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Cationic Amino Acid Transport into Cultured Animal Cells

II. TRANSPORT SYSTEM BARELY PERCEPTIBLE IN ORDINARY HEPATOCYTES, BUT ACTIVE IN HEPATOMA CELL LINES*

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Morris F. White‡ and Halvor N. Christensen§

From the Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Michigan 48109

The transport of cationic amino acids across the plasma membrane of several hepatoma cell lines (HTC, McA-RH7777, McA-RH8994, characterized in detail in the first of these) occurs by a saturable mediation which we designate System y^+. Identical experiments with cultured rat hepatocytes usually yield nonsaturating kinetic curves. Accordingly, System y^+ contributes little, if at all, to the flux of cationic amino acids in these cells. Analogous to the findings with other tissues, the influx of cationic amino acids into hepatoma cells is Na^+- and pH-independent, stereoselective, inhibitable by neutral amino acids in the presence of Na^+, and stimulated by cationic amino acids inside of the cell. This final characteristic, called *trans*-stimulation, is a kinetic property associated with the cationic amino acid transport system in all other eukaryotic cell types studied and provides evidence supporting the operation of System y^+. Influx of cationic amino acids into hepatocytes displays no significant *trans*-stimulation which strongly suggests the absence or alteration of System y^+ in this cell. Transport of arginine into hepatocytes is the rate-limiting step for its hydrolysis by arginase. Therefore, the relatively low influx of this amino acid under physiologic conditions due to the attenuation of System y^+ activity apparently provides a kinetic barrier separating the extrahepatic arginine pool from the active cytoplasmic enzymes of the hepatic urea cycle. Such a separation may be required for the nutrition and survival of extrahepatic tissues.

Dietary arginine is essential to some mammalian species, less so to others, even though they all generate large quantities of this amino acid in the liver. Obviously, hepatic arginine is not freely and uniformly available to other tissues, nor is extrahepatic arginine freely exposed to the hepatic arginase. In the distinctive case of *Neurospora*, an intracellular compartmentation restricts access of arginine to arginase (1). To enhance our understanding of the barriers involved in these limitations in the higher animal, we have compared the facilities serving for the transport of arginine, lysine, and ornithine in the rat hepatocyte with the transport activity seen in cultured human skin fibroblasts (2), and in cell lines derived by transformation of the hepatocyte (3, 4). Transformation is known to attenuate urea synthesis (5) and to cause some changes in nutrient transport (6-9) by hepatocytes. The preceding paper (2) characterizes for comparison the cationic amino acid transport system in human skin fibroblasts, heretofore called System Ly^+ and described for the Ehrlich cell (10) and red blood cells (11). This abbreviation for the present cases we shorten to y^+ to minimize its association with any single amino acid (2). Similar transport components have already been observed in kidney (12) and brain slices (13), and various other tissues. The brain system for "large basic amino acids" appears to serve for cationic structures; that for small basics we suspect does not (14).

MATERIALS AND METHODS

Human skin fibroblasts (15), HTC[1] cells (3), and isolated rat hepatocytes (16) were cultured as described by Gazzola *et al.* (17). The hepatoma cell lines, McA-RH7777[1] and McA-RH8994[1] (4), were cultured as described for HTC cells (17) except that Medium 199 (GIBCO) was supplemented with 10% calf serum and 5% fetal calf serum (both from KC Biologicals). Buffalo or Sprague-Dawley rat hepatocytes were isolated by collagenase perfusion according to the method of Berry and Friend (18) with some modifications described by Kilberg *et al.* (19).

As detailed elsewhere (2, 17), confluent monolayers in 24-well Costar cluster trays were washed and incubated for 1 h with amino acid-free modified Earle's balanced salt solution adjusted to pH 7.4. Then the cells were exposed to the desired radioactive substrate for 0.5 to 1 min. This time interval was short enough that the rate from the beginning was constant, hence an initial rate. The cells were washed twice with ice-cold 10 mM phosphate-buffered saline, extracted with 5% trichloroacetic acid, and counted in a liquid scintillation spectrometer.

The steady state distribution of [G-^{14}C]3-*O*-methyl-D-glucose (New England Nuclear) was used to estimate the apparent intracellular water as described by Kletzien *et al.* (20). The volumes measured for hepatocytes and HTC cells after a 30-min incubation of the cell monolayers in EBS with 1 mM 3-*O*-methyl-D-glucose (unlabeled carrier from Sigma) at 37 °C were 4.6 and 2.5 $\mu l \cdot mg^{-1}$ of protein, respectively.

Data describing amino acid influxes were fitted to the Michaelis-Menten equation modified to include a first order term to account for nonsaturable uptake:

$$\log v = \log\left(\frac{V_{max} \cdot S}{K_m + S} + K_d \cdot S\right) \quad (1)$$

A FORTRAN program (21) applied the Gauss-Newton nonlinear least squares analysis to Equation 1.

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‡ The experiments reported here have been taken from a dissertation submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy at The University of Michigan.

§ To whom correspondence should be addressed.

[1] HTC, hepatoma tissue culture cell line established by E. Brad Thompson from a Buffalo rat hepatoma, Morris 7288C, and supplied to us by Dr. Thomas D. Gelehrter; McA-RH7777 and McA-RH8994, hepatoma cell line established in culture from Buffalo rat hepatomas, Morris 7777 and 8994, respectively, and supplied to us by Dr. Van R. Potter, McArdle Laboratory, University of Wisconsin, Madison.

Automated amino acid analyses were performed as outlined in the preceding paper (2). Thin layer chromatography was carried out on 250-μ-thick cellulose plates (Analtech No. 7011) employing a solvent system composed of 1-butanol:acetic acid:water (2:1:1). The cells were extracted with 75% ethanol and the extract was evaporated to dryness. The residue was dissolved in water and 5-μl portions were applied to the thin layer plate. After development, each lane was divided into 1-cm sections, scraped into scintillation vials containing 0.2 ml of water and 2 ml of scintillation fluid, and radioactive disintegrations counted.

Other materials used in this work were obtained from the sources identified in the previous paper (2). A standard medium for hepatocytes (16) was prepared from reagents obtained from Sigma and Medium 199 was purchased from Grand Island Biological Co., Grand Island, NY.

RESULTS

Intracellular Amino Acid Levels and the Metabolism of Arginine in Hepatocytes and HTC Cells—Table I compares the intracellular amino acid levels in hepatocytes, HTC cells, and human fibroblasts after 24 h of incubation in Medium 199 supplemented with 10% fetal calf serum. The total apparent intracellular amino acid concentrations were lowest in hepatocytes; arginine appeared absent from these cells. *In vivo* experiments by Herbert *et al.* (22) shows for the rat liver, arginine levels lower than those for any other amino acid or any other tissue, namely about 2 nmol/g fresh weight. Glycine, glutamate, glutamine, and alanine were at the highest concentrations in rat liver, a result consistent with the findings in our Table I. The results summarized in Table II show the distribution of selected amino acids in HTC cells and hepatocytes after incubation of the monolayers with serum-free standard hepatocyte medium containing 1 mM each arginine, 2-amino-4-guanidinobutyric acid, and homoarginine. Glutamate and alanine, initially absent from the medium, are present and at higher levels in the cells than outside after 24 h. Arginine was almost completely eliminated from the medium in contact with hepatocytes, whereas only a small decrease was detected after a parallel incubation with HTC cells. Furthermore, both cell types accumulated the slowly metabolized analogs, 2-amino-4-guanidinobutyric acid and homoarginine, suggesting that guanidino amino acids can enter hepatocytes and HTC cells, although the former cell type metabolizes arginine far more rapidly. Arginase activity is evidently attenuated in HTC cells as shown years ago for other hepatomas (5).

Amino acid analysis of the cellular extract from HTC and hepatocytes after a 1-h incubation with 1 mM arginine revealed no arginine in the hepatocyte, whereas 30 nmol·mg^{-1} of protein were found in HTC cells; the distribution ratio of labeled arginine between the hepatoma cells and the suspending medium reached about 6.5. Fig. 1 shows the thin layer chromatogram of the cell extract following a 2-h incubation of HTC cells and hepatocytes with 1 mM L-[*guanidino*-^{14}C]arginine. More than 90% of the radioactive label from hepatoma cells migrated with the arginine standard and less than 10% was present as [^{14}C]urea. In contrast, a major portion of the radioactivity extracted from hepatocytes moved as fast as urea, although a small portion migrated with the arginine marker. Chromatography of the medium separated after the experiment in both cases indicated in contrast that more than 90% of the ^{14}C moves with the arginine standard. As suggested earlier (23, 24), the hydrolysis of arginine is probably instantaneous once the amino acid enters the cytoplasm of the hepatocyte.

Time Course of Arginine Uptake and Hydrolysis—Time courses describing the uptake of 1 mM L-[*guanidino*-^{14}C]arginine are shown in Fig. 2*A*. The HTC cells accumulate the ^{14}C label to a far larger extent than cultured hepatocytes during the time interval studied. The results obtained with cultured fibroblasts, included for comparison, closely resemble the curve for HTC cells. In Fig. 2*B*, curves describing the uptake of homoarginine and GPA[2] into hepatocytes are compared with those describing homoarginine accumulation into HTC cells. The transport of these slowly hydrolyzed substrates (Table II) is sluggish in normal liver cells relative to

TABLE I

Amino acid pools in ordinary hepatocytes, HTC cells, and human skin fibroblasts

Monolayer cultures of Buffalo rat hepatocytes, HTC cells, or human fibroblasts were maintained in 75-cm^2 Corning flasks and incubated with Medium 199 containing 10% fetal calf serum for 24 h. The cells were extracted with 5 ml of 75% ethanol and this extract was analyzed for amino acids by automated chromatography.

Amino acid	Hepatocytes	HTC cells	Fibroblasts
		nmol·mg^{-1} protein	
Asp		20.2	98.4
Thr, Asn, Gln	25.0	49.4	45.2
Ser	16.3	51.0	48.9
Glu	10.5	83.5	145.0
Gly	15.0	183.0	70.9
Ala	6.7	29.6	55.5
Cys	0.7	22.9	2.2
Val	2.7	7.3	8.8
Met	0.3	7.3	7.3
Ile	0.3	9.8	5.2
Leu	0.9	26.1	21.4
Tyr	0.3	9.6	9.8
Phe	1.1	14.2	9.6
His	0.8	3.8	5.6
Lys + Orn	1.3	5.9	8.8
Arg	0	2.3	7.3
Total amino acids	82	526	550
(Ammonium ion)	6.7	2.5	10.3

TABLE II

Steady state distribution of L-amino acids between ordinary hepatocytes or HTC cells and their suspending medium

Ordinary hepatocytes and HTC cells maintained as monolayers in 75-cm^2 Corning flasks were incubated for 27 h in 5 ml of serum- and hormone-free standard hepatocyte medium supplemented with Arg, homoarginine (HArg), and 2-amino-4-guanidinobutyric acid (GBu). The concentrations of several amino acids were then determined in the medium before any incubation (none) and after the 27-h incubation, and in cell extracts with an amino acid analyzer. The distribution ratios were calculated assuming 4.6 (for HTC cells) and 2.5 (for hepatocytes) μl of H_2O·mg^{-1} of protein, as determined in separate experiments with 3-*O*-methyl-D-glucose. N.D. = not detected.

Amino acid	Medium			Cellular		Distribution ratios	
	None	Ordinary hepatocytes	HTC	Ordinary hepatocytes	HTC	Ordinary hepatocytes	HTC
		mM		*nmol·mg^{-1} protein*			
Glu	N.D.	0.20	2.00	26.4	127.0	53	14
Cys	0.32	0.24	0.07	0.8	52.9	1	164
Gly	0.39	0.18	0.53	16.1	171.0	36	70
Ala	N.D.	0.19	0.93	10.3	121.0	22	28
Val	0.69	0.67	0.48	3.4	17.4	2	8
Ile	0.63	0.54	0.39	2.4	17.7	2	10
Leu	0.54	0.46	0.37	2.2	13.0	2	8
Orn + Lys	0.70	0.53	0.61	2.8	10.2	2	4
GBu	1.00	0.88	0.99	5.6	15.9	3	3
Arg	0.68	0.03	0.64	N.D.	13.3	N.D.	5
HArg	0.95	0.92	0.79	4.3	12.9	2	4

[2] The abbreviations used are: GPA, 4-amino-1-guanylpiperidine-4-carboxylic acid; EBS, modified Earle's balanced salt solution; MeAIB, 2-(methylamino)isobutyric acid; BCH, the racemic 2-aminoendobicyclo[2.2.1]heptane-2-carboxylic acid.

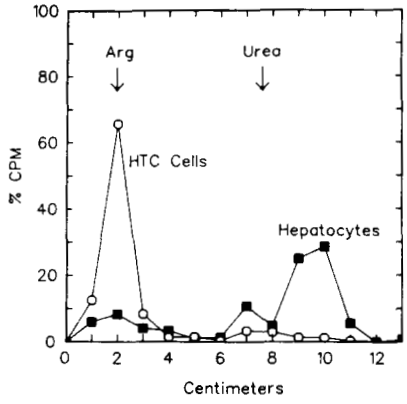

FIG. 1. **Thin layer chromatogram of cell-associated ^{14}C after a 2-h incubation with L-[*guanidino*-^{14}C]arginine.** HTC cells and Buffalo rat hepatocytes maintained in 75-cm^2 Corning flasks were washed 4 times with 5 ml of cold phosphate-buffered saline and extracted with 5 ml of 75% ethanol. The extract was evaporated and dissolved in 0.5 ml of water, and 5 to 10 μl were applied to a cellulose TLC plate and developed with a solvent system composed of 1-butanol:glacial acetic acid:water (2:1:1). The standard was prepared by incubating the labeled arginine with 1 mg/ml of bovine liver arginase, followed by ethanol extraction.

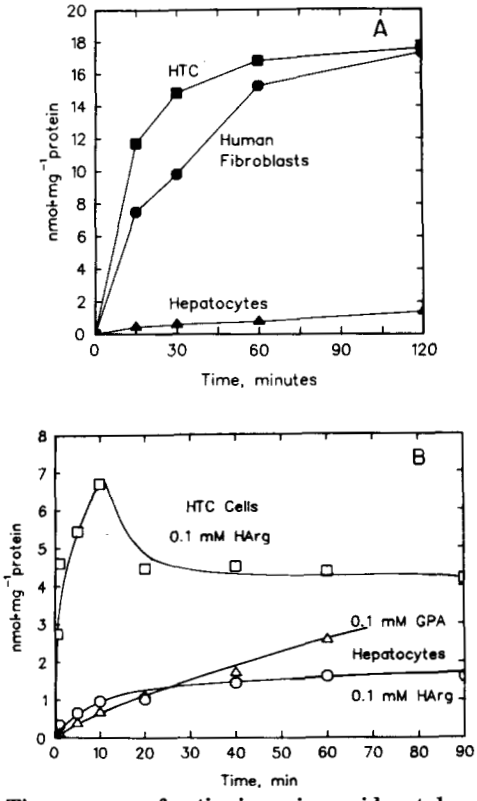

FIG. 2. **Time course of cationic amino acid uptake.** *A* shows progress curves for 1 mM L-[*guanidino*-^{14}C]arginine uptake into HTC cells (■), cultured fibroblasts (●), and cultured hepatocytes isolated from a Buffalo rat (▲), all after incubation of the cells in amino acid-free medium for 90 min. *B* shows progress curves for uptake of [1-^{14}C] GPA (△) and [2,3-^{14}C]homoarginine (○) at 0.1 mM by hepatocytes without previous amino acid depletion. HTC cells were treated in a similar way with 0.1 mM homoarginine (□).

homoarginine uptake into HTC cells. Furthermore, the uptake into HTC cells shows evidence of *trans*-stimulation by endogenous amino acids (note overshoot in Fig. 2*B*). This behavior is not apparent in normal hepatocytes.

The time courses of L-[*guanidino*-^{14}C]arginine hydrolysis by intact hepatocytes and by an equal quantity of homogenized hepatocytes are shown in Fig. 3. The results indicate that the intact cells present a substantial barrier to the rapid hydrolysis of extracellular arginine.

Concentration Dependence of Cationic Amino Acid Up-

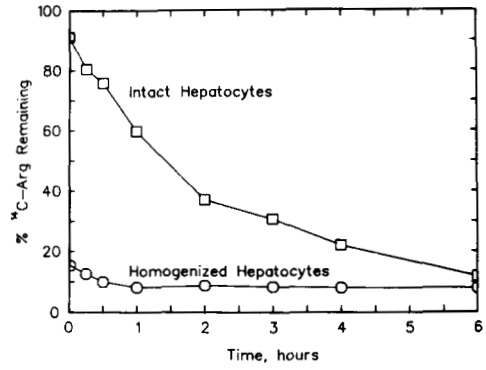

FIG. 3. **Time course for L-arginine hydrolysis.** Hepatocytes maintained in 75-cm^2 Corning flasks were incubated *in situ* with 5 ml of 1 mM L-[*guanidino*-^{14}C]arginine (□) or scraped from the flask and homogenized in a Dounce homogenizer (20 strokes) before the same incubation (○). Samples of 10 μl were removed at various time intervals and diluted with 10 μl of 100% ethanol. The samples were absorbed on cellulose and eluted with a solvent composed of 1-butanol:acetic acid:water (2:1:1). The fraction of total arginine remaining in the sample at the indicated time intervals is shown. The production of [^{14}C]urea was closely reciprocal to the arginine hydrolyzed.

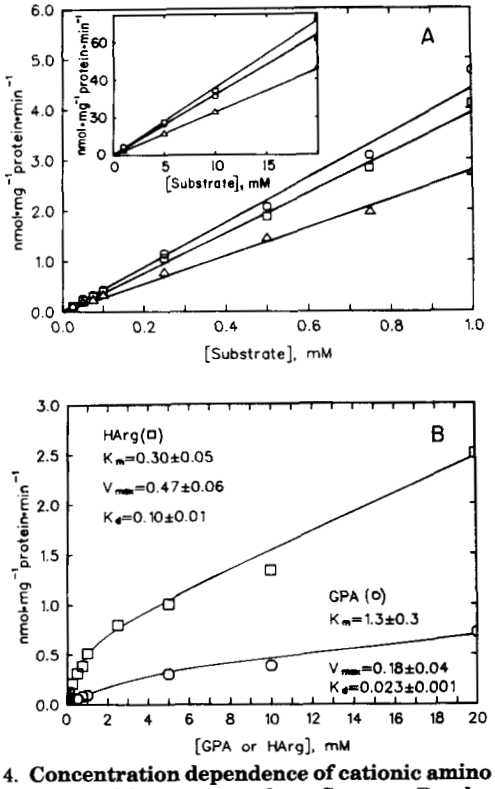

FIG. 4. **Concentration dependence of cationic amino acid uptake into cultured hepatocytes from Sprague-Dawley rats.** *A*, 1-min uptake of [3,4-^3H]arginine (△), [U-^{14}C]lysine (□), and [2,3-^3H] ornithine (○). The *inset* shows that the linear relation of amino acid uptake extends to 20 mM amino acid levels. Slopes (K_d) obtained by fitting the data to a straight line were, in nanomoles·mg^{-1} of protein·min^{-1}·mM^{-1}: Arg, 2.8 ± 2; Lys, 4.0 ± 0.1; Orn, 3.8 ± 0.1. *B*, 10-min uptakes of [2,3-^3H]homoarginine (□) and [1-^{14}C]GPA (○). The kinetic parameters, K_m (mM), V_{max} (nanomoles·mg^{-1} of protein·min^{-1}), and K_d were obtained by fitting the data to Equation 1.

take by Hepatocytes and HTC Cells—Consistent with the slow uptake of [^{14}C]arginine by hepatocytes in culture (Fig. 2) is the apparent absence of saturable uptake of arginine, lysine, and ornithine. Fig. 4*A* discloses no evidence for a hyperbolic relation between velocity and substrate concentration for transport by a specific membrane system as discussed by LeFevre (25). The 1-min uptakes of L-[3,4-^{3}H]arginine, L-[U-^{14}C]lysine, and L-[2,3-^{3}H]ornithine were linear with the extracellular concentration up to 20 mM. These observations may have any of 3 possible explanations: the uptake by System y^+ may be small relative to the nonsaturable component; a specific transport system may be entirely absent from the membrane of hepatocytes; the K_m for uptake may be much larger than the highest tested substrate concentration. Indications that the first explanation is correct were obtained from influx experiments with homoarginine and GPA. The sluggish or absent metabolism of these substrates permits longer incubation intervals, the inward flux being approximated by 10-min incubations between 0.025 and 20 mM. The data fitted satisfactorily into Equation 1 and yielded the kinetic parameters recorded on Fig. 4*B*. Although the long time intervals selected probably provide only a crude estimate of initial velocity, these experiments have succeeded in discriminating a relatively slow, saturable transport component for these cationic amino acids.

One might suppose, for the carnivores showing prompt and acute arginine deficiency on omission of this amino acid from the diet, that the barrier action between hepatic arginase or arginine and extrahepatic arginine might be much weaker. The hepatocytes isolated from a female ferret resembled those from the rat, however, in the apparent absence of System y^+ by kinetic criteria (data not shown).

The HTC cells, like the fibroblasts discussed previously (2), show unambiguous kinetic evidence for a transport system for the cationic amino acids. Fig. 5 directly compares the kinetic curves observed for HTC cells with those obtained for hepatocytes, using arginine labeled with tritium on the carbon chain. The higher rate of arginine uptake seen here for hepatocytes than in Fig. 2*A* may well be explained by our use of [*guanidino*-^{14}C]arginine in the earlier case. Part of this label may well have been lost from the cells in the form of urea during the extended time of that experiment, a difficulty not to be expected for Fig. 5 during 30 s; the ornithine formed presumably retained the tritium label. The uptake of arginine and lysine is well described by the Michaelis-Menten equation modified to include a first order term (26), whereas the uptake of arginine by hepatocytes is satisfactorily characterized by a straight line. The uptake of cationic amino acids by HTC cells and hepatocytes is not affected by equimolar replacement of Na^+ in the medium with choline. The kinetic parameters for lysine and arginine influx into HTC cells are listed in Table III. These results are qualitatively similar to those found for fibroblasts (Table II in Ref. 2) although the kinetic parameters have higher absolute values. Arginine influx into HTC cells, data not shown, is only slightly sensitive to external hydrogen ion concentration, much as shown for fibroblasts (Fig. 4 in Ref. 2). A much larger sensitivity to pH would be expected if most of the uptake occurred for a zwitterionic species by a transport system for neutral amino acids (Fig. 6 in Ref. 14).

To investigate whether using a 15-min collagenase perfusion

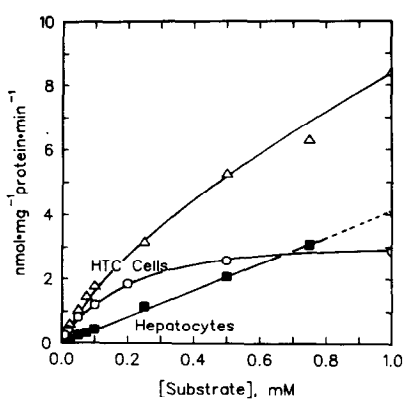

FIG. 5. **A comparison of the concentration dependence of [3,4-^{3}H]arginine (○) and [U-^{14}C]lysine (△) uptake into HTC cells with that of arginine by Sprague-Dawley rat hepatocytes (■).** Uptake was observed during 30-s intervals.

TABLE III

Kinetic constants for L-arginine or L-lysine uptake into HTC cells

Kinetic constants were determined for the initial uptake of [3,4-^{3}H]arginine and [U-^{14}C]lysine in the presence or absence of Na^+. After a 1-h amino acid depletion the monolayers were incubated with Na^+-free EBS for 30 min. Initial velocities were estimated from 30-s (Lys) or 1-min (Arg) incubations over a concentration range of 0.001 to 20 mM in Na^+ or choline-containing EBS. K_m and V_{max} have their usual meanings and K_d is the slope of the nonsaturable component. The parameters ± S.E. were determined as described under "Materials and Methods" using Equation 1.

Substrate	Cation	K_m	V_{max}	K_d
		mM	*nmol·mg^{-1} protein·min^{-1}*	*nmol·mg^{-1} protein·min^{-1}·mM^{-1}*
Arg	Na^+	0.145 ± 0.007	3.3 ± 0.1	0.35 ± 0.03
Arg	Choline	0.112 ± 0.005	2.8 ± 0.1	0.34 ± 0.02
Lys	Na^+	0.146 ± 0.010	3.0 ± 0.2	5.4 ± 0.1
Lys	Choline	0.169 ± 0.020	2.5 ± 0.2	7.6 ± 0.1

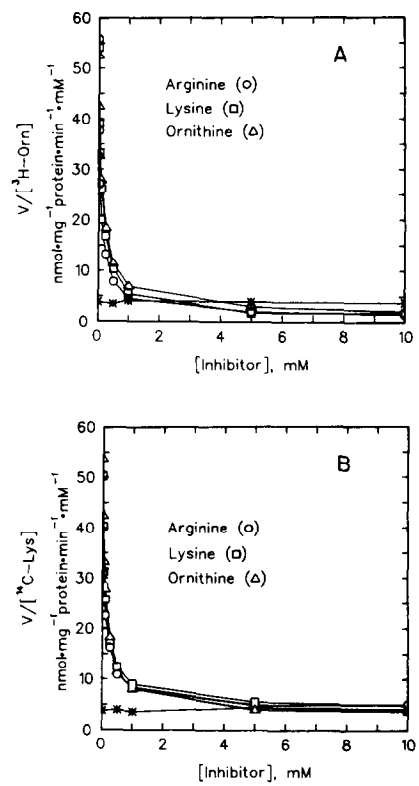

FIG. 6. **Analog inhibition of uptake of trace quantities of ornithine and lysine in HTC cells and cultured Sprague-Dawley hepatocytes.** HTC cells were incubated for 30 s with 3.9 μM [U-^{14}C]lysine (*B*) or 0.11 μM [2,3-^{3}H]ornithine (*A*) in the presence of the indicated concentrations of unlabeled arginine (○), lysine (□), or ornithine (△). For comparison, we include a similar experiment with cultured hepatocytes (*) in which labeled lysine (*B*) or ornithine (*A*) uptake is inhibited with unlabeled excesses of the same amino acid.

to isolate hepatocytes may have damaged the external membrane component of System y^+, HTC cells were incubated for 30 min at 37 °C in the medium used to isolate hepatocytes, containing 50 units/ml of collagenase. The kinetic parameters for arginine uptake measured subsequently (not shown) were unchanged from those reported in Table III.

Analog Inhibition of Cationic Amino Acid Uptake—The inhibition of uptake of [^{14}C]lysine (3 μM) and [^{3}H]ornithine (0.18 μM) by unlabeled arginine, lysine, or ornithine is shown in Fig. 6, *B* and *A*, respectively. The results imply that these cationic amino acids interact with a common membrane transport system in HTC cells. Arginine, lysine, and ornithine inhibited [^{3}H]ornithine or [^{14}C]lysine influx with equal completeness, about 95%. This result is similar to that observed with fibroblasts for inhibition of [^{14}C]lysine uptake (Fig. 6 in Ref. 2). For comparison, a parallel experiment with cultured hepatocytes shows that the 30-s uptakes of trace quantities of [^{14}C]lysine and [^{3}H]ornithine are less than one-tenth as great (Fig. 6, *B* and *A*), and inhibition of this uptake by the unlabeled cognate amino acid is equivocal. The uptake of [^{14}C]GPA by hepatocytes measured during a 10-min interval (Table IV), although very sluggish, is slightly sensitive to inhibition by excesses of unlabeled GPA, arginine, and lysine. MeAIB and BCH, in contrast, have little effect on uptake. These results are consistent with the presence of a minor transport component for cationic amino acids in cultured hepatocytes, but the low activity makes its identification as System y^+ equivocal. Mercury(II) inhibited arginine uptake into HTC cells, whereas it had no effect on influx into hepatocytes (Fig. 7).

Table V lists the competitive inhibition constants for various cationic amino acids on arginine uptake into HTC cells. Consistent with the observations with human fibroblasts (Table III in Ref. 2) and prior results in the Ehrlich cell (14), the

TABLE IV

Analog inhibition of GPA uptake into ordinary hepatocytes

Ordinary hepatocytes not previously depleted in amino acids were incubated for 10 min with 0.1 mM [1-^{14}C]GPA and the concentration of unlabeled amino acid indicated below. The velocity ± S.D. was based on triplicate analyses.

Inhibitor	0 mM	1.0 mM	10.0 mM
	nmol·mg^{-1} protein·min^{-1}		
None	0.055 ± 0.007		
Arg		0.037 ± 0.004	0.015 ± 0.002
Lys		0.034 ± 0.010	0.021 ± 0.03
MeAIB		0.061 ± 0.008	0.049 ± 0.010
BCH		0.051 ± 0.008	0.043 ± 0.006

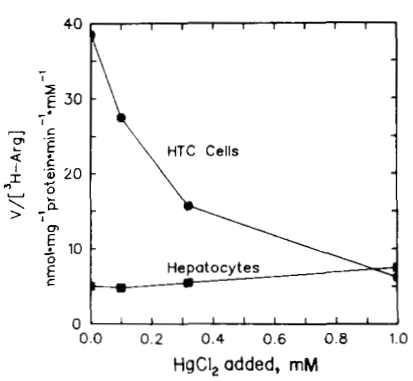

FIG. 7. **Inhibition of arginine uptake by $HgCl_2$.** HTC cells (●) or Sprague-Dawley hepatocytes (■) were incubated for 1 min with 0.17 μM [3,4-^{3}H]arginine and the indicated concentrations of added $HgCl_2$. Each data point represents an average of triplicate determinations.

TABLE V

Analog competitive inhibition constants for arginine uptake into HTC cells

Initial velocities were estimated from 30-s incubations with [3,4-^{3}H]arginine between 0.001 and 1.0 mM in the presence of various concentrations of the amino acids listed below. A constant value for the nonsaturable component, 0.3 nmol·mg^{-1} of protein·min^{-1}·mM^{-1}, was subtracted from all data. K_i ± S.E. was determined by fitting the data to the equation for competitive inhibition (equation 2 in Ref. 2). V_{max} had a constant value of 4.1 ± 0.4 nmol·mg^{-1} of protein·min^{-1}.

Inhibitor	K_i
	mM
L-Homoarginine	0.089 ± 0.008
L-Arginine	0.20 ± 0.04
L-2-Amino-4-guanidinobutyric acid	0.39 ± 0.06
L-2-Amino-3-guanidinopropionic acid	5.3 ± 0.5
L-Lysine	0.14 ± 0.01
D-Arginine	1.0 ± 0.1
N^α-Methyl-L-arginine	30 ± 7
N^α-Methyl-L-lysine	20 ± 4
N^ϵ-Methyl-L-lysine	0.59 ± 0.04
4-Amino-1-guanylpiperidine-4-carboxylic acid	2.3 ± 0.5

TABLE VI

Trans-stimulation of arginine uptake into HTC cells by cationic amino acids

HTC cells were incubated in EBS for 90 min followed by a 1-h incubation with 10 mM solutions of the amino acids listed below. After a brief wash (<5 s) with EBS at 37 °C, 30-s uptakes of [3,4-^{3}H]arginine were measured between 0.05 and 0.5 mM. The kinetic parameters ± S.E. were determined by fitting the data to Equation 1, ignoring the small nonsaturable component.

Amino acid introduced into the cell	Kinetic parameters for [^{3}H]Arg uptake	
	K_m	V_{max}
	mM	*nmol·mg^{-1} protein·min^{-1}*
None	0.120 ± 0.026	4.7 ± 0.3
L-Arginine	0.190 ± 0.024	24.5 ± 1.0
L-Homoarginine	0.167 ± 0.014	16.8 ± 0.4
D-Arginine	0.091 ± 0.015	8.4 ± 0.3
L-Lysine	0.191 ± 0.031	26.8 ± 1.4
L-2-Amino-3-guanidinopropionic acid	0.186 ± 0.027	9.8 ± 0.4
L-2-Amino-4-guanidinobutyric acid	0.160 ± 0.019	24.5 ± 0.9
1-Guanyl-4-aminopiperidine-4-carboxylic acid	0.119 ± 0.059	3.4 ± 0.6

K_i increases as the number of methylene groups separating the α-carbon atom from the guanidino group decreases, homoarginine being the most effective inhibitor studied. As for the fibroblast (2) and other cells (27), selected neutral amino acids differentially inhibit cationic amino acid uptake if Na^+ is present. For example, arginine uptake by HTC cells incubated in EBS containing 150 mM Na^+ was inhibited 3 times more effectively by homoserine than by serine (data not shown). Serine in turn was twice as effective as alanine. System y^+ in the HTC cells is stereospecific; inhibition by D-arginine displayed a 10-fold higher K_i than L-arginine did. N^α-methylation of L-arginine or L-lysine was not tolerated by System y^+, although methylation of the terminal amino group of lysine only increased the apparent K_i 4-fold. These analogs caused no apparent inhibition of arginine uptake by hepatocytes.

Trans-Stimulation of Arginine Influx by Cationic Amino Acids—The acceleration of a unidirectional flux of cationic amino acids by the presence of a transported analog on the opposite side of the membrane is a characteristic of the corresponding transport systems in Ehrlich cells (10), rabbit reticulocytes (11), and human fibroblasts (Tables IV and V in Ref. 2). Table VI in this report lists the kinetic constants for

[^{3}H]arginine uptake following incubation and presumed loading of HTC cells with unlabeled cationic amino acids. L-Arginine, L-homoarginine, L-lysine, and L-2-amino-4-guanidinobutyric acid increase the observed V_{max} 4- to 6-fold; small but reproducible increases in K_m^{obs} were also recorded. As for Ehrlich cells (14) and fibroblasts (2), intracellular GPA does not stimulate arginine influx, although extracellular GPA stimulates efflux. Instead, a slight but equivocal *trans*-inhibitory effect was noticed. Fig. 8*A* compares the initial velocity of L-[3,4-^{3}H]arginine uptake into Buffalo rat hepatocytes and HTC cells following a 60-min incubation with 10 mM homoarginine. The hepatocytes showed nonsaturable uptake and insignificant *trans*-stimulation. Similar results from a separate experiment shown in Fig. 8*B* were observed for Sprague-Dawley hepatocytes after loading with L-2-amino-4-guanidinobutyric acid or homoarginine. As expected (28), the homoarginine entering the ordinary hepatocyte was not destroyed to a sufficient extent (last line of Table II) to compromise these tests for countertransport. The influx of homoarginine into hepatocytes prepared from Sprague-Dawley rats likewise was not stimulated significantly by intracellular cationic amino acids (Fig. 9). When compared with results for human fibroblasts (2), for the HTC cells, or for other cells (10, 29, 30), these results fail to support transport of nutrients into hepatocytes by System y^+.

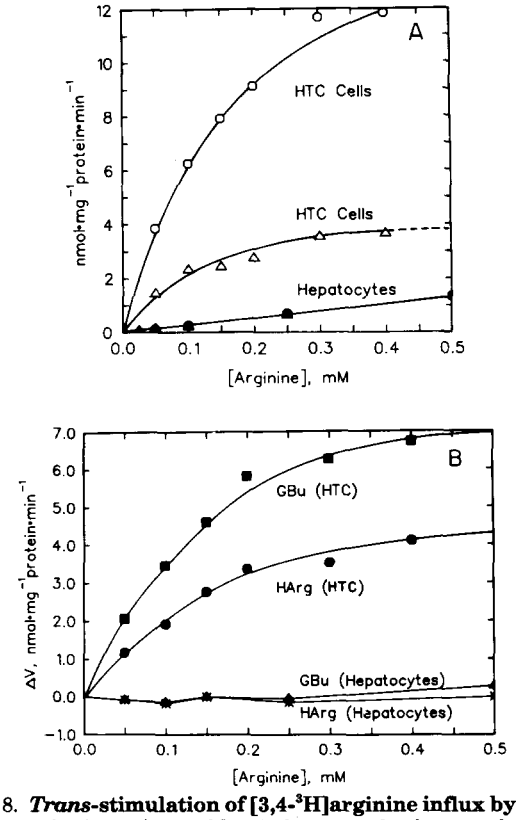

FIG. 8. ***Trans*-stimulation of [3,4-^{3}H]arginine influx by intracellular cationic amino acids.** *A* shows results for monolayers of Buffalo rat hepatocytes or HTC cells. After amino acid depletion, the cells were incubated for 1 h with EBS containing 10 mM homoarginine (HTC, ○; hepatocytes, ●) or amino acid-free EBS (HTC, △; hepatocytes, ▲). *B* shows the difference of [2,3-^{3}H]arginine influx, ΔV, into Sprague-Dawley hepatocytes or HTC cells incubated with homoarginine (*HArg*) or L-2-amino-4-guanidinobutyric acid (*GBu*) on the one hand, and into the same cells depleted in their cellular amino acids, on the other hand. After amino acid depletion, cells were maintained in amino acid-free EBS or incubated for 1 h with EBS containing 10 mM homoarginine (HTC, ●; hepatocytes, ▲) or 10 mM L-2-amino-4-guanidinobutyric acid (HTC, ■; hepatocyte, *). Before the 1-min uptake measurements, the monolayers were washed (<5 s) with EBS at 37 °C. Each point is an average of the triplicate determinations of the difference between the stimulated and unstimulated influx.

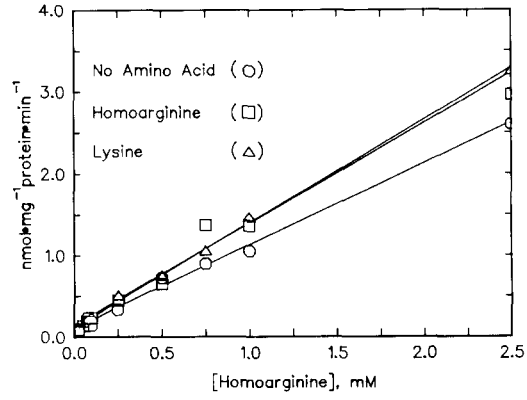

FIG. 9. ***Trans*-stimulation of [2,3-^{3}H]homoarginine uptake into ordinary hepatocytes.** Hepatocytes isolated from a Sprague-Dawley rat were cultured for 24 h and then incubated for 60 min with EBS containing no amino acids (○), 10 mM homoarginine (□), or 10 mM lysine (△). Before the 1-min uptake measurements, the monolayers were washed (<5 s) with EBS at 37 °C. Each point in the figure represents an average of duplicate determinations. The corresponding kinetic parameters ± S.E. for homoarginine influx between 0.025 and 10 mM, K_m (mM), V_{max} (nanomoles·mg^{-1} of protein·min^{-1}), and K_d (nanomoles·mg^{-1} of protein·min^{-1}·mM^{-1}), respectively, were determined by fitting these data to Equation 1: no amino acid, 0.05 ± 0.05, 0.14 ± 0.09, 1.0 ± 0.1; homoarginine-loaded, 0.04 ± 0.03, 0.14 ± 0.06, 1.3 ± 0.01; lysine-loaded, 0.03 ± 0.02, 0.17 ± 0.05, 1.2 ± 0.1. The values for K_m and V_{max} are statistically equivocal.

TABLE VII

Steady state and zero-trans kinetic parameters for arginine transport determined in various cell lines

McA-RH7777 or McA-RH8994 hepatoma cells, HTC, and fibroblasts depleted of endogenous amino acids for 1 h were incubated an additional hour with amino acid-free EBS or with EBS containing various concentrations of arginine corresponding exactly to those used for the influx measurements. Prior to uptake, the monolayers were washed (<5 s) and the influx of [3,4-^{3}H]arginine was estimated from 30-s incubations between 0.01 and 20 mM. The resulting data were fitted to Equation 1. K_m and V_{max} are the approximate zero-*trans* kinetic parameters measured in the near absence of intracellular arginine. K_m^{ss} and V_{max}^{ss} are the estimated steady state kinetic parameters. K_d, the first order rate constant representing the nonsaturable transport component, was consistently identical in steady state and zero-*trans* experiments. V_{max}^{ss}/V_{max} represents an index of the maximum *trans*-stimulation measured. The kinetic parameter for ordinary hepatocytes is included for comparison.

Cell line	K_m	K_m^{ss}	V_{max}	V_{max}^{ss}	K_d	V_{max}^{ss}/V_{max}
	mM		*nmol·mg^{-1} protein·min^{-1}*		*nmol·mg^{-1} protein·min^{-1}·mM^{-1}*	
7777	0.13 ± 0.04	2.5 ± 0.7	0.29 ± 0.07	9.1 ± 2	2.60 ± 0.20	32
8994	0.06 ± 0.02	0.51 ± 0.10	0.24 ± 0.05	2.3 ± 0.7	1.87 ± 0.04	10
HTC	0.13 ± 0.02	1.4 ± 0.1	3.3 ± 0.1	36 ± 3	0.30 ± 0.01	11
Fibroblasts	0.040 ± 0.005	0.31 ± 0.08	0.75 ± 0.05	5.0 ± 1	4.0 ± 0.4	9
Ordinary hepatocytes					2.8 ± 0.3	

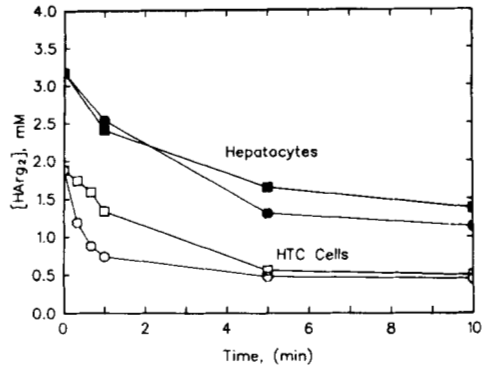

FIG. 10. **Time courses for [2,3-^{3}H]homoarginine exodus from HTC cells and Sprague-Dawley hepatocytes.** HTC cells (○, □) and ordinary hepatocytes (●, ■) were incubated for 2 h with 0.2 mM or 1 mM [2,3-^{3}H]homoarginine, respectively. Exodus was initiated by quickly washing (<5 s) the cells with 2 ml of EBS followed by incubation of the monolayers with fresh amino acid-free EBS (□, ■) or EBS containing 10 mM unlabeled homoarginine (○, ●) for the indicated time interval. The data points represent averages of triplicate determinations of the homoarginine remaining associated with the cells.

The zero-*trans* and steady state[3] influx of arginine into the McA-RH7777 and McA-RH8994 hepatoma cell lines (4, 9) derived from the Buffalo rat were studied to explore whether the appearance of System y^+ is a general characteristic of hepatic transformation. Table VII lists the kinetic parameters obtained from these two cell lines and compares them with the analogous values for hepatocytes, HTC cells, and human fibroblasts. In addition to the ubiquitous nonsaturable component, a sodium-independent saturable uptake of arginine was observed in both the 7777 and the 8994 hepatoma cell. Although the value of V_{max}/K_m for these cells was about 1/10 as great as that measured in HTC cells and fibroblasts, accelerative countertransport was unequivocally detected in them as shown by the values of V^{ss}_{max}/V_{max} computed from influx measurements at the steady state (for V^{ss}_{max}) and under zero-*trans* (for V_{max}) conditions (see legend, Table VII). Therefore, the three hepatoma cell lines and fibroblasts are similar in showing a *trans*-stimulatable membrane component for cationic amino acid transport, a behavior not detected in normal cultured hepatocytes.

Exodus of Arginine and Homoarginine from HTC Cells and Hepatocytes—Fig. 10 shows the time course of homoarginine exodus from HTC cells and cultured hepatocytes. In the absence of extracellular amino acids, the progress curves are remarkably similar for the two cell types. External homoarginine also accelerates exodus from the HTC cells, a result consistent with observations in fibroblasts (2) and other cells (31). Similar to the results for influx (Fig. 9), accelerative countertransport is, however, not apparent for exodus of homoarginine from cultured hepatocytes.

DISCUSSION

Of the five enzymes of the hepatic urea cycle, arginase shows the highest activity, both in the rat and man. In nonhepatic tissues, arginase is less than one-twentieth as active (32). Consequently, arginine has a dramatically different fate in the liver cell. Very little arginine (2 nmol/g fresh weight) can be extracted from rat liver, whereas other tissues contain from 7 to 65 times as much (22). Arginine supplied to the liver is completely hydrolyzed (33). Circulating arginine therefore appears to arise elsewhere. Net arginine uptake by the human splanchnic bed (gut, pancreas, spleen, liver) represents only 1% of the total of all the other amino acids added and may be insignificant (34). Citrulline is the only amino acid showing significant net release from the human splanchnic region, whereas muscle and kidney release net quantities of arginine into the capillary blood. For the case of kidney, arginine represents 20% of the amino acid molecules released (35). This observation correlates with the presence in the kidney of appreciable quantities of the enzymes forming arginine from citrulline, but rather little arginase (36, 37). The ability of young rats to grow, albeit slowly when fed an arginine-free diet (37), suggests that the kidney may supply important net quantities of arginine. Arginine deficiency lowers hepatic arginase activity only slightly (38), probably not enough to make the liver a major source of arginine for growth.

The plasma membrane of the hepatocyte appears then from the results of Fig. 3 to be a functional barrier, largely excluding extrahepatic arginine from reaction with intrahepatic arginase. In disagreement, Simell (39) proposed two saturable transport systems to explain the kinetics observed for homoarginine uptake by rat liver slices during 15 min. His data seem rather limited, however, to warrant his interpretation. Cornford *et al.* (40) reported more recently a K_m of 3 mM for the hepatic uptake of arginine after a single circulatory passage in the mouse. Restricting our attention here to the homogeneous rat hepatocyte, we failed to observe corresponding uptake. Schimke observed that the rapid intraperitoneal injection of arginine into rats led to the swift appearance of the intrahepatic fraction of this label in urea (41). No free hepatic [^{14}C]arginine could be isolated after 20 or 30 min and by 20 min hepatic proteins were maximally labeled. Poole *et al.* (42) showed that leucine, an amino acid little catabolized by liver, is incorporated into proteins 7 times more efficiently than [*guanidino*-^{14}C]L-arginine.

In contrast, a lively transport activity for cationic amino acids into various other cells has been shown, meeting the criteria of saturability and participation in accelerative countertransport (24, 43). We cannot, as we have noted, entirely exclude a weak transport of these amino acids into liver cells by the same system, although these criteria are not uniformly met. Even if System y^+ were totally absent, we still could not exclude a relatively slow migration of these amino acids by one or more routes not saturating perceptibly in our tests. Even at neutral pH, lysine, along with other diamino acids, shows measurable transport by neutral systems (11, 14, 44), not only by System L in the case of lysine and the Ehrlich cell (44), but also by a Na^+-independent use of Na^+-dependent systems in various cells. As a dietary essential, lysine must be captured by the liver, perhaps by one or more of these routes as indicated by the accumulation of [^{14}C]lysine in hepatic proteins (41). Relative to other tissues of the rat, however, the incorporation of this labeled amino acid into liver tissue is very low (45).

The order of activity for arginine influx (V_{max}/K_m) into cells by System y^+ was, according to Table VII,

hepatocytes $\ll$ 7777; 8994 < fibroblasts; HTC.

The degree to which arginine uptake was *trans*-stimulated took a slightly different order:

hepatocytes $\ll$ 8994; HTC, fibroblasts < 7777.

[3] Zero-*trans* influx concerns an ideal situation in which the initial velocity of substrate uptake is measured in the absence of transport-reactive compounds inside of the cell. Steady state influx is essentially an isotope exchange experiment in which the initial uptake of various concentrations of labeled substrate is measured after the distribution of unlabeled substrate has reached a steady state at the identical concentrations.

The nonsaturable component as measured by K_d showed a different trend, namely,

HTC $\ll$ 8994; 7777; hepatocytes $\leq$ fibroblasts.

We have no evidence whether the latter sequence arises from inherent differences in the plasma membrane, possible mechanical effects of the adhesion of cells to the plastic layer, or what. Gap junctions (46–48) are less likely for the transformed cells, so these structures could offer an explanation.

We may summarize by concluding that extracellular arginine can enter the liver slowly, although mainly not by the usual route, and serve there for hepatic protein synthesis. The major part of the entering arginine is, however, destined to be hydrolyzed.

We have noted previously that the detailed properties of transport systems of a given type vary somewhat from one occurrence to another (49, 50). What we face here, however, appears to be the complete or nearly complete absence of a system. For this finding, we also have precedents: no transport system for anionic amino acids was detected in the Ehrlich cell (51), although such systems have been described in various other cells; no System A has been detected in nucleated or reticulated red blood cells (52, 53).

An active hepatic System y^+ is probably incompatible with the co-existence in an animal of an efficient urea cycle and a complete extrahepatic nutrition. The appearance of System y^+ activity may be a critical characteristic of successful hepatic transformations, at least in the rat.

REFERENCES

1. Davis, R. H., Bowman, B. J., and Weiss, R. L. (1978) *J. Supramol. Struct.* **9,** 473–488
2. White, M. F., Gazzola, G. C., and Christensen, H. N. (1982) *J. Biol. Chem.* **257,** 4443–4449
3. Thompson, E. B. (1979) *Methods Enzymol.* **58,** 544–552
4. Becker, J. E., de Nechaud, B., and Potter, V. R. (1976) in *Onco-Developmental Gene Expression* (Fishman, W. H., and Sell, S., eds) pp. 259–270, Academic press, New York
5. Knox, W. E. (1967) *Adv. Cancer Res.* **10,** 117–161
6. Craik, J. D., and Elliott, K. R. F. (1979) *Biochem. J.* **182,** 503–508
7. Graff, J. C., Wohlhueter, R. M., and Plagemann, P. G. W. (1981) *Biochim. Biophys. Acta* **641,** 320–333
8. Handlogten, M. E., Garcia-Cañero, R., Lancaster, K. T., and Christensen, H. N. (1981) *J. Biol. Chem.* **256,** 7905–7909
9. Kelley, D. S., and Potter, V. R. (1979) *J. Biol. Chem.* **254,** 6691–6697
10. Christensen, H. N. (1964) *Proc. Natl. Acad. Sci. U. S. A.* **51,** 337–344
11. Christensen, H. N., and Antonioli, J. A. (1969) *J. Biol. Chem.* **244,** 1497–1504
12. Segal, S., Schwartzman, L., Blair, A., and Bertolli, O. (1967) *Biochim. Biophys. Acta* **135,** 127–135
13. Blasberg, R., and Lajtha, H. (1966) *Brain Res.* **1,** 86–104
14. Christensen, H. N. (1973) in *Membrane Structure and Mechanisms of Biological Energy Transductions* (Avery, J., ed) pp. 233–263, Plenum Press, London
15. Sly, W. S., and Grubb, J. (1979) *Methods Enzymol.* **58,** 444–450
16. Leffert, H. L., Koch, K. S., Moran, T., and Williams, M. (1979) *Methods Enzymol.* **58,** 536–544
17. Gazzola, G. C., Dall'Asta, V., Franchi-Gazzola, R., and White, M. F. (1981) *Anal. Biochem.* **115,** 368–374
18. Berry, M. N., and Friend, D. S. (1969) *J. Cell. Biol.* **43,** 506–520
19. Kilberg, M. S., Handlogten, M. E., and Christensen, H. N. (1980) *J. Biol. Chem.* **255,** 4011–4019
20. Kletzien, R. F., Pariza, M. W., Becker, J. E., and Potter, V. R. (1975) *Anal. Biochem.* **68,** 537–544
21. Cleland, W. W. (1979) *Methods Enzymol.* **63A,** 103–138
22. Herbert, J. D., Coulson, R. A., and Hernandez, T. (1966) *Comp. Biochem. Physiol.* **17,** 583–598
23. Bloch, K. (1946) *J. Biol. Chem.* **165,** 469–476
24. Bloch, K. (1946) *J. Biol. Chem.* **165,** 477–484
25. LeFevre, P. G. (1975) in *Current Topics in Membranes and Transport* (Bronner, F., and Kleinzeller, A., eds) Vol. 7, pp. 109–215, Academic Press, New York
26. Akedo, H., and Christensen, H. N. (1962) *J. Biol. Chem.* **237,** 118–122
27. Thomas, E. L., Shao, T.-C., and Christensen, H. N. (1971) *J. Biol. Chem.* **246,** 1677–1681
28. Stevens, C. M., and Bush, J. A. (1950) *J. Biol. Chem.* **183,** 139–147
29. Christensen, H. N., and Handlogten, M. E. (1969) *FEBS Lett.* **3,** 14–17
30. Schwartzman, L., Blair, A., and Segal, S. (1967) *Biochim. Biophys. Acta* **135,** 120–126
31. Christensen, H. N. (1975) in *Current Topics in Membranes and Transport* (Bronner, F., and Kleinzeller, A., eds) Vol. 6, pp. 227–258, Academic Press, New York
32. Ratner, S. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol.* **39,** 1–90
33. Miller, L. L. (1962) in *Amino Acid Pools* (Holden, J. T., ed) pp. 708–721, Elsevier, New York
34. Felig, P., and Wahren, J. (1971) *J. Clin. Invest.* **50,** 2703–2714
35. Owen, E. E., and Robinson, R. R. (1963) *J. Clin. Invest.* **42,** 263–276
36. Greengard, O., Sahib, M. J., and Knox, W. E. (1970) *Arch. Biochem. Biophys.* **137,** 477–482
37. Meister, A. (1965) *Biochemistry of the Amino Acids,* Vol. 2, p. 696, Academic Press, New York
38. Schimke, R. T. (1963) *J. Biol. Chem.* **238,** 1012–1018
39. Simell, O. (1975) *Pediat. Res.* **9,** 504–508
40. Cornford, E. M., Braun, L. D., Pardridge, W. M., and Oldendorf, W. H. (1980) *Am. J. Physiol.* **238,** H553–H560
41. Schimke, R. T. (1964) *J. Biol. Chem.* **239,** 3808–3817
42. Poole, B., Leighton, F., and de Duve, C. (1969) *J. Cell. Biol.* **41,** 536–546
43. Stein, W. D. (1967) *The Movement of Molecules across Cell Membrane,* pp. 126–176, Academic Press, New York
44. Christensen, H. N., Handlogten, M. E., and Thomas, E. L. (1969) *Proc. Natl. Acad. Sci. U. S. A.* **63,** 948–955
45. Davis, J. R., Baietto, E. B., and Delisi, S. D. (1966) *Comp. Biochem. Physiol.* **17,** 697–700
46. Flag-Newton, J., Simpson, I., and Loewenstein, W. R. (1979) *Science* **205,** 404–407
47. Loewenstein, W. R. (1979) *Biochim. Biophys. Acta* **560,** 1–65
48. Brostoff, S. W., Powers, J. M., and Weise, M. J. (1980) *Nature* **285,** 103–104
49. Kilberg, M. S., Handlogten, M. E., and Christensen, H. N. (1981) *J. Biol. Chem.* **256,** 3304–3312
50. Christensen, H. N. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol.* **49,** 41–101
51. Garcia-Sancho, J., Sanchez, A., and Christensen, H. N. (1977) *Biochim. Biophys. Acta* **464,** 295–312
52. Eavenson, E., and Christensen, H. N. (1967) *J. Biol. Chem.* **242,** 5386–5396
53. Christensen, H. N., Liang, M., and Archer, E. G. (1967) *J. Biol. Chem.* **242,** 5237–5246