Biochimica et Biophysica Acta, 507 (1978) 165—174

BBA 77918

ADAPTIVE REGULATION OF AMINO ACID TRANSPORT IN CULTURED AVIAN FIBROBLASTS

INFLUENCE OF THE AMINO ACID COMPOSITION OF THE CULTURE MEDIA

VALERIA DALL'ASTA, GIAN C. GAZZOLA and GUIDO G. GUIDOTTI

Istituto di Patologia Generale, Universitá di Parma, Via Gramsci, 14 — 43100 Parma (Italy)

(Received July 18th, 1977)

Summary

The regulation of amino acid transport across the cell membrane by adaptive mechanisms has been studied in cultured chick embryo fibroblasts.

Time-dependent changes of transport activity by the A system (a Na^+-dependent agency with affinity for a discrete group of neutral amino acids), as a function of the composition in amino acids of the culture medium, have been evaluated by measurements of ^{14}C-labelled L-proline uptake under conditions approaching initial entry rates.

Results and conclusions based on the adopted experimental procedures include the following:

(1) Transport of L-proline in cultured avian fibroblasts is an inverse function of the concentration of amino acid substrates of system A in the medium used for cell culturing before uptake assay.

(2) Cells grown in media containing amino acids that are substrates for system A (repressive conditions) exhibit a marked increase of L-proline uptake upon incubation in media devoid of these amino acids (derepressive conditions).

(3) Cells grown in media containing amino acids which are not typical substrates of system A (derepressive conditions) undergo a definite decrease of L-proline uptake upon incubation in media supplemented with amino acid substrates of this agency (repressive conditions).

(4) The adaptive increases in the transport of L-proline observed when 'repressed' cells are incubated under depressive conditions, are abolished in the presence of cycloheximide.

(5) The results presented suggest that adaptive regulation of amino acid transport by system A is an intrinsic property of the cells and has a normal function in vivo. Its occurrence under culture conditions demands that one must consider the actual composition of amino acids in the growth medium when investigating amino acid transport in cultured cells.

Introduction

Nutritional regulations of amino acid transport have been found to operate in a variety of mesenchymal and epithelial cells from avian and mammalian tissues [1—7]. Among these regulations, amino acid-dependent adaptive control mechanisms appear to modulate the activity of the A transport system [8—11], a Na^+-requiring agency which is devoted to translocate neutral amino acids across the cell membrane [12,13]. Recent studies have emphasized the central role of system A as the transport system on which widely different factors and conditions may converge to regulate amino acid transport in eukaryotic cells [14,15].

Cells in culture are grown in media which differ extensively in the composition of their constituent amino acids. Some of them contain all the natural amino acids, others lack the 'non essential' amino acids (particularly those which are preferential substrates of transport system A). If adaptive regulation occurs in cultured cells, the concentration of relevant substrate amino acids in the cell environment should affect transport activity of the A system. When exposed to conditions of amino acid shortage the cells would respond with an increase of transport activity (adaptive derepression); when matched by conditions of abundant amino acid supply the cells would react with a decrease in transport activity (adaptive repression).

The purpose of this work was to verify the occurrence of the mechanism of adaptive regulation for amino acid transport by the A system in cultured avian fibroblasts and to investigate the relationship between amino acid composition of the medium and amino acid transport activity of the cells during their growth in culture.

Materials and Methods

The sources for most of the materials used are listed in preceding papers [5,8]. The following media (obtained from Gibco, N.Y., U.S.A. or made up in our laboratory) were used: (a) Medium 199 (M199) which contains all twenty of the naturally occurring amino acids and the amide derivative L-glutamine [16]; in this medium the total concentration of amino acids (L-form) which are taken up primarily or appreciably by transport system A in mesenchymal chick embryo cells [5] is approximately 2 mM. (b) Minimal essential medium which contains the twelve amino acids (plus glutamine) 'essential' for survival and growth of cultured cells [17]; this medium does not contain the amino acids which are considered typical substrates of the A system. (c) Minimal essential medium supplemented with the non-essential amino acids glycine, L-proline, L-alanine, L-serine, L-aspartic acid, L-glutamic acid and the amide derivative L-asparagine at the same concentrations present (as L-form) in M199; asparagine was 0.4 mM. (d) Minimal essential medium supplemented with a group of neutral amino acids: L-proline, glycine, L-alanine and L-serine (minimal essential medium + Pro-Gly-Ala-Ser) at the same concentrations present (as L-form) in M199. (e) Minimal essential medium supplemented with the non-metabolizable amino acid analogue α-aminoisobutyric acid at a concentration of 2 mM. (f) Earle's balanced salt solution, a salt mixture which contains

glucose (5.5 mM) but not amino acids [18]. Specific additions to these media are described in Results.

Chick embryo fibroblasts were obtained from 11-day old embryos by the method of Rubin [19]. Primary cultures were grown in plastic Roux flasks (Corning) in M199 with Earle's balanced salt solution containing 2% chicken serum. Conditions of culturing were: pH 7.5, atmosphere air/CO_2 (95 : 5, v/v), temperature 37°C. All experiments were performed with secondary cultures prepared by trypsinization and replating of 3-day old primary cultures. Secondary cultures were seeded at $4 \cdot 10^5$ cells per 25 cm^2 culture flasks and grown (a) for 48 h, as described above for primary cultures in either M199 or minimal essential medium supplemented with non-essential amino acids; and (b) for a subsequent 24 h in media of the desired amino acid composition supplemented with 2% dialysed chicken serum. The volumes of the media were such that decrements in the concentration of individual amino acids during 24 h culturing were less than 5% (as assessed by analysis on a Tecnicon amino acid analyzer). In some experiments, cell monolayers were rinsed free from growth medium and incubated for an additional 3 h at 37°C with the medium under study (see Results).

Initial rates of amino acid uptake were measured as follows: culture flasks with attached cells were washed three times with Earle's balanced salt solution to remove completely the amino acids present in the culture medium and incubated for 3 min at 37°C in Earle's balanced salt solution containing L-[^{14}C]proline (0.1 mM, final concentration). After two successive rinses with ice-cold Earle's balanced salt solution, monolayers were dissolved in 1 M NaOH solution containing 0.3% sodium-deoxycholate at room temperature for 30 min. Aliquots of the resulting solution were used for radioactivity measurements in a Tri-Carb scintillation spectrometer and for protein determination [20]. Radiochromatogram scanning after thin-layer chromatography of cell extracts always gave a single peak which corresponded to proline. The methods for determining the intracellular accumulation of the tracer amino acid and for evaluating the proper corrections to be introduced were as described by Guidotti et al. [21,22].

Changes in the concentration of individual amino acids in culture media were monitored from samples subject to complete analysis by the amino acid analyzer (Tecnicon).

Results

Choice of the tracer amino acid

Among a number of amino acids which are known to be taken up, primarily or appreciably, by the A system in mesenchymal chick embryo cells [1,5], L-proline was found to be the best natural substrate for this transport agency in cultured avian fibroblasts. Such identification was formally attained by means of competition experiments among natural amino acids, inhibition analysis by transport-specific model substrates (α-(methylamino) isobutyric acid, 2-amino-bicyclo(2,2,1)heptane-2-carboxylic acid), uptake measurements in the presence and absence of Na^+ (or Li^+) and definition of pH profiles for uptake (cf. ref. 14). Within the limits of this discriminating analysis, L-proline was adopted

as a tracer amino acid to evaluate adaptive changes of amino acid transport activity by the A system, as a function of the different experimental conditions.

Amino acid composition of culture media affects amino acid transport

The composition of amino acids in different culture media in which avian fibroblasts have been grown for 24 h before measurements of L-[^{14}C]proline uptake (performed in Earle's balanced salt solution under conditions approaching initial rates of entry), had a profound influence on the transport of this amino acid. As shown in Table I, transport activity was higher in cells cultured in minimal essential medium (devoid of the typical amino acid substrates of system A) than in cells cultured in M199 (containing all the naturally occurring amino acids). When minimal essential medium was supplemented with the neutral amino acids L-proline, glycine, L-alanine and L-serine (substrates of the A system with a total concentration of 1.5 mM) transport activity decreased by about 30%, and became comparable to that observed in fibroblasts cultured in M199 when all non-essential amino acids were added to minimal essential medium (total concentration of added amino acids, 2.6 mM). The addition to minimal essential medium of α-aminoisobutyric acid (a non-metabolizable analogue that is transported appreciably by system A

TABLE I

L-PROLINE UPTAKE BY CHICK EMBRYO FIBROBLASTS CULTURED IN MEDIA OF DIFFERENT AMINO ACID COMPOSITIONS

Secondary cultures of chick embryo fibroblasts were grown (a) for 72 h in M199 with or without (M199 (72 h)) a change of medium 24 h before uptake assay; (b) for 48 h in minimal essential medium supplemented with non-essential amino acids and for a subsequent 24 h in the following media of different amino acid compositions: minimal essential medium plus Pro-Gly-Ara-Ser, plus α-aminoisobutyric acid and alone; all these culture media contained 2% dialysed chicken serum; (c) for 72 h in minimal essential medium supplemented with non-essential amino acids without medium changes. Cell monolayers were rinsed free from growth medium with Earle's balanced salt solution. Amino acid transport was assayed by measurements of L-[^{14}C]proline uptake in 3-min incubations (initial velocity) at 37°C in Earle's balanced salt solution containing the labelled amino acid (0.1 mM, final concentration). The values are mean ± S.E. of 4 separate determinations.

Culture medium *	Uptake (μmol/min per ml cell water)	Percent difference from incubation in:	
		M199	Minimal essential medium
M199	0.22 ± 0.01	—	−48 ***
Minimal essential medium	0.42 ± 0.02	+91 ***	—
+Pro-Gly-Ala-Ser	0.28 ± 0.02	—	−33 ***
Supplemented with non-essential amino acids (48 h)	0.22 ± 0.01	—	−48 ***
+α-Aminobutyric acid	0.20 ± 0.02	—	−52 ***
Supplemented with non-essential amino acids (72 h)	0.32 ± 0.04	—	−24
M199 (72 h)	0.32 ± 0.03	+45 **	—

* Last 24 h of cell culturing except for conditions (6) and (7).

** $P < 0.05$.

*** $P < 0.01$.

in cultured fibroblasts, cf. ref. 15) at a concentration of 2 mM depressed transport activity even further.

When cells were grown for 72 h without medium changes in minimal essential medium supplemented with non-essential amino acids (or in M199), the concentration of amino acid substrates of system A in the culture medium (as assayed by means of the amino acid analyzer) decreased as a result of cellular utilization. Under these conditions, the uptake of L-proline was higher than that measured in cells cultured in the same media renewed 24 h before uptake assay (Table I) in order to minimize any change in amino acid concentration (see Materials and Methods). Decrements of less than 10% in the average concentration of amino acid substrates of system A in the culture medium were sufficient to sustain a definite stimulation of transport activity ($P < 0.05$). However, the concentration of amino acids in the vicinity of the cells might have decreased more than in the medium as a whole, due to the formation of unstirred layers; if this were the case, larger decreases could have been responsible for the observed adaptive changes. This possibility was ruled out by experiments in which cell culturing was carried out under continuous mild swinging. Stepwise increases of the swinging rate (compatible with medium stirs that did not detach cells from the plastic surface of the culture flasks) did not affect changes in amino acid concentration in the medium, or alter adaptive changes of amino acid transport as determined in motionless cultures.

In attempt to reproduce a situation of amino acid shortage comparable to that described in the preceding paragraph, but under more strictly controlled experimental conditions, cells were grown for 24 h before uptake assay in the presence of decreasing concentrations of amino acid substrates of system A. The volume ratios of culture-medium to cell mass were such that the selected amino acid concentrations (obtained by mixing minimal essential medium unsupplemented and supplemented with non-essential amino acids in different proportions) were quite constant during the 24 h culturing. This approach showed that the transport of L-proline increased progressively as the concentration of amino acid substrates of system A during cell culturing was lowered. A statistically significant effect on transport activity ($P < 0.05$) was first obtained with a 10% decrease of substrate concentrations in the culture medium and became highly significant ($P < 0.01$) for larger decreases.

Adaptive regulation

When cells grown in M199 (repressive condition) were subsequently incubated in media devoid of amino acids (Earle's balanced salt solution) or containing amino acids which are not typical substrates of the A system (minimal essential medium), the transport of L-proline increased markedly and attained comparably high values (Table II). This increase was prevented by the addition of natural (L-alanine) or analogue (α-aminoisobutyric acid) amino acid substrates of system A to the amino acid-free Earle's balanced salt solution.

When cells grown in minimal essential medium (derepressive condition) were subsequently incubated in a medium devoid of amino acids (Earle's balanced salt solution), no substantial changes in L-proline uptake were detected (Table III). The addition of the model amino acid α-(methylamino)isobutyric

TABLE II

CHICK EMBRYO FIBROBLASTS CULTURED IN M199. CHANGES IN L-PROLINE UPTAKE UPON INCUBATION IN MEDIA OF DIFFERENT AMINO ACID COMPOSITIONS

Secondary cultures of chick embryo fibroblasts were grown for 72 h in M199 (with a medium change at 48 h, see Materials and Methods). Cell monolayers were then rinsed free from growth medium and incubated for 3 h in the different media (all supplemented with 2% dialysed chicken serum). Incubations were at 37°C in an atmosphere of air/CO_2 (95 : 5, v/v). At the end of incubation, cultures were washed exhaustively with Earle's balanced salt solution and amino acid transport was assayed by L-[^{14}C]proline uptake measurements in 3-min incubations (initial velocity) at 37°C in Earle's balanced salt solution containing the labelled amino acid (0.1 mM, final concentration). The values are mean ± S.E. of 3 separate determinations.

Incubation medium	Uptake (μmol/min per ml cell water)	Percent difference from incubation in M199	P
M199	0.24 ± 0.01	—	—
Minimal essentital medium	0.41 ± 0.02	+71	<0.01
Earle's balanced salt solution	0.40 ± 0.02	+67	<0.01
+L-Alanine (5 mM)	0.22 ± 0.01	−8	(n.s.) *
+α-Aminoisobutyric acid (2 mM)	0.18 ± 0.02	−25	(n.s.)

* n.s., not significant.

acid (typical substrate of the A system in cultured avian fibroblasts) to minimal essential medium or of the natural amino acid L-alanine to amino acid-free Earle's balanced salt solution markedly depressed transport activity. On the contrary, the addition of L-leucine or L-lysine (preferential substrates of transport systems L and Ly^+, respectively), did not affect L-proline uptake.

When cells were frown in minimal essential medium plus α-aminoisobutyric acid (a culture medium in which repressive conditions were obtained by the addition of the non-metabolizable amino acid analogue α-aminoisobutyric acid) (cf. refs. 1 and 5), a subsequent incubation in minimal essential medium or Earle's balanced salt solution (both devoid of the pertinent substrates of system A) enhanced transport activity (Table IV). Incubations in media containing all

TABLE III

CHICK EMBRYO FIBROBLASTS CULTURED IN MINIMAL ESSENTIAL MEDIUM. CHANGES IN L-PROLINE UPTAKE UPON INCUBATION IN MEDIA OF DIFFERENT AMINO ACID COMPOSITIONS

Secondary cultures of chick embryo fibroblasts were grown for 48 h in M199 and for a subsequent 24 h in minimal essential medium (see Materials and Methods). Cell monolayers were then rinsed free from growth medium and incubated for 3 h in the different media (all supplemented with 2% dialysed chicken serum). Incubation conditions and assay of L-[^{14}C]proline uptake were as described in the legend of Table II. The values are mean ± S.E. of 3 separate determinations.

Incubation medium	Uptake (μmol/min per ml cell water)	Percent difference from incubation in minimal essential medium	P
Minimal essentital medium	0.41 ± 0.02	—	—
+α-(Methylamino)isobutyric acid (2 mM)	0.25 ± 0.02	−39	<0.01
Earle's balanced salt solution	0.43 ± 0.01	+5	(n.s.)
+L-Alanine (5 mM)	0.26 ± 0.02	−37	<0.01
+L-Leucine (5 mM)	0.42 ± 0.03	+2	(n.s.)
+L-Lysine (5 mM)	0.41 ± 0.03	0	(n.s.)

TABLE IV

CHICK EMBRYO FIBROBLASTS CULTURED IN MINIMAL ESSENTIAL MEDIUM SUPPLEMENTED WITH α-AMINOISOBUTYRIC ACID. CHANGES IN L-PROLINE UPTAKE UPON INCUBATION IN MEDIA OF DIFFERENT AMINO ACID COMPOSITIONS

Secondary cultures of chick embryo fibroblasts were grown for 48 h in M199 and for a subsequent 24 h in minimal essential medium plus α-aminobutyric acid (see Materials and Methods). Cell monolayers were then rinsed free from growth medium and incubated for 3 h in the different media (all supplemented with 2% dialysed chicken serum). Incubation conditions and assay of L-[^{14}C]proline uptake were as described in the legend of Table II. The values are mean ± S.E. of 3 separate determinations.

Incubation medium	Uptake (μmol/min per ml cell water)	Percent difference from incubation in minimal essential medium + α-aminoisobutyric acid	*P*
Minimal essential medium + α-aminoisobutyric acid	0.20 ± 0.02	—	—
Minimal essential medium	0.39 ± 0.04	+95	<0.01
M199	0.23 ± 0.04	+7	(n.s.)
Earle's balanced salt solution	0.39 ± 0.04	+95	<0.02
+ α-Aminoisobutyric acid (2 mM)	0.19 ± 0.02	−4	(n.s.)

naturally occurring amino acids (M199) or a sufficient concentration of α-aminoisobutyric acid (Earle's balanced salt solution plus α-aminobutyric acid) did not alter appreciably the activity of transport observed under repressive conditions.

TABLE V

EFFECTS OF CYCLOHEXIMIDE ON CHANGES IN L-PROLINE UPTAKE BY CULTURED FIBROBLASTS INCUBATED IN MEDIA OF DIFFERENT AMINO ACID COMPOSITIONS

Secondary cultures of chick embryo fibroblasts were grown for 48 h in M199 and for a subsequent 24 h in M199 or in minimal essential medium plus α-aminoisobutyric acid (see Materials and Methods). Cell monolayers were then rinsed free from growth medium and incubated for 3 h in the different media (all supplemented with 2% dialysed chicken serum) in the absence and presence of cycloheximide (5 μg/ml, final concentration). Incubation conditions and assay of L-[^{14}C]proline uptake were as described in the legend of Table II. The values are mean ± S.E. of 3 separate determinations.

Incubation medium	Uptake (μmol/min per ml cell water)	Percent difference from incubation in growth medium	*P*
Cells grown in M199			
M199	0.24 ± 0.02	—	—
Earle's balanced salt solution	0.40 ± 0.02	+67	<0.01
+Cycloheximide	0.21 ± 0.04	−12	(n.s.)
+Cycloheximide + α-amino-isobutyric acid (2 mM)	0.15 ± 0.01	−37	<0.05
Cells grown in minimal essential medium + α-aminoisobutyric acid			
Minimal essential medium + α-aminoisobutyric acid	0.20 ± 0.01	—	—
Earle's balanced salt solution	0.39 ± 0.02	+95	<0.01
+Cycloheximide	0.19 ± 0.02	−4	(n.s.)
+Cycloheximide + α-amino-isobutyric acid (2 mM)	0.16 ± 0.01	−20	(n.s.)

Effects of cycloheximide

Cells grown under repressive conditions (M199 or minimal essential medium plus α-aminoisobutyric acid) exhibited the usual increases in transport activity after subsequent incubation in a medium (Earle's balanced salt solution) devoid of amino acids (Table V). When cycloheximide was added to the latter medium at a concentration sufficient to abolish protein synthesis, these increases in transport activity were prevented. When α-aminoisobutyric acid was added together with cycloheximide, transport activity was repressed even further, suggesting that the degradation (or inactivation) of specific protein components was accelerated during operation of the transport system (cf. ref. 23).

Discussion

The foregoing experiments demonstrate that alterations in the amino acid composition of media during cell culturing result in dramatic changes in the activity of transport system A, an Na^+-dependent agency with affinity for a discrete group of neutral amino acids [13,24,25]. These changes appear to reflect the occurrence in cultured fibroblasts of amino acid-dependent adaptive control mechanisms comparable to those formerly described in a variety of mesenchymal and epithelial cells obtained directly from avian and mammalian tissues [1,5,8—11].

An adaptive regulation of amino acid transport in fibroblasts brought into suspension by a collagenase treatment from growing and quiescent cultures has been described recently [26]. Elsas and co-workers were unable to detect this control mechanism in aggregates of cultured chicken heart cells and suggested, as a tentative interpretation, that the rapidly changing transport properties observed in collagenase-dissociated cells upon incubation in amino acid-free media could have resulted from repair processes initiated by proteolytic enzymes [27]. That this is not the case has been shown by experiments in which an adaptive regulation of amino acid transport has been found to occur in intact tissues [1—4,6,9] and in isolated cells never exposed to proteolytic enzymes [5,11,28]. The results of the present experiments, performed with cultured fibroblasts still attached to the plastic surface of the flask (therefore without any exposure to collagenase), indicate that adaptive regulation is retained under culture conditions. It must be realized that amino acid uptake by cultured cell monolayers is also subject to a serum-mediated control mechanism [7,15]. The activity of transport system A is markedly stimulated after the addition of serum to serum-deprived cultured cells and the resulting enhancement declines rapidly after serum withdrawal [15]. In Elsas' experiments the cardiac cell aggregates, removed from a complete culture medium containing serum, were incubated in an amino acid-free balanced salt solution in the absence of serum [27]. Under these conditions, it is likely that the expected adaptive enhancement of amino acid uptake was superseded by the marked decrease of transport activity which follows serum withdrawal.

Evidence has been presented recently showing that the concentration of such amino acids as glutamine and α-aminoisobutyric acid in the growth medium affects the transport of glycine (a substrate of the A system) in cultured HeLa cells and in *Xenopus laevis* kidney cells [29]. Glutamine is known to be

reactive with systems A, L and ASC [30]. A slow regulation of the transport of cycloleucine (an amino acid analogue taken up in appreciable way by transport system A) by glutamine in BHK polyoma transformed cells (GIV variant) capable of growing in the absence of glutamine has been reported by Kalckar et al. [31].

The results presented in this paper show that careful attention must be paid to the preceding 'history' of the culture when amino acid transport is studied in cultured cells. Availability of serum factors [15,32], conditions of cell density [33,34] and composition of amino acids in the culture medium (this paper) are all parameters that modulate the activity of transport system A.

The occurrence of adaptive control mechanisms for amino acid transport by system A in cells freshly isolated from tissues, i.e. in biological preparations of limited viability in vitro, can be revealed under extreme conditions (absence or presence of highly concentrated relevant amino acids for short time intervals, cf. refs. 1 and 5) which are far from those operating in vivo. In cell cultures, i.e., biological preparations of prolonged viability on which stimuli of low intensity can modulate cellular functions over extended periods of time, small alterations in the composition of pertinent amino acids (comparable to those which are likely to occur in vivo) are sufficient to elicit large changes in amino acid transport by means of system A. The results presented in this paper suggest that adaptive regulation of amino acid transport is an intrinsic property of the cells and has a normal function in vivo which, in combination with other control mechanisms (involving hormones, mediators, mitogens, etc.), enables the cell to adapt itself to the actual conditions of its environment.

Acknowledgements

The authors are indebted to Drs. G. Serventi and F. Ferrari for performing the measurements with the amino acid analyzer. This investigation was supported by the Consiglio Nazionale delle Ricerche and by the Ministero della Pubblica Istruzione, Roma, Italy.

References

1 Gazzola, G.C., Franchi, R., Saibene, V., Ronchi, P. and Guidotti, G.G. (1972) Biochim. Biophys. Acta 266, 407—421
2 Riggs, T.R. and Pan, M.W. (1972) Biochem. J. 128, 19—27
3 Smith, C.H., Adcock, E.W., Teasdale, F., Meschia, G. and Battaglia, F.C. (1973) Am. J. Physiol. 244, 558—564
4 Reynolds, R., Rea, C. and Segal, S. (1974) Science 184, 68—69
5 Guidotti, G.G., Gazzola, G.C., Borghetti, A.F. and Franchi-Gazzola, R. (1975) Biochim. Biophys. Acta 406, 264—279
6 Gusseck, D.J., Yuen, P. and Longo, L.D. (1975) Biochim. Biophys. Acta 401, 278—284
7 Borghetti, A.F., Tramacere, M. and Guidotti, G.G. (1977) Biochem. Soc. Trans. 5, 921—923
8 Gazzola, G.C., Franchi-Gazzola, R., Ronchi, P. and Guidotti, G.G. (1973) Biochim. Biophys. Acta 311, 292—301
9 Reynolds, R.A. and Segal, S. (1976) Biochim. Biophys. Acta 426, 513—525
10 Enders, R.H., Judd, M., Donohue, T.M. and Smith, C.H. (1976) Am. J. Physiol. 230, 706—710
11 Peck, W.A., Rockwell, L.H. and Lichtman, M.A. (1976) J. Cell. Physiol. 89, 417—428
12 Oxender, D. and Christensen, H.N. (1963) J. Biol. Chem. 238, 3686—3699
13 Christensen, H.N. (1969) Adv. Enzymol. 32, 1—20
14 Christensen, H.N. (1976) J. Cell. Physiol. 89, 603—604

15 Tramacere, M., Borghetti, A.F. and Guidotti, G.G. (1977) J. Cell. Physiol., in the press
16 Morgan, J.F., Morton, H.J. and Parker, R.C. (1950) Proc. Soc. Exp. Biol. Med. 73, 1—8
17 Eagle, H. (1959) Science 130, 432—437
18 Earle, W.R. (1943) J. Natl. Cancer Inst. 4, 165—212
19 Rubin, H. (1973) in Tissue Culture, Methods and Applications (Kruse, Jr., P.F. and Patterson, Jr., M.K., eds.), pp. 119—122, Academic Press, New York
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (151) J. Biol. Chem. 193, 265—275
21 Guidotti, G.G., Lüneburg, B. and Borghetti, A.F. (1969) Biochem. J. 114, 97—105
22 Guidotti, G.G., Borghetti, A.F., Lüneburg, B. and Gazzola, G.C. (1971) Biochem. J. 122, 409—414
23 Franchi-Gazzola, R., Gazzola, G.C., Ronchi, P., Saibene, V. and Guidotti, G.G. (1973) Biochim. Biophys. Acta 291, 545—556
24 Guidotti, G.G., Gazzola, G.C. and Franchi-Gazzola, R. (1974) Adv. Cytopharmacol. 2, 155—162
25 Christensen, H.N. (1975) in Current Topics in Membrane and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 6, pp. 227—258, Academic Press, New York
26 Tramacere, M., Dall'Asta, V., Franchi-Gazzola, R., Borghetti, A.F., Gazzola, G.C. and Guidotti, G.G (1976) Ateneo Parm. Acta Biomed. 47, 59—67
27 Elsas, L.J., Wheeler, F.B., Danner, D.J. and DeHaan, R.L. (1975) J. Biol. Chem. 250, 9381—9390
28 Gazzola, G.C., Franchi-Gazzola, R. and Guidotti, G.G. (1973) Atti Riun. Sci. Plen., Soc. Ital. Biofis., pp. 393—399, Camogli, Italy
29 Hume, S.P. and Lamb, J.F. (1976) J. Physiol. Lond. 259, 83—101
30 Christensen, H.N. (1977) in Biochemistry of Membrane Transport (Semenza, G. and Carafoli, E., eds.), pp. 222—235, Springer-Verlag, Berlin
31 Kalckar, H.M., Christopher, C.W. and Ullrey, D. (1976) J. Cell. Physiol. 89, 765—767
32 Holley, R.W. (1975) Nature 258, 487—490
33 Robinson, J.H. (1976) J. Cell. Physiol. 89, 101—110
34 Oxender, D.L., Lee, M. and Cecchini, G. (1977) J. Biol. Chem. 252, 2680—2683