A Stereoselective Anomaly in Dicarboxylic Amino Acid Transport*

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Using L-cysteate and L-cysteine sulfinate as model substrates, we characterize here a transport system, both in cultured rat hepatocytes and human skin fibroblasts, serving for the anions glutamate and aspartate, but not for the dipolar species glutamic and asparagine acids. This system appears to be accompanied by a second, lower affinity system for the anionic forms, which is also Na+-dependent; this lower affinity system applies at least to glutamate. These systems show the usual degree of preference for L- over D-glutamate and, in the fibroblast, for L- over DL-α-amino adipate. D-Aspartate proved nearly as inhibitory to the uptake of L-cysteate or L-aspartate, however, as did L-aspartate itself, a comparison recalling a similar stereoselective anomaly discovered by Pall in Neurospora (Pall, M. (1976) Biochim. Biophys. Acta 211, 513–520). We conclude that this anomaly arises from the ability of the two substrate carboxylate groups to bond in the spatial order either αβ for the L-isomer or βα for the D-isomer and also to bond in the order αγ for L-glutamate, but scarcely in the order γα for D-glutamate. A major lack of inhibition by D-cysteate, which might be expected to bind like aspartate in the inverted order, shows, however, that the two anionic groups are not recognized in identical manners by the two corresponding subsites. Precedent for a chemical difference in these two subsites is available from transport systems for neutral α- and β-amino acids. A strong transport inhibition of the hepatic system by 3-aminoglutarate shows that an α,α relation between the amino group and either of the carboxylate groups of the anionic amino acid is not required. The above anomaly in stereoselectivity is compared with a corresponding one, applying to the reactions of aspartic acid and asparagine, versus glutamic acid and glutamine, with System L for neutral amino acid transport in the Ehrlich cell. A weak pH-dependent inhibition of the uptake of anionic amino acids by cysteine can be associated with its unique mode of conversion to an anionic species.

The transport of the dicarboxylic amino acids across cellular membranes is marked by two interesting problems. First, they may be transported by systems for neutral amino acids, e.g., by the Na+-dependent System A and by Na+-independent

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† Terms such as aspartate and cyssteeate refer specifically to the anionic species of these amino acids. As usual, aspartic acid and the like may refer to the amino acid in general or, when the context indicates specifically, to the dipolar species without net charge.

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but in a contrasting case the anomaly was inconspicuous (see Table II in Ref. 8). We investigate here new cases where the dicarboxylic amino acids must be anions, selecting the rat hepatocyte to represent an epithelial tissue and the human skin fibroblast to represent a mesenchymal tissue for contrast.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Rat hepatocytes were isolated as previously described (9), seeded in collagen-treated 4-compartment (24 x 67 mm each) plastic trays (LUX), and cultured in Waymouth's medium (MB752/1) containing 0.2% bovine serum albumin, 5 µg/ml of sodium oleate, 0.41 mM L-alanine, 0.53 mM L-serine, 65.5 µg/ml of penicillin, 5.8 µg/ml of streptomycin, 22.4 µg/ml of gentamicin, and 500 micrograms/ml of insulin. This medium was replaced after 4 h of culturing with the same medium lacking the insulin. Cells were used for transport assay 24 h after plating.

Human fibroblasts, obtained from skin biopsy explants as described previously (10), were routinely grown in 10-cm diameter dishes (Costar) in Medium 199 containing 10% fetal calf serum. The conditions of culturing were: pH 7.4; atmosphere, 5% CO₂ in air; temperature, 37°C. For uptake experiments cells were seeded in 24-well plates (Costar) and used when cell density reached 25 ± 3 µg of protein/cm². All points out of this range were discarded. The culture medium was always renewed 24 h before the experiment.

**Uptake Assay**—Amino acid uptake was measured under conditions approaching initial entry rates (1 min for fibroblasts, 5 or 10 min for hepatocytes) as follows. After appropriate incubation in bicarbonate-buffered solutions (90 min in Earle's salt solution containing 10% dialyzed fetal calf serum for human fibroblasts and 30 min in Krebs-Ringer solution for hepatocytes) at pH 7.4 and 37 °C, cell layers were washed and incubated for the required time in the same salt solution containing the labeled amino acid under study and any other designated compounds. In some experiments, a medium in which choline replaced Na⁺ was used. During experiments in which the pH of the medium differed from 7.4, Tris(hydroxymethyl)aminomethane hydrochloride or ε-amincaproic acid was used as buffer. The incubations were terminated by rapidly rinsing the cell layers twice with ice-cold saline. The soluble contents were extracted from the cells with 10% trichloroacetic acid and counted in a liquid scintillation spectrometer. Cells were dissolved in 1 N NaOH and assayed for protein by the method of Lowry et al. (11).

**Calculations**—In all cases, uptake was linear over the indicated time intervals. Kinetic parameters were determined by a computer (Hewlett-Packard 9845A) using the Marquardt's algorithm, a general procedure for least squares estimation of nonlinear parameters (12). All uptake data are expressed in nanomoles or micromoles (as indicated) of tested amino acid/ml of intracellular water-min.

**Materials**—All sera, growth media, antibiotics, and trypsin solution were from Gibco, New York, L-[3,3'-H]Aspartic acid (specific activity, 12 Ci/mmol) and L-[6,2-3H]-Glutamic acid (27 Ci/mmol) were obtained from Amersham, Bucks, England. 2-(Methylamino)isobutyric acid was from Aldrich-Chemie, Beerse, Belgium. Unlabeled L- and D-cysteinesulfinic and L-homocysteic acids were generous gifts to one of us (G. C. G.) from Professor Ferdinando Palmieri, University of Bari, Bari, Italy. L-Cysteate and L-cysteinesulfinate were prepared synthetically from both L-cysteine and L-3,3,3′-H₂cysteine for the experiments at Michigan. L- and DL-Aminoacidic acids were obtained from ICN Pharmaceuticals, Cleveland, 3-Aminoglutaric acid (13) was the gift of Dr. Alton Meister, Department of Biochemistry, Cornell University Medical School, New York (to H. N. C.). All other unlabeled natural or analogous amino acids were synthesized in the laboratory of one of us (H. N. C.) or purchased from Sigma.

**RESULTS**

Cysteic acid has been titrated potentiometrically and shown to have a pK₁ value rather lower than that for taurine (14), perhaps about 1.5. Cysteinesulfinic and homocysteinesulfinic acids showed similar pK₁ values, set at 1.50 and 1.66 (pK₂ = 2.98 and 2.60) at 0.2 M concentrations (15). We will...
obtained at substrate levels too low to include a significant contribution by the second component.

Fig. 3 shows that the Na⁺-dependent uptake of 0.01 mM aspartate by skin fibroblasts was little affected by the presence of 2 mM BCH or MeAIB, even at decreased pH values.

**TABLE I**

Lack of inhibition of rate of cysteate uptake into the rat hepatocyte by some neutral amino acids

Uptake of tritium-labeled L-cysteate at 0.1 mM measured during 1 min at 37 °C and pH 7.4 from the bicarbonate buffered Krebs-Ringer medium with the inhibitor replacing part of the NaCl isoosmotically. The raw data were corrected for the corresponding nonsaturable fraction according to Akedo and Christensen (1). The transport assay was made during (B), and of [3H]glutamate (C) in cultured human fibroblasts. The curves in the center panel corresponds to a half-maximal action at [Na⁺] = approximately 45

![Graph](image1)

**FIG. 2.** Kinetic parameters by Hofstee plots for the uptake of [3H]cysteinesulfinate by hepatocytes (A), of L-[3H]aspartate (B), and of L-[3H]glutamate (C) in cultured human fibroblasts. The transport assay was made during 1 min for the hepatocytes and 1 min for the fibroblasts, all at pH 7.4 and 37 °C and over the indicated ranges of values of v/[S] in the presence of either 142 mM Na⁺ (total uptake) or zero added Na⁺ (Na⁺-independent uptake). The raw data were corrected for the corresponding nonsaturable fraction according to Akedo and Christensen (17). The Na⁺-dependent fraction (A) was then obtained by difference. The data were fitted by computer to two rectangular hyperbolas as the Na⁺-dependent component and to only one hyperbola in the case of aspartate. Parameters obtained for the individual components of transport thus presumed were as follows (within 68% confidence intervals). For A and the hepatocytes, the lines drawn by a different computer program correspond to KA values of 0.021 and 3.1 mM and Vmax values of 0.029 and 0.23 μM·mL⁻¹·cell water⁻¹. For B and C and aspartate uptake by the fibroblasts: Na⁺-dependent component values were Vmax = 0.042 ± 0.01 μM·mL⁻¹·cell water⁻¹ and KA = 0.015 ± 0.002; (Na⁺-independent component) Vmax = 0.061 ± 0.005; KA = 1.63 ± 0.17; glutamate: (Na⁺-dependent component) Vmax = 0.020 ± 0.006; KA = 0.016 ± 0.005; Vmax = 0.83 ± 0.075; KA = 3.24 ± 0.55; (Na⁺-independent component) Vmax = 0.139 ± 0.003; KA = 0.90 ± 0.043.

**TABLE II**

Initial rates (during 1 min) of uptake of [3H]aspartate at 0.01 mM by the human skin fibroblast at pH 7.4 and 37 °C in the presence of increasing concentrations of selected nonanionic amino acids

The values are shown with the standard errors for three determinations within two to five complete experiments. GPA, the arginine analog, 1-guanil-4-aminopiperidine-4-carboxylic acid (16). The values are shown with the standard errors for three determinations within two to five complete experiments. GPA, the arginine analog, 1-guanil-4-aminopiperidine-4-carboxylic acid (16).

| Inhibitor | Uninhibited uptake | % |
|-----------|--------------------|---|
| Taurine (25 mM) | 108 | |
| Alanine (25 mM) | 101 | |
| Glutamine (25 mM) | 94 | |
| Cystine (0.5 mM) | 104 | |
| BCH (20 mM) | 96 | |
| MeAIB (20 mM) | 102 | |

Furthermore, lowering the pH of the medium largely eliminated the Na⁺-dependent uptake of aspartate but sharply increased (as shown by the widest separation of the curves in the center panel) its BCH-inhibitable, Na⁺-independent uptake, as would be expected if the neutral form, aspartic acid, were taken up by System L as is it is in the Ehrlich cell (2). Fig. 4 shows the effect of [Na⁺] on the uptake rate for aspartate at pH 7.4, again illustrated for the fibroblast. The curve corresponds to a half-maximal action at [Na⁺] = approximately 45

![Graph](image2)

**FIG. 3.** Effect of pH on initial uptake rates of 0.01 mM L-[3H]aspartic acid in the human skin fibroblast. The gradual conversion of the anionic to the zwitterionic form resulted in an increase in total uptake rate (B, left). The increase shows itself to be Na⁺-independent (C, center). This fraction was not inhibited by excess MeAIB (2 mM; A, left and center) but was strongly inhibited by BCH (2 mM; B, left and center). Accordingly, it undoubtedly represents the contribution of System L, which accepts well the zwitterionic form of this substrate in the Ehrlich cell (2). The Na⁺-dependent component, as estimated by subtracting the Na⁺-independent component from the total uptake rate, was instead decreased by lowering the pH (B, right) but was not affected by MeAIB (2 mM; A, right) or BCH (2 mM; B, right). The pH effect supports operation of this component on the anionic form of the substrate.
The following studies concern the predominating high-affinity transport system of the respective cultured cells.

Fig. 5 shows the uptake of L-cysteate at 0.1 mM into cultured hepatocytes at pH 7.4 and 37 °C under inhibition by various anionic amino acids, and Fig. 6 shows corresponding effects on the uptake of L-aspartate at 0.01 mM by the skin fibroblasts. L-Cysteinesulfinate showed a similar pattern of inhibition (data not included in Fig. 5). Table III summarizes the $K_i$ and maximal inhibition values derived from the data of these figures. Uptake by the fibroblast tended to half-saturate with aspartate (not shown in Table III) and to be half-maximally inhibited by it at concentrations about one-tenth those required for the hepatocyte. This difference might reflect differences in the range of concentrations of extracellular amino acids to which the two cells are exposed in their organismal contexts. Except for the high affinities seen with fibroblasts for neutral (10), as well as for anionic amino acids, other

**Fig. 4.** Sodium dependence of the uptake of aspartate at 0.01 mM by cultured human fibroblasts at pH 7.4. Na+-containing medium in the form of Earle's balanced salt solution, and a Na+-free medium in which choline replaced that cation in Earle's solution were mixed to obtain intermediate concentrations of Na⁺.

**Fig. 5.** Inhibition of the 1-min uptake of 0.1 mM tritium-labeled cysteate by rat hepatocytes from 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered Ringer medium, pH 7.4, by various anionic amino acids.

**Fig. 6.** Inhibition of uptake rates of 0.01 mM l-[3H]aspartate in the human skin fibroblast in the presence of increasing concentrations of several analogs, as specified in each panel. ---, best computer-derived fit for a rectangular hyperbola; -- - , drawn between experimental points when no fit of a rectangular hyperbola was justified.
**Contrasts between the epithelial hepatocyte and the mesenchymal fibroblast include the presence in only the former of cysteate. D-Cysteate produced a much weaker inhibition than the acid, proved essentially as effective an inhibitor of cysteate as an agency transporting cysteate into Neurospora mycelial pads, whereby n-aspartate was distinctly preferred to the amino form (Fig. 6, upper right).**

**Pall (4) first reported an anomaly in the stereoselectivity of an agency transporting cysteate into Neurospora mycelial pads, whereby D-aspartate was distinctly preferred to the L-isomer. Subsequently, Garcia-Sancho et al. (2) discovered a corresponding anomaly between the same two amino acids but applying to the totally different transport system L, tested at pH values of about 4.2, low enough to convert glutamate and aspartate extensively to their neutral forms, HOOC(CH₂)₇CH(CH₃)₂-COO⁻.**

**The present anomaly corresponds in both cell types more closely to that described by Kleinzeller, personal communication as cited in Ref. 5 (p. 62),**

| Inhibitor                  | Rat hepatocytes | Human fibroblasts |
|----------------------------|-----------------|-------------------|
|                            | Control         | Human fibroblasts  |
|                            | I_{max}         | K, (K_{i}
| Rat hepatocytes            | (nmol-m⁻¹ cell water-min⁻¹) | (nmol-m⁻¹ cell water-min⁻¹) |
| L-Aspartate                | 23.3            | 12.4              |
| D-Aspartate                | 23.3            | 12.4              |
| L-Cysteinesulfinate        | N.D.*           | N.D.              |
| D-Cysteinesulfinate        | N.D.            | N.D.              |
| L-Cysteate                 | 23.3            | 12.4              |
| D-Cysteate                 | 23.3            | 12.4              |
| L-Glutamate                | 23.3            | 12.4              |
| D-Glutamate                | 23.3            | 12.4              |
| L-Aminoadipate             | N.D.            | N.D.              |
| D-Aminoadipate             | N.D.            | N.D.              |
| L-Aminopimelate            | N.D.            | N.D.              |
| L-Homocysteate             | 23.3            | 12.4              |
| 3-Aminoglutarate           | 23.3            | 12.4              |

*N.D.*, not determined.

The higher homolog of cysteate, namely homocysteate (Fig. 5, lower right; Fig. 6, lower right), produced little or no inhibition in either cell, in agreement with an unpublished observation by Kleinzeller for the freshly separated hepatocyte. D-Cysteate produced a much weaker inhibition than the L-isomer in both cell types (Fig. 5, upper left panel; Fig. 6, upper right). The same distinction was seen for D- and L-glutamate (Fig. 5, lower left panel; Fig. 6, lower left; D-glutamate failing to cause perceptible inhibition even at 1 mM) and for D- and L-cysteinesulfinate (compared in the fibroblasts only, Fig. 6, upper center), whereas little difference was seen between the effect of D- and L-aspartate (Fig. 5, upper right; Fig. 6, upper left). In the fibroblast, the K, of DL-a-aminoacidopimelate showed twice the value seen for the L-isomer (Fig. 6, lower center; Table III), whereas D-a-aminoacidopimelate failed to show inhibitory action (Fig. 6, lower right). The optically inactive structural isomer of glutamic acid, 3-aminoacetic acid, proved essentially as effective an inhibitor of cysteate uptake by the hepatocyte as did glutamic acid, each in its anionic form (Fig. 5, lower right).

**The apparent unsuitability of homocysteate as a test substrate for the low K_{in} system for anionic amino acids in either cell type (Figs. 5 and 6) does not exclude the possibility that this homolog may prove effective as a model substrate for the high K_{in} glutamate-prefering system (Fig. 2). Cysteate and homocysteate each has proved to be a selective inhibitor for a system, one for aspartate and one for glutamate, respectively, in the hepatoma cell line, HTC.**

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4. A. Kleinzeller, personal communication as cited in Ref. 5 (p. 62).

5. M. Makowske and H. N. Christensen, unpublished results.
The anomaly in the stereoselectivity of the L-system of the Ehrlich cell in the transport of the ω-protonated forms, namely glutamic and aspartic acids at low pH (2), bears a provocative relation to that just discussed. (The L system apparently also transports aspartic acid into the fibroblast at decreased pH (Fig. 3, central panel.) In this case the two carboxyl groups of the substrate are no longer alike since one is protonated and the other is charged. Furthermore, we have a priori no reason for expecting a second carboxyl group-recognition structure to serve as a part of the transport acceptor site of System L. Therefore, we cannot logically insist that D-aspartic acid binds in an inverted position at the site for the anionic system, nor does it follow that this ligand is disfavored by the presence and configuration of a side chain group common to these substrates, perhaps the carbonyl group. Although we accept the idea that D-aspartate combines in an inverted way at the site for the anionic system, nevertheless we note as a caution that a different explanation is needed for the anomalous behavior of D-asparagine with System L.

Where tested, Na⁺ dependency has been a usual finding for membrane transport systems by which aspartate and glutamate move in the form of those anions and, in some cases as summarized elsewhere (Ref. 5, pp. 82-83), two sodium ions have been reported to migrate with each anionic molecule. Renal brush border membranes appear, however, to take up glutamate with only one sodium ion since the uptake process is electrically neutral (20). Here too, both D- and L-aspartate, but not D-glutamate, are taken up.

In both the hepatocyte (see Fig. 4 in Ref. 22) and the skin fibroblast (Table II), inhibition of anionic amino acid transport was shown by cysteine. Cysteine inhibition has also been observed for the glutamate transport system in the mouse lymphocyte (in Ref. 6, see final footnote). In the present work (data not shown), this inhibition rises with pH in a manner consistent with reaction by the anionic form, "SCH₂CH(NH₂)COO⁻", which appears to be present already at pH 7.3 to the extent of about 2% of the total cysteine present, and to increase in abundance, along with the presumably inert anion, HSCH₂CH(NH₂)COO⁻, as the pH is raised (21). Considered, however, as to their making a contribution to cysteine transport, the anionic systems may apparently be neglected except at unphysiologically high pH values (22). Whether cysteine levels rise high enough to give this amino acid a regulatory function for anionic amino acid movements may also be questioned.

Our experiments have not yet shown differences in glutamic acid transport of a kind we might expect from the great functional differences between the hepatocyte and a peripheral tissue in handling this amino acid.

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