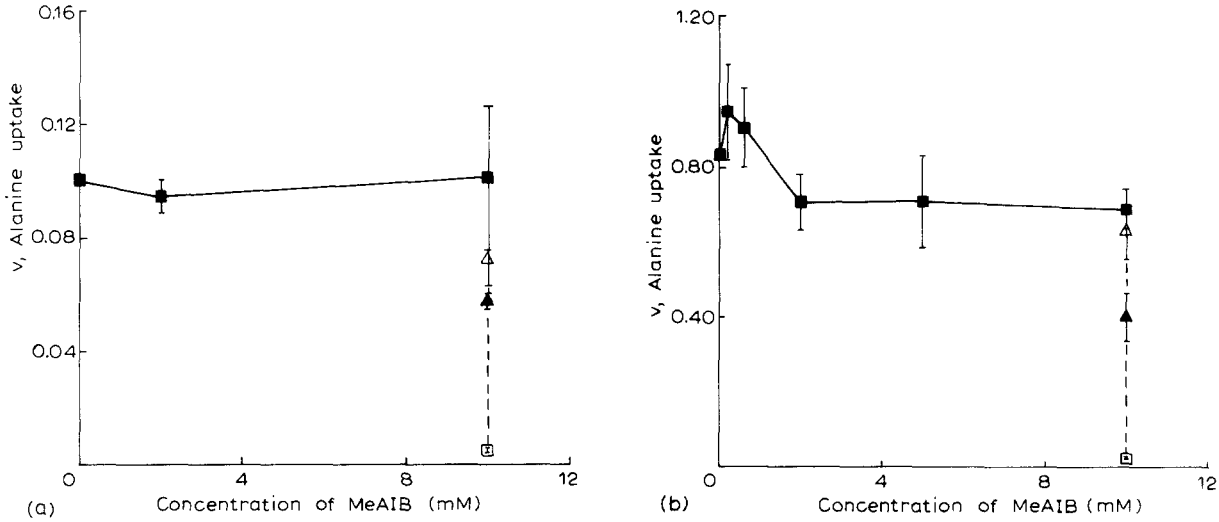

Fig. 2. Evidence for a two-carrier transfer system of alanine uptake. Apparent saturation of part of the uptake of alanine into quiescent (a) and into 20 h phytohaemagglutinin-P-activated (b) pig lymphocytes by methylaminoisobutyric acid (MeAIB) (■) at high extracellular concentrations, and inhibition of the unaffected part of uptake by further addition of 10 mM serine (□), or phenylalanine (▲) or lysine (△). The initial rate of 0.1 mM alanine uptake was measured for 1 min at 37°C. The values are shown with the standard deviation of the mean for three determinations.

uptake of 0.2 mM alanine significantly in quiescent cells, but reduces the uptake by one-sixth in stimulated lymphocytes. Increasing the inhibitor concentration to 10 mM had no obvious further effect, suggesting that the methylaminoisobutyric acid-inhibitable component of alanine transport (System A) had been saturated. The remaining Na^+-dependent component could be inhibited by the presence of other amino acids: the most effective appeared to be serine, known to be transported mainly by the ASC system in these cells (cf. Table I) as in other cell types [11]. Because a small component of uptake (less than 10% in quiescent and activated lymphocytes) was measured in the absence of Na^+ for alanine entry, these results suggest that one system (ASC) in quiescent and two systems (A and ASC) in stimulated cells contribute to the Na^+-dependent uptake of this amino acid, the residual uptake being accountable by the activity of Na^+-independent route (presumably system L). These conclusions should be taken to apply only at the low alanine concentrations used in this experiment. At higher substrate concentrations the contribution of different amino acid transport systems to the uptake of alanine might diverge from those mentioned above.

Effect of external pH

It has been reported that in Ehrlich ascites tumour cells [18] and rat hepatocytes [16,19] the greater sensitivity of System A to increases of $[H^+]$ can be used to differentiate System A and ASC. In Fig. 3 results are presented which compare the sensitivity to external pH of transport System A (estimated as Na^+-dependent methylaminoisobutyric acid uptake), ASC (as Na^+-dependent methylaminoisobutyric acid non-inhibitable component of alanine uptake) and L (as Na^+-independent component of alanine uptake) in activated lymphocytes. As shown, alanine uptake by System L decreases as the pH increases, while System A activity shows a remarkable sensitivity toward $[H^+]$ in lymphocytes, confirming previous results obtained in Ehrlich cells [20]. In contrast to expectation, the pH sensitivity of System ASC was rather high in lymphocytes: indeed the profile of alanine uptake by System ASC can be almost superimposed on the profile of System A. Thus the pH sensitivity does not appear to be a good criterion for differentiating the A and ASC transport systems in stimulated lymphocytes. The findings reported herein on the behaviour of System A and ASC toward change of $[H^+]$ are similar to those of Edmondson et

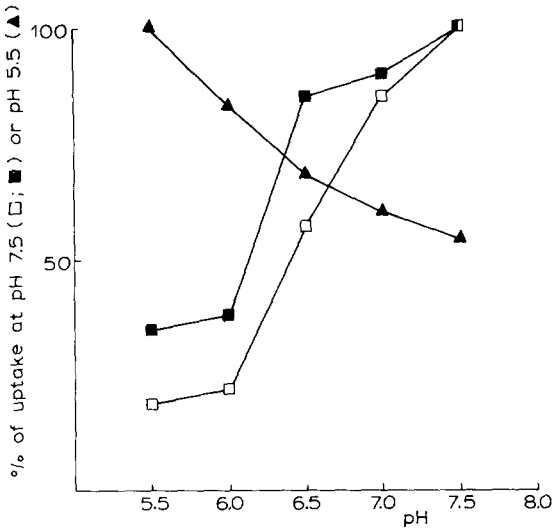

Fig. 3. Differences in pH sensitivity of A (□), ASC (■) and L (▲) components of neutral amino acid uptake in phytohaemagglutinin-P-activated pig lymphocytes. Uptake, during 1 min, of 0.1 mM alanine or methylaminoisobutyric acid was performed at 37°C in the incubation medium, with pH varying between 5.5 and 7.5. A, Na^+-dependent component of methylaminoisobutyric acid uptake. ASC, Na^+-dependent component of alanine uptake in the presence of 10 mM methylaminoisobutyric acid. L, Na^+-independent component of alanine uptake.

al. [15] who found similar pH sensitivity of both the A and ASC systems in freshly isolated rat liver cells. In quiescent pig lymphocytes the profiles of pH sensitivity of System L and ASC were almost identical to those obtained with stimulated lymphocytes.

Inhibition of alanine uptake by other amino acids

The specificity of transport System ASC was investigated by examining the ability of a number of naturally occurring amino acids and certain analogues to inhibit alanine uptake both in quiescent and activated lymphocytes. The results presented in Table II represent, under the conditions used, uptake through the ASC transport system. Generally, all amino acids, the side-chains of which are uncharged at neutral pH were good inhibitors. Amino acids with side-chains bearing a positive charge at neutral pH were very poor inhibitors, as were aminoisobutyric acid and histidine. Serine, cysteine and threonine caused the strongest inhibition, as expected for preferential substrates of the ASC route in several systems. Methionine, which has been considered an amino acid utilizing A and L systems in other cells, behaved as a strong inhibitor of ASC transport in lymphocytes. Substantial inhibition was also produced by amino acids with hydrophobic side-chains, such as leucine, tryptophan, valine and phenylalanine, all of which utilize preferentially the L transport system [11]. As expected, proline, which uses primarily the A transport route in pig lymphocytes, caused modest inhibition of alanine uptake. In agreement with our previous observations on the properties of the A system, the amino acid transport system ASC presents an unusually broad specificity in pig lymphocytes.

Initial rate kinetics

Initial velocities of alanine transport as a function of substrate concentration were measured in unstimulated and activated lymphocytes over a broad range of substrate concentrations. Fig. 4 shows the dependence of transport on substrate concentration for alanine in stimulated cells. A Eadie-Hofstee plot of the initial velocity of transport, v, against $v/[S]$ was

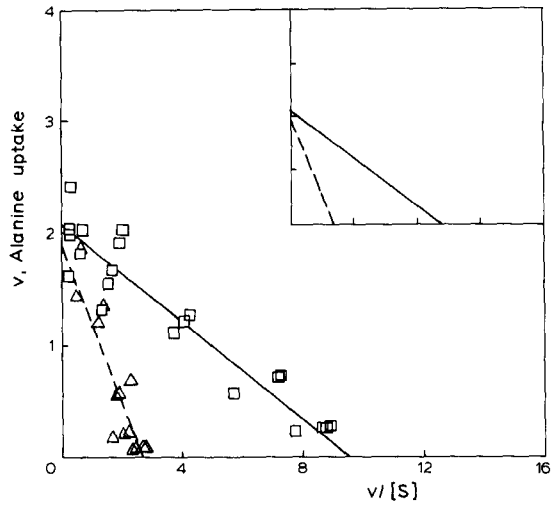

Fig. 4. Kinetic analysis of alanine uptake in 20 h phytohaemagglutinin-P-activated peripheral pig lymphocytes. Initial rates of uptake of alanine were determined in the absence (□) and in the presence (△) of inhibiting amino acid, and data, corrected for the diffusion component as described in the Experimental section, were analyzed by the Eadie-Hofstee method. Computer drawn lines were calculated by linear regression analysis. The range of alanine concentrations tested was 0.03 to 50 mM. Solid lines represent control and broken lines alanine uptake in the presence of 0.5 mM serine.

TABLE II

COMPARISON OF INHIBITORY EFFECTS OF AMINO ACIDS ON THE UPTAKE OF ALANINE (ASC SYSTEM) BY UNSTIMULATED AND PHYTOHAEMAGGLUTININ-P-ACTIVATED LYMPHOCYTES

The effects of individual amino acids, each added at 10 mM, on the initial rate of uptake of 0.1 mM labelled alanine in the presence of 10 mM methylaminoisobutyric acid are shown. Mean values (±S.D.) for three determinations are given.

Inhibitor	Alanine uptake (nmol/min per 10^7 cells)			
	Unstimulated		20 h phytohaemagglutinin-P-stimulated	
		Inhibition (%)		Inhibition (%)
None	0.125 ± 0.014	–	1.045 ± 0.075	–
Threonine	0.006 ± 0.001	95	0.030 ± 0.001	97
Cysteine	0.008 ± 0.003	94	0.030 ± 0.001	97
Serine	0.009 ± 0.001	93	0.050 ± 0.004	95
Methionine	0.018 ± 0.002	86	0.116 ± 0.014	89
Leucine	0.031 ± 0.002	75	0.200 ± 0.028	81
Glycine	0.040 ± 0.015	68	0.350 ± 0.123	67
Tryptophan	0.057 ± 0.007	54	0.339 ± 0.014	68
Valine	0.048 ± 0.003	62	0.364 ± 0.020	65
Proline	0.056 ± 0.009	55	0.551 ± 0.051	47
Phenylalanine	0.061 ± 0.001	51	0.556 ± 0.064	47
Histidine	0.084 ± 0.012	33	0.630 ± 0.019	40
Aminoisobutyric acid	0.103 ± 0.010	18	0.703 ± 0.048	33
Arginine	0.100 ± 0.008	20	0.798 ± 0.093	24
Lysine	0.111 ± 0.009	11	0.987 ± 0.160	6

linear over the entire range of substrate concentrations tested. This result is consistent with the hypothesis of alanine uptake by a single transport agency (ASC system) in activated lymphocytes. Analysis of the data gives an apparent K_m of 0.218 mM and a V of 2.074 nmol/min per 10^7 cells (mean ± S.D. of nine experiments: $K_m = 0.202 \pm 0.061$ mM and $V = 2.043 \pm 0.610$ nmol/min per 10^7 cells). In the same figure it is reported the plot of alanine uptake in the presence of inhibiting amino acid serine (pertinent substrate of ASC system) that appeared of the competitive type, indicating that in these cells serine and alanine share a common Na^+-dependent transport system. Similar results, not presented here, have been obtained with cysteine and threonine, ASC system-specific substrates that behaved as strong competitive inhibitors of alanine uptake in these cells. Negligible or none effect at all was obtained by the presence of methylaminoisobutyric acid (characterizing substrate of the A system), phenylalanine (preferential substrate of the L system) or arginine (which utilizes the Ly^+ system *) on both kinetic constants of alanine transport (results not shown).

In quiescent lymphocytes, in contrast, the plot of v against $v/[S]$ was curvilinear. This can be explained by the contribution of at least two independent families of carriers with different affinities, as described previously for amino acid transport in embryonic heart cells [21] and freshly isolated rat hepatocytes [22]. Fig. 5a shows the resolution of the experimental curve for alanine transport into two linear components: the computer analysis revealed that a high affinity-low capacity and a low affinity-low capacity component contribute to the total saturable transport. Analysis of the data of Fig. 5a gives an apparent K_m of 0.219 mM and a V of 0.392 nmol/min per 10^7 cells (mean ± S.D. of five experiments: K_m 0.178 ± 0.053 mM and $V = 0.260 \pm 0.086$ nmol/min per 10^7 cells) for the high affinity component

* Ly^+ system, Na^+-dependent transport system for basic amino acids such as lysine and arginine (see Ref. 11).

and an apparent K_m of 7.01 mM and V of 0.460 nmol/min per 10^7 cells (mean ± S.D. of five experiments: K_m 4.019 ± 1.778 and $V = 0.353 \pm 0.154$ nmol/min per 10^7 cells) for the low affinity component of alanine transport. The presence of methylaminoisobutyric acid produced negligible effects on alanine entry (data not presented). In the same figure it is shown how serine modified in a competitive manner the high affinity components of alanine transport, indicating that this amino acid shares the same transport route as alanine in quiescent cells (in the inset the effect of the inhibiting amino acid on the two resolved linear components of total uptake in presented). In Fig. 5b it is noteworthy that phenylalanine modified in a non-compititive manner the high affinity component. The reason for the discrepancy between results of phenylalanine inhibition of kinetic constants of alanine entry in quiescent and in activated cells is not readily apparent, since in both cell types we are dealing with a presumptive ASC component of alanine transport; it might be explained by considering that the several-fold greater capacity of the ASC component in phytohaemagglutinin-P-treated cells was too large to be modified in any significant way by a phenylalanine non-competitive effect, while in quiescent cells the transport of alanine through the ASC system (i.e., the high affinity component) displayed low capacity, thus allowing the phenylalanine effect to become visible. Furthermore, the competitive inhibition by phenylalanine on the low affinity component of alanine transport might suggest that this low affinity component resembles System L. Arginine showed only modest competitive effects on both the kinetic parameters of the two transport components of alanine entry (result not shown).

Taken together, the results presented in this section suggest that the affinity of the amino acid transport system ASC did not change appreciably following lectin stimulation (from a K_m value of 0.178 mM in quiescent to 0.202 mM in stimulated cells for the high affinity component). On the other hand, a large increase in the value of maximal velocity of alanine transport (from a V value of 0.260 in quiescent cells to 2.043 nmol/min per 10^7 cells in activated lymphocytes) may explain the large stimulation of amino acid uptake through the ASC system observed following activation.

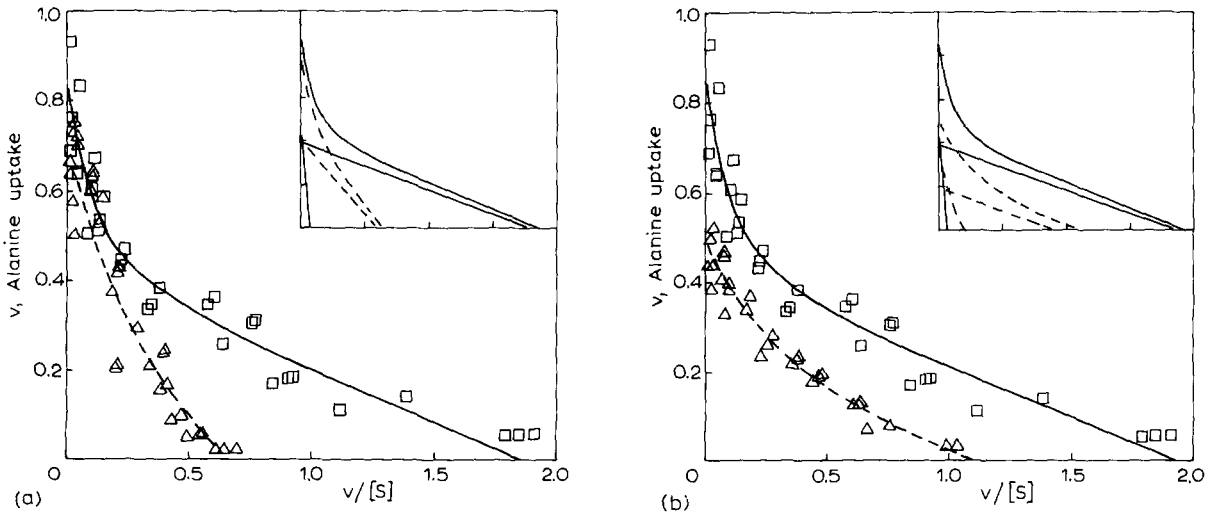

Fig. 5. Kinetic analysis of alanine uptake in quiescent peripheral pig lymphocytes. Initial rates of uptake of alanine were determined in the absence (□) and in the presence (△) of the inhibiting amino acid, and data were corrected for the diffusion component as described in the experimental section. The range of alanine concentrations tested was 0.03 to 50 mM. Lines relating the variation of initial velocity of alanine transport to the ratio of velocity to substrate concentration ($v/[S]$) were drawn according to the fitting of the data obtained by computer analysis (see the Experimental section). Inset: the two Michaelis-Menten components obtained after resolution of the curvilinear plots by computer analysis were presented. For both curvilinear and linear plots, solid lines represent control and broken lines alanine uptake in the presence of 0.5 mM serine (a) and 5 mM phenylalanine (b).

Effect of Na^+ on alanine entry

Fig. 6 shows the relationship between the external Na^+ concentration and the entry of alanine measured at low substrate concentration, a condition in which alanine transport occurs predominantly through the high affinity-low capacity component (ASC route) in quiescent cells and entirely through the high affinity-high capacity component (ASC system) in activated lymphocytes. The linear relationship observed when v was plotted against $v/[Na^+]$ indicates a first-order dependence of alanine transport on $[Na^+]$ in both cell types. Upon more detailed analysis of the data, the comparison between quiescent and stimulated lymphocytes shows a strong difference in V (from 0.267 in quiescent to 1.430 nmol/min per 10^7 cells in stimulated cells) but only a modest change in the K_m for Na^+, from 59 to 33 mM, was observed following activation.

Fig. 7a depicts the effect of reducing the $[Na^+]$ on the two components of alanine influx in quiescent cells. When Na^+ has been reduced from 136 to 36 mM, the K_m values of both components were slightly modified (see inset of this figure); on the other hand, the V values were not modified by varying the exter-

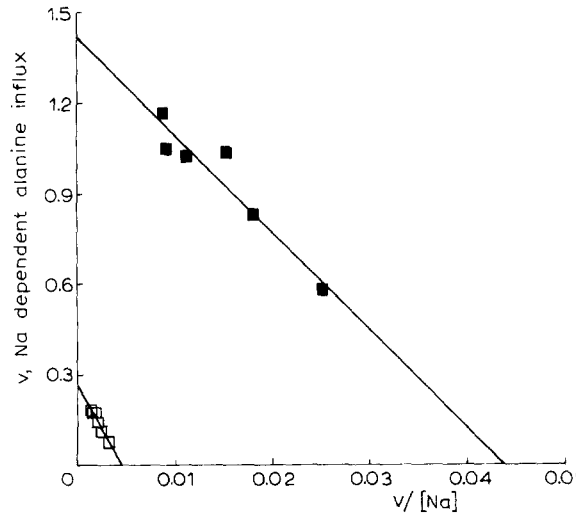

Fig. 6. Dependence of alanine transport of the Na^+ concentration in quiescent (□) and 20 h-activated (■) peripheral pig lymphocytes. The concentration of Na^+ was varied between 23 and 132 mM, and choline chloride replaced Na^+ to maintain the correct osmolarity. Alanine uptake measured at 0.1 mM for 1 min, has been plotted against the ratio of velocity to Na^+ concentration. Each point is the mean of triplicate determinations.

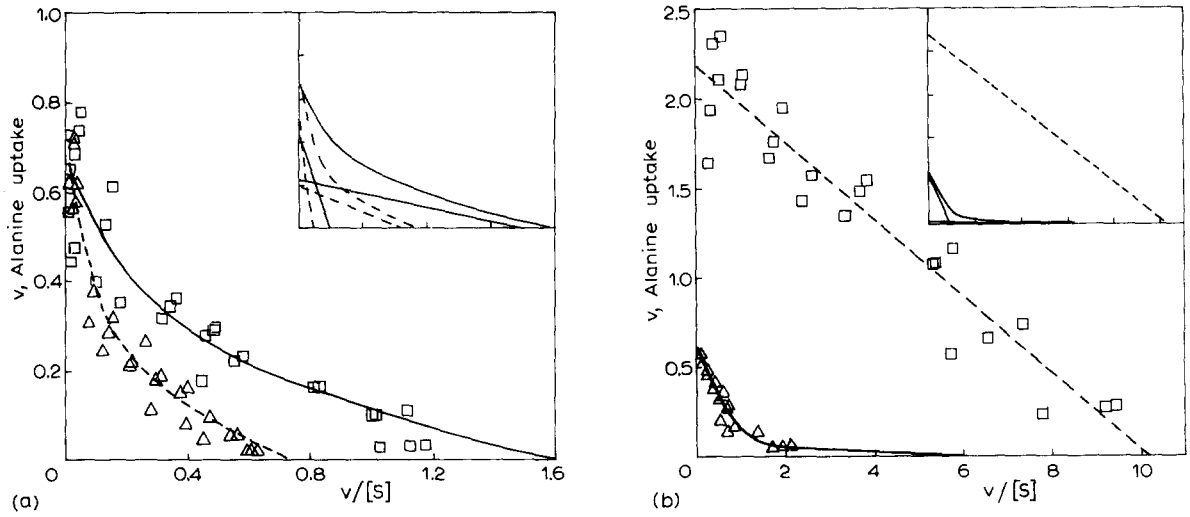

Fig. 7. Effect of Na^+ on the substrate concentration dependence of alanine transport in quiescent (a) and 20 h-activated (b) peripheral pig lymphocytes. The relationship between alanine entry and external substrate concentration was studied as described in the legend of Figs. 4 and 5, in media containing 36 (△) or 136 (□) mM Na^+. Osmolarity was kept constant by replacing Na^+ with choline. Inset: the two Michaelis-Menten components obtained after resolution of the curvilinear plots by computer analysis for quiescent or activated cells were presented. Solid lines represent alanine uptake at 136 (a) or 36 (b) mM Na^+ concentrations and broken lines represent alanine uptake at 36 (a) or 136 (b) mM Na^+ concentrations.

nal [Na^+]. In activated lymphocytes (Fig. 7b) with decreasing [Na^+] the transport of alanine changed abruptly: the V value of the high affinity component decreased in this experiment from 2.188 to 0.041 nmol/min per 10^7 cells. It is worth noticing that in stimulated lymphocytes the apparent single linear component of alanine entry resulted, at low Na^+ concentration, in two components, one with high (K_m = 0.04 mM) and the other with low (K_m = 0.61 mM) affinity. It should also be recognized that these two component emerging at low [Na^+] had kinetic constants different from those of stimulated or quiescent cells as measured at physiological [Na^+]. Thus sodium appears to affect both the affinity of the carrier site for alanine molecules and the rate of translocation of a ternary complex (carrier-substrate-Na^+) across the plasma membrane.

Exodus of alanine

The increased rate of alanine uptake in stimulated lymphocytes may be due to a decreased exodus from the cells. The effect of the lectin-treatment on the exodus of alanine was examined by preloading the cells for ten minutes with labelled alanine. Following washing and resuspending the cells, the remaining intracellular alanine was determined as a function of time. Fig. 8 shows the fractional exodus of alanine from quiescent and activated pig lymphocytes preloaded with alanine. The plot of the natural logarithm of the remaining intracellular alanine versus time of exodus yielded straight lines, suggesting that exodus processes followed first-order kinetics in both types of cell, and indicating that a single transport system mediated alanine exodus or, if two transport routes were involved, the rate constants should be almost identical for both components of exodus. The same figure shows also that phytohaemagglutinin-P treatment did not decrease the exodus of alanine in these cells: on the contrary, a slight but definite increment in the rate of exodus was regularly found following mitogenic activation. A corresponding difference in the rate of amino acid exodus between unstimulated and concanavalin A-activated rat lymphocytes has been reported previously [4].

Exchange properties of the ASC system

Because the amount of the labelled amino acid taken up by a cell may be the sum of net entry and exchange phenomena, we investigated the feed-back controls due to the internal amino acid level on alanine transport in lymphocytes and whether or not phytohaemagglutinin-P treatment had any effect on it. As shown in Fig. 9, in the presence of alanine or serine preaccumulated intracellularly, a trans-stimulation of labelled alanine transport was evident in phytohaemagglutinin-P-treated cells. With cells preloaded with proline (substrate of the A system) or arginine (System Ly^+) such an effect was not obtained, but a slight transinhibition of alanine entry was found. With preaccumulated phenylalanine (System L) no difference in uptake of alanine resulted in comparison to control. In conclusion, these results show that exchange phenomena are operative in these cells and lymphocytes show trans-stimulatory effects due to preaccumulated amino acids provided they are transported by the same transport system ASC. Since alanine uptake in our cells has usually been measured after extensive depletion of intracellular amino acids (see the Experimental section), i.e., when exchange phenomena are not operative, the enhanced activity of amino acid transport system ASC seen following

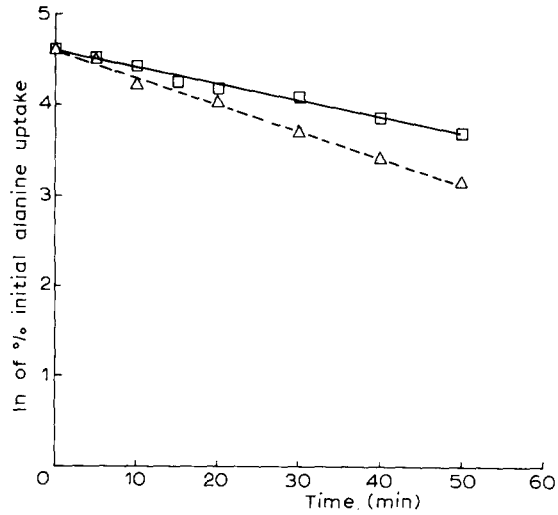

Fig. 8. Time course of alanine efflux. Quiescent (□) and 20 h-stimulated lymphocytes (△) were preincubated for 10 min with 0.2 mM labelled alanine. Cells were washed once and immediately resuspended in excess of alanine-free medium to minimize reuptake. The amount of alanine remaining in the cells was determined at the time indicated, and is expressed as the natural logarithm of the percentage of the amount of alanine present in lymphocytes at zero times. Each point is the mean of triplicate determinations.

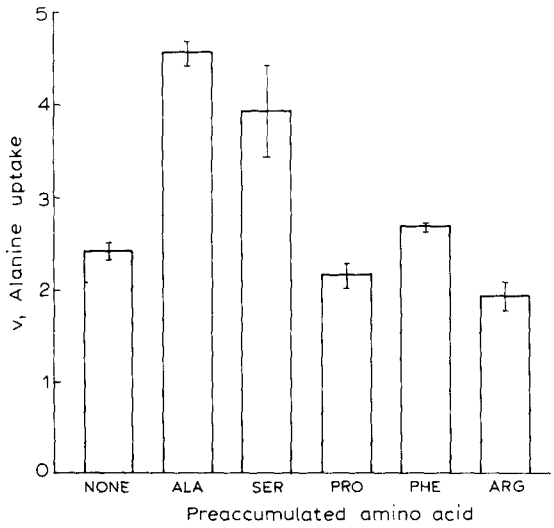

Fig. 9. Dependence of alanine transport by preaccumulated amino acids in activated lymphocytes. 20 h phytohaemagglutinin-P-treated cells have been preincubated for 15 min at 37°C with unlabelled alanine, serine, proline, phenylalanine or arginine all at 2 mM. Following washing at 0°C and resuspension of the cells in standard buffer, the initial velocity assay (1 min) of labelled alanine uptake at 0.2 mM was determined on 10^7 cells, as described in the Experimental section. The values shown are the mean ± S.D. of four independent determinations.

mitogenic stimulation should be considered the result of increased entry of alanine.

Discussion

Our previous results [6] have indirectly suggested that a second Na^+-dependent transport system for neutral amino acids other that the A system was active in unstimulated pig lymphocytes and that entry by this route was accelerated after incubation with lectins. The present study was designated to investigate further this process in quiescent and stimulated cells and to characterize the role and the properties of this transport system. The results of the present investigation formally indicate the presence of an ASC transport system in these cells, as demonstrated by the following observations: (a) transport through this agency was strictly dependent on the presence of Na^+ and remarkably sensitive to cation substitution; (b) good substrates for this system were alanine, serine, cysteine and threonine, known pertinent amino acids for the ASC system in other cells [11,18]; (c) the value of the ASC apparent affinity constant (K_m) is greatly different (almost one order of magnitude lower) from that of the A system; (d) kinetic analysis of inhibition experiments have indi-

TABLE III

A SUMMARY OF THE DIFFERENTIATING CHARACTERISTICS OF SYSTEMS A AND ASC IN PIG LYMPHOCYTES

The transport Systems A and ASC have been compared by using as substrates the two system-specific amino acids listed.

Properties under test	A (proline)	ASC (alanine)
1 Ion dependence:		
(a) Na^+-dependence	Yes	Yes
(b) tolerance to Li^+ substitution	No	No
2 Inhibition by 2-methylaminoisobutyric acid	Yes	No
3 Inhibition by increasing H^+	Yes	Yes
4 Sensitivity to feedback controls:		
(a) transtimulation	No	Yes
(b) transinhibition	Yes	No
5 Enhancement after mitogenic activation	Yes	Yes
6 Apparent affinity constant (K_m) values (mM)	1.5–2	0.15–0.25
7 Substrate specificity	broad	broad

cated that methylaminoisobutyric acid behaved as non-competitive but serine, cysteine and threonine acted as strong competitive inhibitors of alanine uptake; (e) in contrast to System A, which appears to be modulated by transinhibition phenomena in a variety of animal cells [11] including pig lymphocytes, System ASC is regulated by trans-stimulatory phenomena in the course of both entry and exodus of pertinent substrates. This is another property, besides intolerance to *N*-methylation, different kinetic constant and substrate specificity, that characterizes the activity of the ASC system in pig lymphocytes and allows its discrimination from the A system.

In contrast with properties first shown in other cells [17,18], System ASC of lymphocytes displays a marked sensitivity to $[H^+]$ which was very similar to the well recognized sensitivity of System A. Furthermore, we could not use lithium to replace sodium to elicit directly the contribution of the ASC system in lymphocytes, as claimed for the ASC system in freshly isolated liver cells [14], because no cation-dependent uptake of alanine was evident in the presence of lithium. In spite of the high substrate specificity found for the ASC system in other cells [11], we confirmed our previous observation on the wide specificity of the Na^+-dependent amino acid systems in these cells.

Table III summarizes the cumulative evidence that System ASC may be separated from the A system and displays unique properties in peripheral pig lymphocytes.

It should also be recognized that the ASC system of the quiescent cells was almost entirely responsible for the Na^+-dependent uptake of neutral amino acids, the activity of System A being extremely low or absent (Table I). In 20 h-stimulated cells, the activity of the ASC system increased several-fold after mitogen addition, with the concomitant emergency of the A transport system activity.

In quiescent cells, analysis of the relationship between initial velocity of alanine transport and substrate concentration revealed that more that one Michaelis-Menten component contributes to alanine transport. Quantitative treatment of the data using a curve-fitting method and a computer analysis allowed for a model compatible with two transport components that contribute to total uptake. By inhibition analysis it was possible to ascribe to System ASC (because markedly inhibited in a competitive way by pertinent substrates as serine, cysteine and threonine) the high affinity component. On the other hand, the large increase in the capacity of the high affinity component without substantial change in the apparent affinity constant K_m could have masked, in stimulated cells, the actual contribution carried to total uptake by the low affinity component, if still present: therefore, computer-made kinetic analysis resulted, in activated cells, in only one detectable linear component, the ASC system.

These results compare well with the accelerated activity of the ASC system previously observed by us [6] but utilizing non pertinent substrates, and may suggest that the large increases in System A and ASC activities are important or even essential for subsequent steps in lymphocyte activation. The possible causal or coincidental relation of the accelerated Na^+-dependent transport of neutral amino acids with the proliferative programme requires further investigation.

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References

1 Ling, N.R. and Kay, J.E. (1975) Lymphocyte Stimulation, 2nd edn., pp. 270–274, North-Holland Publishing Co., Amsterdam
2 Resch, K. (1976) in Receptors and Recognition (Cuatrecasas, P. and Greaves, M.F., eds.), pp. 59–117, Chapman and Hall, London
3 Mendelshon, J., Skinner, A. and Kornfeld, S. (1971) J. Clin. Invest. 50, 818–826
4 Van den Berg, K.J. and Betel, I. (1973) Exp. Cell Res. 76, 63–72

5 Greene, W.C., Parker, C.M. and Parker, C.W. (1976) J. Biol. Chem. 242, 4017–4025
6 Borghetti, A.F., Kay, J.E. and Wheeler, K.P. (1979) Biochem. J. 182, 27–32
7 Borghetti, A.F., Girit, E.Z. and Kay, J.E. (1979) Biochem. Soc. Trans. 7, 1007–1009
8 Wise, W.C. (1978) J. Cell. Physiol. 97, 161–168
9 Kay, J.E., Ahern, T., Lindsay, V.J. and Sampson, J. (1975) Biochim. Biophys. Acta 378, 241–250
10 Gazzola, G.C., Dall'Asta, V. and Guidotti, G.G. (1980) J. Biol. Chem. 255, 929–936
11 Guidotti, G.G., Borghetti, A.F. and Gazzola, G.C. (1978) Biochim. Biophys. Acta 515, 329–366
12 Feldman, H.A. (1972) Anal. Biochem. 48, 317–338
13 Christensen, H.N. (1979) Adv. Enzymol. Relat. Areas Mol. Biol. 49, 41–101
14 Christensen, H.N. and Handlogten, M.E. (1977) J. Membrane Biol. 37, 193–211
15 Edmondson, J.W., Lumeng, L. and Li, T.K. (1979) J. Biol. Chem. 254, 1653–1658
16 Kilberg, M.S., Handlogten, M.E. and Christensen, H.N. (1980) J. Biol. Chem. 255, 4011–4019
17 Thomas, E.L. and Christensen, H.N. (1967) J. Biol. Chem. 246, 1682–1688
18 Christensen, H.N., Liang, M. and Archer, E.G. (1967) J. Biol. Chem. 242, 5237–5246
19 Kilberg, M.S., Christensen, H.N. and Handlogten, M.E. (1979) Biochem. Biophys. Res. Commun. 88, 744–751
20 Garcia-Sancho, J., Sanchez, A. and Christensen, H.N. (1977) Biochim. Biophys. Acta 464, 295–312
21 Guidotti, G.G., Borghetti, A.F., Lüneburg, B. and Gazzola, G.C. (1971) Biochem. J. 122, 409–414
22 Fehlmann, M., Le Cam, A., Kitabgi, P., Rey, J.F. and Freychet, P. (1978) J. Biol. Chem. 254, 401–407