

Facilitated Transport of Amino Acids Across Organic Phases and the Human Erythrocyte Membrane

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1. An artificial facilitated amino-acid-transfer process operating across a chloroform phase is reported. 2. This process utilizes a family of bis(salicylamidato)copper(II) complexes. 3. A mechanism is proposed for this process and for its sensitivity towards cyanide and bathophenanthroline sulphonate. 4. Facilitated transfer of L-leucine in human erythrocytes has been shown to be inhibited by bathophenanthroline sulphonate.

In order to transfer an amino acid across a membrane, hydrogen-bonded water must be substituted by alternate groups, giving an interaction of approximately the same free energy and yet rendering the substrate lipophilic. Such a transition does not necessarily have to involve covalent bonds, for instance macrocyclic ethers are well able to solvate positively charged nitrogen atoms (Pedersen & Frensdorff, 1972; Pressman, 1973), and chelate formation between an amino acid and a metal ion can render the amino acid more lipophilic. In the work reported in the present paper the latter possibility of a metal-chelating centre being involved in amino acid transport is investigated. Pal & Christensen (1959) have previously considered this problem and showed that, in Ehrlich ascites cells, Mn^{2+} but not Fe^{2+} , Cu^{2+} or Zn^{2+} enhanced the uptake of amino acids. However, more recently a number of workers have demonstrated increased uptake of Cu^{2+} in the presence of a range of amino acids in Ehrlich ascites cells, brain, liver and kidney (Neumann & Silverberg, 1966; Harris & Sass-Kortsak, 1967; Chutkow, 1978).

Of the metals known to be essential for biological function, copper and zinc are the only two that are relatively abundant in biological material and that possess a high affinity for amino acids. Copper(II) has a greater affinity for α -amino acids than zinc(II) [$\log K_1$ for glycine: $Cu(II)$, $>10^8$; $Zn(II)$, 10^5]. Furthermore, copper(II)-amino acid complexes are kinetically labile, in fact more labile than those of zinc(II) (Irving & Williams, 1953). The extreme lability of copper(II) complexes results from the $3d^9$ electron configuration imposing a lack of spherical symmetry on the copper(II) ion. In contrast, the $3d^{10}$ non-bonding core of zinc(II) is spherically symmetrical. Thus of the two possible candidates for involvement in facilitated transport, copper(II) would

appear to be more suited. In the present study it has been established that despite the extremely high affinity of copper(II) for amino acids, it is possible for the amino acids to dissociate from such a metal centre, thus rendering the chelate mechanism a variable proposal. Bathophenanthroline sulphonate, a powerful copper(II)-chelating ligand, has also been used in erythrocyte-amino-acid-transport studies in an attempt to assess its inhibitory properties on the process.

Materials and Methods

Materials

Glycine, alanine, D- and L-leucine and bathophenanthroline sulphonate were purchased from Sigma. 1-Bromododecane, n-butylamine, 4-aminobutyric acid, ethylenediamine, diethylacetamidomalonic acid were purchased from BDH; [U - ^{14}C]glycine (10 mCi/mmol), D-[4,5- 3H]leucine (1 Ci/mmol), L-[U - ^{14}C]leucine (10 mCi/mmol) and L-[U - ^{14}C]alanine (10 mCi/mmol) were purchased from The Radiochemical Centre. 2-Aminotetradecanoic acid was prepared by condensation of molar quantities of 1-bromododecane and diethylacetamidomalonic acid in the presence of sodium metal in ethanol. Acid hydrolysis of the condensation product yielded 2-aminotetradecanoic acid hydrochloride (m.p. 206–208°C).

Preparation of Schiff-base copper chelates (Fig. 1)

The general method of Houghton & Pointer (1964) was used throughout and is described for the preparation of bis(*N*-ethoxycarbonylpropylsalicylamidato)copper(II). The amino acid ethyl esters were prepared by reflux in ethanolic HCl (16h) recrystallized from ethanol and treated with ammoniacal chloroform (1h) to regenerate the free base.

Table 1. *The series of bis(salicylamidato)copper(II) complexes used in the present study*

The structures of complexes I–V are probably of the type indicated in Fig. 1. Complex VI has a *cis* structure, owing to the tetradentate nature of the ligand.

Compound	R	M.p. (°C)	Infrared ν_{\max} . (cm ⁻¹)		Colour
I	-CH ₂ -CH ₂ -CH ₂ -CO ₂ -CH ₂ -CH ₃	94.5–95.5	1710	1620	Olive green
II	-CH ₂ -CO ₂ -CH ₂ -CH ₃	174–175	1735	1620	Olive green
III	-CH{CH ₂ -CH(CH ₂) ₂ }CO ₂ -CH ₂ -CH ₃	Oil	1735	1620	Olive green
IV	-CH[n-C ₁₄ H ₂₉]CO ₂ -CH ₂ -CH ₃	84–86	1720	1640	Olive green
V	-CH ₂ -CH ₂ -CH ₂ -CH ₃	81–82		1620	Brown
VI	(-CH ₂ -CH ₂ -) ₄	268–270		1630	Light green

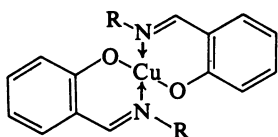


Fig. 1. *Bidentate bis(salicylamidato)copper(II) complex*

The majority of bidentate bis(salicylamidato)-copper(II) complexes possess a slightly distorted square planar configuration.

The precipitated NH₄Cl was filtered off and the filtrate was evaporated to dryness under reduced pressure to give a yellow oil.

Bis(salicylaldehydato)copper(II) (1.5 g) was suspended in benzene (15 ml) under N₂. To this suspension was added dropwise a solution of ethyl 4-aminobutanoate (1 g) in benzene (5 ml). The resulting mixture was stirred vigorously at 40°C for 5 min, cooled in ice and filtered. The filtrate was evaporated to dryness under reduced pressure and the resulting oil was dissolved in dichloromethane and filtered. Subsequent evaporation of dichloromethane and trituration with light petroleum (b.p. 60–80°C) yielded a solid. Recrystallization from benzene/light petroleum (b.p. 60–80°C) yielded bis-(*N*-ethoxycarbonylpropylsalicylamidato)-copper(II) [m.p. 367–368°C (368–369°C; Houghton & Pointer, 1964); 77%]. The range of compounds prepared in this study is given in Table 1.

Measurement of amino-acid flux across a chloroform barrier

U-tube assemblies, at 20°C, were utilized throughout the present study, in which the chloroform layer (6 ml) was stirred by a magnetic follower and the two aqueous layers (20 mM Tris/HCl, pH 7.0; 5 ml) were mechanically stirred by glass propellers (20 rev./min). Radiolabelled amino acids were added to one aqueous compartment (source) and samples

(0.1 ml) were removed from the second aqueous compartment (sink) at timed intervals. Samples were immediately replaced by the same volume of buffer. Samples were taken from the source at the beginning and end of the experiment. All samples were subjected to liquid-scintillation counting of radioactivity, and representative samples were chromatographed.

Measurement of amino acid flux across intact erythrocyte membranes

Freshly outdated blood was centrifuged at 2000 g for 15 min at 2°C, the plasma was subsequently removed and the cells were resuspended in NaCl (150 mM). The centrifuge step was repeated. After two such washes, the packed cells were resuspended in NaCl (150 mM) containing D-glucose (5 mM). Amino-acid-uptake rates were identical when erythrocytes were incubated in either Krebs–Ringer bicarbonate (Umbreit *et al.*, 1946) or in NaCl (150 mM). Samples of this preparation were used for dry-weight determination. The erythrocyte preparation (1 ml) was added to a 25-ml conical flask containing NaCl (150 mM, 1 ml) in the presence or absence of inhibitor. The contents of the flask were warmed to 37°C and shaken for 5 min. The experiment was then initiated by the addition of ¹⁴C-labelled amino acid that has been previously diluted with unlabelled (¹²C) amino acid. After a predetermined time, the incubation was terminated by the addition of ice-cold NaCl (150 mM, 2 ml) and immediate placement of the flask into an ice bath. The chilled incubation mixture was centrifuged at 2000 g for 5 min, followed by removal of the supernatant, which was retained for liquid-scintillation counting of radioactivity. The packed cells were washed with NaCl (150 mM) twice and then haemolysed by suspension in water (2 ml). The haemoglobin was denatured by standing the tubes in boiling water for 5 min. After centrifugation the supernatant was used for liquid-scintillation counting of radioactivity. By using an intracellular-fluid fraction for human erythrocytes of 68%, it was possible to determine the amino-

acid-distribution ratios. Amino-acid accumulation by Extensor digitorum longus muscle of the rat was determined as previously reported by Hider *et al.* (1971).

Determination of amino-acid-permeability coefficients

The basic method used in the present study was that described by Winter & Christensen (1964). The time-dependency of amino-acid uptake is assumed to follow first-order kinetics, thus the permeability coefficient, *P*, can be calculated from the following relationship:

$$P = k \frac{V}{A}$$

where *k* is the first-order rate constant calculated from the time-dependency of amino-acid uptake (Lossen, 1972), *V* the volume of a human erythrocyte (87 μm³; Whittam, 1964), and *A* the surface area of a human erythrocyte (155 μm²; Whittam, 1964).

Measurement of radioactivity in samples by liquid-scintillation counting

Aqueous samples (0.1 or 0.2 ml) were diluted with 5 ml of 0.2% 2,5-bis-(5-t-butylbenzoxazol-2-yl)-thiophen ('BBOT') in Triton/water/toluene (5:1:10, by vol.). Samples from partition studies, involving chloroform, were evaporated under reduced pressure before the addition of ethanol and scintillant. Quenching due to the green chelates was corrected by the use of an internal standard.

Determination of membrane-bound copper

Washed packed cells (20ml) prepared as above were haemolysed by the addition of water (30ml), followed by stirring at 2°C for 2h. The lysed cells were then centrifuged at 400g for 2h at 2°C, the supernatant being discarded. The pellet, consisting mainly of membrane material, was washed free of cellular contaminants by suspension in water, followed by centrifugation at 4000g for 30min. This procedure was repeated three times. The pellet was suspended in 2mM-NH₃ (6ml) and the copper content determined by atomic absorption with a Perkin-Elmer model 103 atomic-absorption spectrophotometer.

Results

Facilitated transfer of amino acids across a chloroform barrier

The effect of bis(salicylamidato)copper(II) complexes on the transfer of amino acids across a chloroform barrier is typified by the experiments depicted in Fig. 2. In the presence of bis-(*N*-ethoxy-

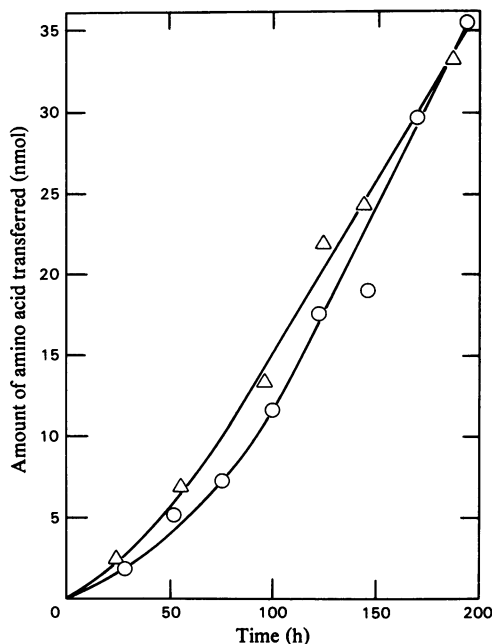


Fig. 2. Transfer of L-leucine (O) and L-alanine (Δ) across a chloroform barrier

The concentration of complex I was 6 mM in both cases. The amino acid concentration of the sources was 1 mM. The long period required for the system to reach a steady state was due to the time necessary for the concentration of complexed amino acid in the chloroform phase to reach a maximum value.

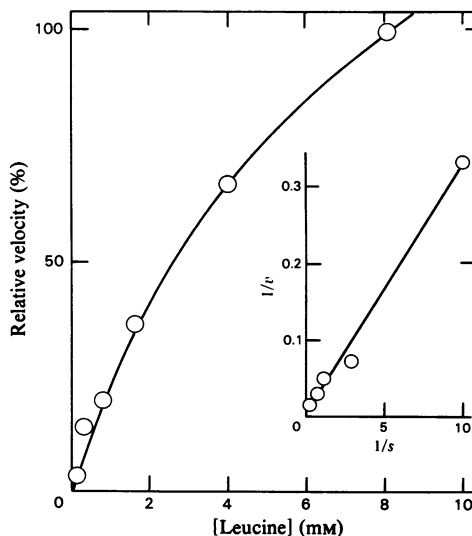


Fig. 3. Concentration-dependence of the copper-chelate (I)-facilitated transfer of L-leucine

The relative velocity was measured for each concentration as indicated in Fig. 2. The inset shows the data treated by the Lineweaver-Burk method.

Table 2. Steady-state flux of L-leucine across a chloroform phase separating two aqueous solutions. The concentration of all the complexes was 6 mM. The leucine concentration of the source was 1 mM. The fluxes were determined as indicated in Fig. 2.

Chelate	Organic phase	ϵ	Additive to the aqueous phases	$10^{15} \times$ Steady-state flux (mol/h)
I	Carbon tetrachloride	2.0	—	1200
I	Chloroform	4.6	—	1300
I	Dichloromethane	9.0	—	2000
I	Dichloroethane	10.5	—	2100
II	Chloroform	4.6	—	870
III	Chloroform	4.6	—	1000
IV	Chloroform	4.6	—	220
V	Chloroform	4.6	—	130
VI	Chloroform	4.6	—	80
No chelate	Chloroform	4.6	—	4.0
I	Chloroform	4.6	NaCN (2 mM)	1400
I	Chloroform	4.6	Bathophenanthroline sulphonate (2.5 mM)	93

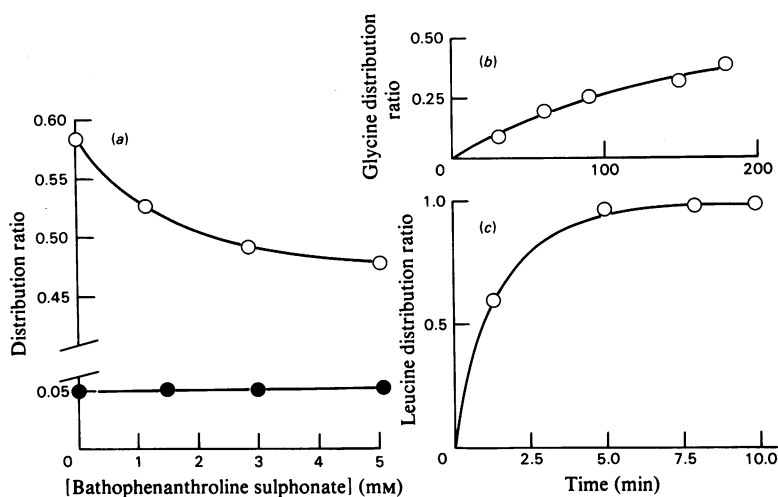


Fig. 4. Effect of bathophenanthroline sulphonate on the uptake of L-leucine (O) and glycine (●) by human erythrocytes. (a) The concentration of each amino acid in the medium was 1 mM, and the incubation times were: L-leucine, 3 min; glycine, 30 min. Each value is the mean of three determinations. (b) and (c) show the time-dependence of uptake of glycine and L-leucine. The permeability coefficients for glycine and L-leucine were calculated as 5×10^{-9} and $4.8 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ respectively. Distribution ratios were determined as the ratio of the concentrations of intracellular and extracellular radioactivity.

carbonylpropylsalicylamidato)copper(II) (compound I, Table 1) (6 mM), L-alanine ($1 \mu\text{M}$) and L-leucine ($1 \mu\text{M}$) are both transferred from one aqueous compartment to the second, the rates of transfer reaching a steady state after approx. 80 h. The rates of transfer were: L-leucine, 1.3 pmol/h; L-alanine, 1.1 pmol/h. This amino-acid transfer showed an indication of saturation kinetics (Fig. 3) and was influenced by the dielectric constant of the organic phase (Table 2). Amino-acid transfer was demonstrated directly by chromatography of the aqueous sink, when it was shown that neither

^{14}C -labelled alanine nor ^{14}C -labelled leucine had undergone chemical modification. A range of bis(salicylamidato)copper(II) complexes were originally studied with a view to establish the optimum complex type for amino-acid facilitated transport (Table 2). The rates of leucine transfer were found to reflect the tendency of leucine to partition into the chloroform phase in the presence of the chelate, the relative sequence of partition coefficients being $\text{I} > \text{III} > \text{II} \gg \text{IV} > \text{V} > \text{VI}$ (compounds in Tables 1 and 2). There was little difference between the activity of complexes I, II and III (Tables 1 and 2);

however, the other complexes were far less effective at facilitating leucine transfer (Table 2).

The presence of NaCN in the aqueous media produced no significant change in the facilitated flux of L-leucine, in contrast with bathophenanthroline sulphonate, which had a profound effect on L-leucine transfer (Table 2). In the presence of bathophenanthroline sulphonate there was no appreciable change in the colour of the chloroform solution of the copper chelate (I, Table 2).

Effect of bathophenanthroline sulphate on erythrocyte amino-acid transport

In view of the results with the synthetic copper chelates, bathophenanthroline sulphate was selected as a possible inhibitor of leucine transport because of its high affinity for copper(II). A problem with most relatively specific copper(II) chelators, for instance unsubstituted bathophenanthroline, is that they are extremely hydrophobic and are thus able to penetrate membranes in a non-specific manner. Such a problem is much less likely with bathophenanthroline sulphonate, owing to its charged nature. Bathophenanthroline sulphonate was found to inhibit the transport of L-leucine by human erythrocytes without influencing glycine uptake, the effect increasing with inhibitor concentration (Fig. 4). The proportion of leucine entering the erythrocyte via simple diffusion was determined by measuring the uptake of L-leucine under condi-

tions of carrier saturation (Fig. 5). The leucine permeability coefficient calculated for this process was $1.4 \times 10^{-8} \text{ cm} \cdot \text{s}^{-1}$, the corresponding value for erythrocytes was $4.8 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$, which is of similar magnitude to that determined by Winter & Christensen (1964). The inhibition of leucine uptake was investigated by measuring V_{max} and K_m values (Fig. 6) in the presence and absence of bathophenanthroline sulphonate. The data clearly correspond to inhibition of a mixed type. A correction for the simple diffusion component is included in Fig. 6 thus it can be estimated that, at a leucine concentration equal to that of the inhibitor (2.5 mM), the accumulation of leucine via the facilitated transfer mechanism after 3 min is 62% of that of the control.

The quantity of copper associated with erythrocyte membranes used in the present study was found to be $0.013 \mu\text{g}$ of copper/ml of blood. As the average human erythrocyte count is $5 \times 10^6/\text{ml}$ (Wintrobe, 1952), it can be estimated that there are 10^7 membrane-bound copper atoms per human erythrocyte.

Specificity of leucine transport

Incubation of erythrocytes with L- and D-leucine

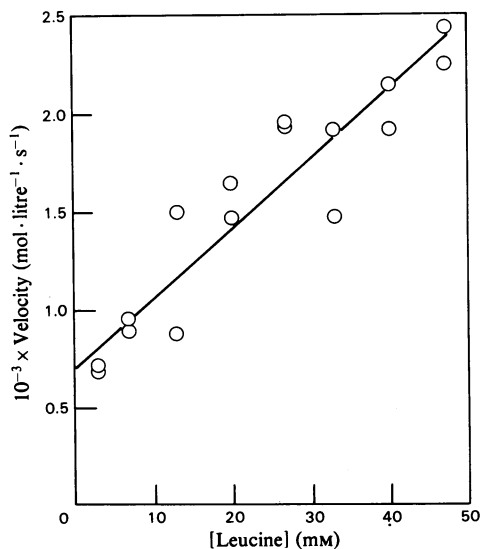


Fig. 5. Uptake of L-leucine by human erythrocytes under conditions of carrier saturation

The concentration of NaCl in the incubation media was adjusted to give iso-osmotic solutions. The incubation time was 3 min.

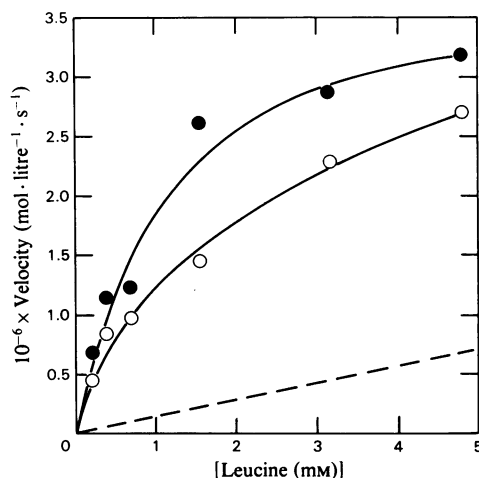


Fig. 6. Uptake of L-leucine by erythrocytes in the absence (●) and presence (○) of bathophenanthroline sulphonate (2.5 mM)

----, Estimated contribution by simple diffusion. The incubation time was 3 min. Each point is the mean of three individual determinations. V_{max} and K_m values (\pm s.d.) in the presence and absence of the inhibitor were determined from an Eadie-Hofstee plot:

	K_m (mM)	V_{max} (mmol . litre ⁻¹ . s ⁻¹)
+ Inhibitor	0.98 ± 0.12	2.28 ± 0.16
- Inhibitor	0.67 ± 0.10	2.78 ± 0.19

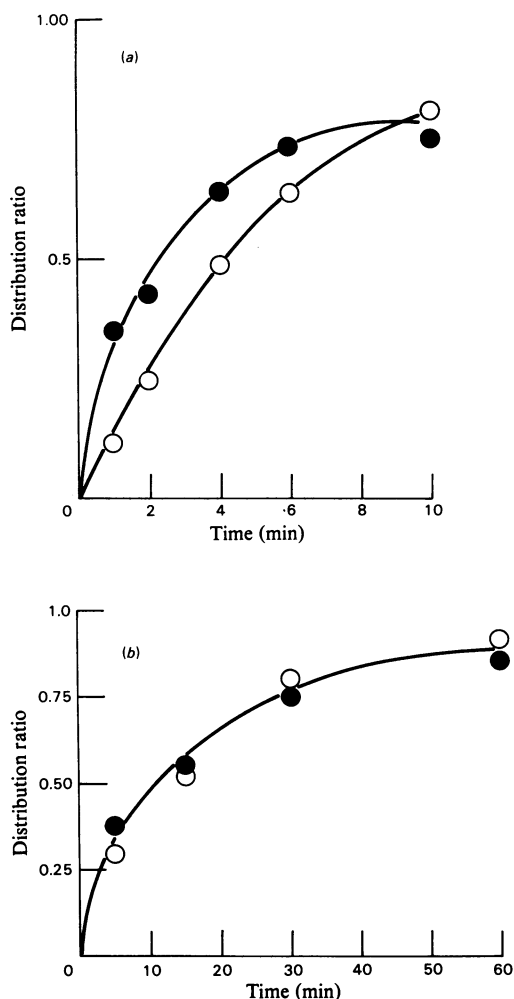


Fig. 7. Transport of D-leucine (O) and L-leucine (●) by (a) human erythrocytes and (b) rat extensor digitorum longus muscle

The concentration of leucine in the medium was $50\mu\text{M}$.

was followed at a low amino-acid concentration ($50\mu\text{M}$) in order to minimize the contribution due to simple diffusion processes. There was found to be little difference between the rates of facilitated transfer of the two enantiomers (Fig. 7a). As leucine is transported by the L-system (Oxender & Christensen, 1963), it was decided to establish whether or not the poor selection of enantiomers was also observed in other tissues reported to possess the same transport mechanism. The data obtained from rat extensor digitorum longus muscle were found to be very similar to that from erythrocytes (Fig. 7b).

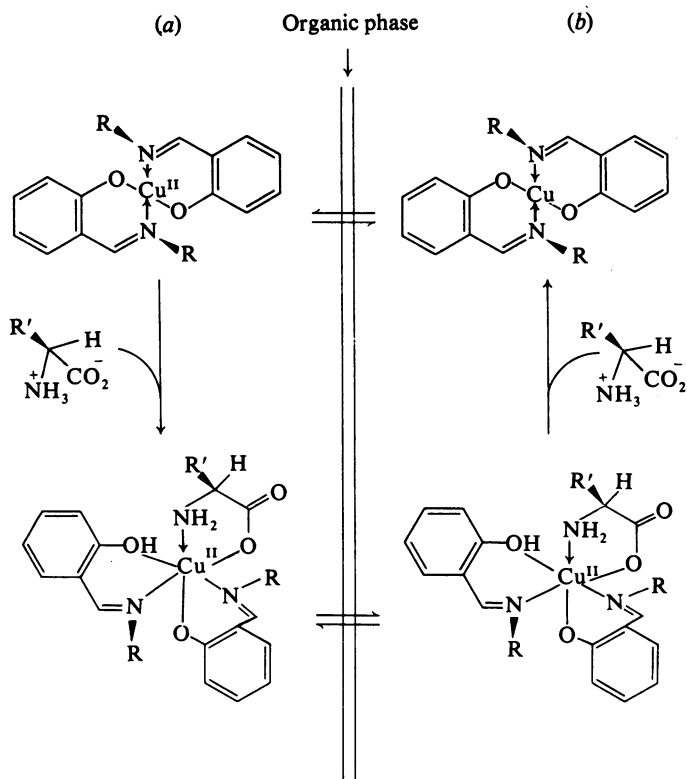
Discussion

The initial work with lipid-soluble copper(II) chelates demonstrates that although the affinity of copper(II) for amino acids is extremely high, it is possible for such a centre to transfer amino acids from one aqueous compartment to another, i.e. amino acids dissociate from such complexes at appreciable rates. This is presumably a direct result of the kinetic lability of copper(II) complexes (Poplewell & Wilkins, 1955; Pearson & Ellgen, 1975). Thus the concept of chelate-mediated amino-acid translocation outlined in the introduction appears to be a viable possibility. The mechanism of bis(salicylamidato)copper(II)-facilitated transfer of amino acids is likely to be of the type indicated in Scheme 1. Such a mechanism is quite feasible for copper(II), owing to its extremely flexible stereochemistry (Österberg, 1974). The process has been shown to function in media of dielectric constant approaching 2 (Table 2), a value typical of the lipid environment of biological membranes. This mechanism also accounts for the inhibitory properties of bathophenanthroline sulphonate, which by competing with the chelates (Fig. 1) for the copper ion would decrease the rate of amino-acid transfer. The lack of inhibitory properties of the cyanide anion (Table 2) is an important result, as many studies involving amino-acid transport utilize cyanide to inhibit respiration (Eddy & Hogg, 1969). Although non-complexed copper(II) readily disproportionates to copper(0) and copper(I) in the presence of cyanide, when co-ordinated by a multi-dentate ligand, cyanide only binds loosely and will not reduce copper(II) (Curtis & Curtis, 1965). Thus under the circumstances of this experiment, cyanide is unlikely to compete effectively with bidentate amino-acid ligands.

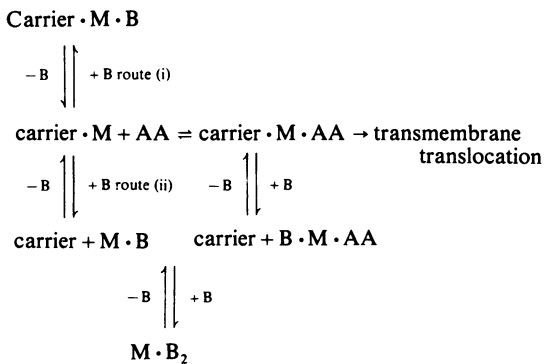
The sequence of bis(salicylamidato)copper(II) chelate activity with respect to leucine translocation is noteworthy in that the most effective complexes (I, II, III and IV) all possess an oxygen atom in the R function (Fig. 1, Table 1). This oxygen atom may be involved in hydrogen-bonding with the protonated salicylato oxygen of the proposed octahedral complex (Scheme 1). The extremely low activity of complex VI is probably related to its tetradentate nature, all the ligand atoms being firmly held in the same plane (Hall *et al.*, 1966).

Facilitated transfer in erythrocytes

Leucine was found to enter erythrocytes mainly via a facilitated transfer system (Figs. 4 and 5) in agreement with the results of Winter & Christensen (1964) and Hoare (1972). This process has been found to be inhibited by bathophenanthroline sulphonate (Fig. 6). Glycine has little or no affinity for the neutral-amino-acid facilitated carrier system



Scheme 1. Possible sequence of molecular events associated with the copper(II)-chelate-facilitated transfer of amino acids across an organic phase



Scheme 2. Proposed mode of bathophenanthroline sulphonate inhibition of the facilitated transfer of amino acids in erythrocytes

Abbreviations used: M, metal ion at binding site of carrier; AA, amino acid; B, bathophenanthroline sulphonate. Inhibition via route (i) would give competitive kinetics, inhibition via route (ii) would give non-competitive kinetics. The presence of bathophenanthroline sulphonate induces a change in both V_{max} and K_m (Fig. 6), indicating that a contribution from both routes (i) and (ii) is involved in the inhibition.

(Winter & Christensen, 1964) and thus, not surprisingly, bathophenanthroline sulphonate has no effect on the uptake of this amino acid. The lack of effect on glycine permeability indicates that non-specific damage to the membrane does not occur in the presence of this phenanthroline derivative. A possible mode of bathophenanthroline sulphonate inhibition is shown in Scheme 2. Preliminary experiments in the present study have demonstrated that there is sufficient membrane-bound copper in the erythrocyte membrane to account for such a system. However, another possibility is that, despite its hydrophilic nature, bathophenanthroline sulphonate could facilitate the oxidation of thiol groups associated with the carrier molecule.

The finding that both erythrocytes and skeletal muscles accumulate L- and D-leucine at approximately the same rate (Fig. 7) adds weight to the concept of a relatively simple binding site. Chelates are able to preferentially select enantiomers, but the differences are usually slight (Basolo & Pearson, 1967). Although Winter & Christensen (1964) demonstrated a distinct preferential affinity of the facilitated amino-acid carrier for L-valine over the corresponding D-amino acid, valine is branched at

the β -carbon atom and is thus likely to experience greater selectivity on chelation to a metal cation. Indeed, (L-valine-*N*-monoacetato)copper(II) has been reported to show a selectivity for L-amino acids over the corresponding D-enantiomers (Leach & Angelici, 1969). In a related transport study, Schultz *et al.* (1972) have reported that, with the exception of valine, there is a relatively poor selectivity of the rabbit ileum towards accumulation of a range of amino-acid enantiomers.

One interpretation of the present data is that a metal cation, possibly copper(II) or zinc(II), is involved at the amino-acid binding site of the L-system (Oxender & Christensen, 1963).

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