Phenceptin: a biomimetic model of the phenytoin receptor

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5,5-Diphenylhydanytoin (phenytoin) is the most widely used anticonvulsant drug, but has many side effects. Although its chemical mode of action is unknown, phenytoin is believed to function primarily by interference with the transport of sodium ions across the neuronal membrane. Structure–activity and lipophilicity–activity studies suggest that the drug interacts with its receptor through hydrogen bonding to the N3—C4 amide bond, and an aromatic–aromatic interaction with the C5 substituent. Since sodium channels are cysteine-rich peptides, whose function depends upon the cysteine⇄cystine redox process, it has been hypothesized that the action of the phenytoin receptor may be mimicked by a properly designed cyclodepsipeptide containing a cystinyl moiety, a cavity lined with five oxygen atoms oriented in the trigonal-bipyramidal manner appropriate for selective transport of sodium ions, and a site for the binding of phenytoin. A computer programme and strategy were developed to permit the three-dimensional structures of potential target molecules to be viewed, prior to synthesis. Use of this programme led to the discovery of Boc-L-cystinyl-glycyl-L-prolyl-glycyl-L-prolyl-L-cystine-$OCHPh_2$. This compound, termed phenceptin, was synthesized from a linear precursor containing *tert*-butoxycarbonyl protection at the N-terminus, benzhydryl ester protection at the C-terminus, and trityl protection at sulfur. Detritylation and cyclization to phenceptin were accomplished with iodine in methanol–pyridine. Using an *n*-octanol membrane to study the kinetics of ion transport, phenceptin was found to transport sodium ions selectively, but only in its oxidized, cyclic form. This transport was inhibited significantly by one mol-equiv. of phenytoin, and not at all by biologically inactive analogs of the drug. The nature of the binding of phenytoin to phenceptin was examined by nuclear magnetic resonance, in *n*-C_8D_{17}-OH solvent, and found to involve hydrogen bonding of the drug to a glycine residue whose oxygen atom is involved in complexation to sodium ions.

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La diphényl-5,5 hydantoïne (phénytoïne) est le médicament le plus utilisé comme anticonvulsivant; toutefois, il provoque de nombreuses réactions secondaires. Même si son mode d'action chimique est inconnu, on croit que la phénytoïne agit principalement par interférence avec le transport des ions sodium à travers les membranes neuronales. Des études de structure/activité et caractère liphophilique/activité suggèrent que la drogue interagit avec son récepteur par le biais de liaisons hydrogènes avec la liaison amide N3—C4 et par une interaction aromatique/aromatique avec le substituant en C5. Puisque les canaux du sodium sont des peptides riches en cystéine dont la fonction dépend du processus redox cystéine/cystine, il a été suggéré que l'on pourrait répliquer l'action du récepteur de la phénytoïne à l'aide d'un cyclodepsipeptide approprié contenant une portion cystinyle, une cavité contenant cinq atomes d'oxygène orientés d'une façon trigonale/bipyramidale appropriée au transport sélectif d'ions sodium et d'un site pour la liaison de la phénytoïne. On a mis au point un programme d'ordinateur et une stratégie qui permettent de voir les structures tri-dimensionnelles des molécules cibles potentielles avant d'en réaliser la synthèse. L'utilisation de ce programme a conduit a la découverte du Boc-L-cystinyl-glycyl-L-prolyl-L-cystine-$OCHP_2$. On a synthétisé ce composé, appelé phenceptine, à partir d'un précurseur linéaire contenant le groupement protecteur *tert*-butoxycaronyle sur l'acide portant le N-terminal, un groupement protecteur ester benzhydryle sur l'acide du C-terminal et une protection trityle sur le soufre. On a réalise la détritylation et la cyclisation de la phenceptine à l'aide d'iode dans un mélange de méthanol et de pyridine. Utilisant un membrane de *n*-octanol pour étudier la cinétique du transport ionique, on a trouvé que la phenceptine transporte sélectivement les ions sodium uniquement dans sa forme oxydée, cyclique. Ce transport subit une inhibition importante par un équivalent molaire de phénytoïne; les analogues biologiquement inactifs de la drogue ne causent toutefois pas d'inhibition. Opérant dans le *n*-C_8H_{17}-OH comme solvant et faisant appel à la résonance magnétique nucléaire, on a examiné la nature de la liaison de la phénytoïne à la phenceptine; on a trouvé que celle-ci implique une liaison hydrogène de la drogue avec un résidu glycine dont l'atome d'oxygène est impliqué dans la complexation avec les ions sodium.

[Traduit par la revue]

Introduction

The epilepsies are a group of diverse disorders with a common central nervous system manifestation, the occurrence of seizures (convulsions). An accurate epidemiology of the illness is difficult, but various studies suggest an overall incidence of 1% in the general population (1). Ten times this number of persons require medical attention at some time during their lives as the result of a seizure.

A seizure is primarily an electrical event: a high voltage discharge of an assemblage of brain cells (neurons), and aberrant neuronal excitability is associated mainly with the ion transport behaviour of sodium channels (2). As these open and close sequentially throughout the cerebral cortex, an electrical discharge spreads from an initial focus, and the symptoms of the epileptic state become manifest.

Treatment of epilepsy is imperative, because brain damage and (or) death are not infrequent sequelae of untreated abnormal electrical activity. Any successful drug therapy for the control of seizures must function by interference with the development and propagation of this activity.

The most important drug for the control of seizures is 5,5-diphenylhydantoin (**1**), known in medicine as Dilantin or phenytoin (3). However, phenytoin and all other anticonvulsant drugs have deficiencies associated with their use: only 60% of epileptic patients can be controlled adequately (5) and each of the drugs exhibits many side effects.

Consequently, there is a need to develop new drugs for the

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treatment of epilepsy, a point emphasized repeatedly in the medical literature (4, 5). The present work is concerned with the development and experimental evaluation of a biomimetic model of the activity of phenytoin. This is the first stage of a programme that, it is hoped, will eventually allow the design of novel anticonvulsants, whose activity is based on the mechanism of action of phenytoin.

Structure–activity and lipophilicity–activity relationships of phenytoin analogs

A search of the literature yielded data for over 800 analogs of phenytoin, about one quarter of which had been evaluated comparably using maximal electroshock techniques. Structure–activity studies have led to the view (6) that the phenytoin receptor interacts with the drug primarily through the formation of intermolecular hydrogen bonds, but also possesses a hydrophobic pocket that interacts with the C5 substituent. In addition, within the hydantoin ring, an unsubstituted N3—C4 amide bond is the most important, one phenyl group or a bulky aliphatic group is necessary at C5, and the introduction of one or more benzyl substituents at C5 causes loss of activity.

Conformational studies on 5-phenyl and 5-benzyl substituted hydantoins provide insight into the origin of the benzyl effect. In phenytoin, the phenyl groups are approximately perpendicular to the hydantoin ring (7); in 5-benzylhydantoin the phenyl group is approximately parallel to the hydantoin ring (8), in harmony with an early suggestion (9) concerning the existence of aromatic–amide interactions in phenylalanyl-containing peptides. To probe this point further, 3-ethyl-5-phenyl- (**2**) and 3-ethyl-5-benzyl-hydantoin (**3**) were synthesized (see Experimental), and their ^{1}Hmr spectra examined. In the 5-benzyl compound, both the methylene and the methyl hydrogens of the 3-ethyl group are shifted upfield by 0.3 ppm, as expected for a structure in which the aromatic ring lies over the ethyl group.

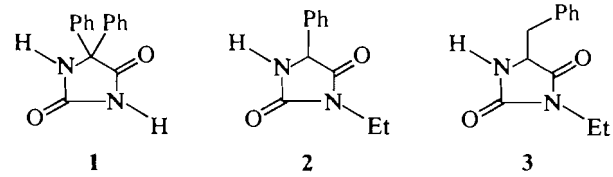
The existence of an aromatic–N3C4 amide interaction in a 5-benzylhydantoin would have two consequences: access by a receptor to the N3—C4 amide bond would be impaired; and the aromatic ring would no longer possess the proper orientation for interaction with the hydrophobic pocket of the receptor (cf. Fig. 12 of ref. 7).

It is also necessary that the drug reach this receptor, and this capability will depend upon the lipophilicity of the hydantoin (10, 11). Our examination of the literature (12) suggests that, although the contribution of lipophilicity cannot be ignored (13), the anticonvulsant activity of a hydantoin depends primarily upon its ability to bind to the phenytoin receptor. Any attempt to model this process requires information concerning the nature of this receptor and its chemical constitution.

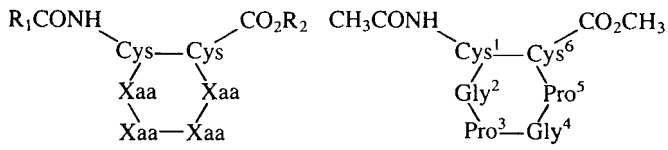
Phenytoin and the sodium channel

The anticonvulsant activity of phenytoin can be ascribed to a single demonstrable primary event: the specific inactivation of sodium channels (14, 15).

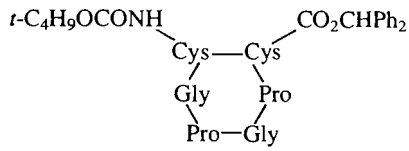
Recent studies (see ref. 16 for a review) have demonstrated that sodium channels are glycoproteins, containing up to 36% by weight of carbohydrate, principally sialic acid (7). Studies of the function of these channels suggest that the carbohydrate portion is directed extracellularly, and the peptide portion is directed intracellularly; the movement of ions occurs through a fixed pore or channel, rather than as part of a larger membrane macromolecule, and is selective for sodium. A model of the ion-selectivity filter, i.e., the narrowest region of the ion-conducting pore of the sodium channel, has been proposed (18).



1 **2** **3**



4 **5**



6

This consists of a 3 × 5 Å orifice *lined by oxygen atoms* (italics added).

All sodium channels contain a ca. 260 kD unit, and the primary structure of the peptide portion of this unit (M_r 208,321) in the electric eel, *Electrophorus electricus*, has been determined by Noda *et al.* (19). The peptide contains 36 cysteine residues, and shows sequence homology to the corresponding peptide of rat brain (20).

Hypothesis

Noda *et al.* (19) and Kosower (21) have developed models for the sodium channel of *Electrophorus electricus*, based on slightly different analyses of the structure of the 180-residue protein. In both models, the sodium binding site consists of a series of negatively charged (−) residues (Asp^-, Glu^-), and is surrounded by a series of positively charged (+) residues (Arg^+, Lys^+). Opening (activation) and closing (inactivation) of the channel is determined by conformational motion of side chains, e.g., (−, −, −, −) (open) ⇄ (−, −, −, +) (closed). In Kosower's model, possible (−) residues are Asp-908, Asp-910, Glu-943, and Glu-944; possible (+) residues are Arg-210, Arg-657, and Arg-1417.

If we accept such models as plausible representations of the chemical events associated with normal electrical behaviour, is it possible that aberrant behaviour is the result of the creation of additional sodium binding sites?

A number of reagents have been found to slow or prevent sodium channel inactivation, including *N*-bromoacetamide, *N*-chlorosuccinimide, *N*-bromosuccinimide, and *N*-chloro-*p*-toluenesulfonamide (chloramine-T) (22). All of these are mild oxidants; in a molecule containing an abundance of cysteine residues, the primary effect of such reagents must be to maintain the cysteine–cystine couple in its oxidized form. Significantly, sulfhydryl-specific reagents, such as *N*-ethylmaleimide and 5,5′-dithiobis-2-nitrobenzoic acid, neither slow nor prevent inactivation of sodium channels (22).

The simplest reagent for the oxidation of a mercaptan to a disulfide is O_2 (23), and it is known that hyperventilation (24) or hyperbaric oxygen (25) precipitate seizures, which can be suppressed with phenytoin.

We are, therefore, led to the hypothesis that the opening of

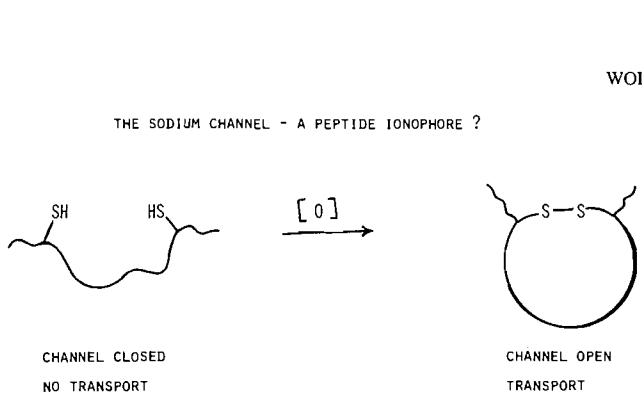


FIG. 1. Hypothesis of the present work: the opening and closing of sodium channels associated with abnormal electrical behaviour is a redox process.

sodium channels that initiates a seizure corresponds chemically to the creation of a sodium-selective *cyclodepsipeptide ionophore* (26), by an oxidative process, as depicted schematically in Fig. 1.[3]

The concept of "redox-switched" ionophores is not new. Shinkai and co-workers (28) and Raban *et al.* (29) have reported the syntheses of α,ω-dimercapto polyoxy compounds, which behave as pro-ionophores. In their reduced forms these compounds are poor ionophores; oxidation generates effective crown ether ionophores, whose cation selectivity depends on ring size. The oxidation reaction exhibits a marked template effect (28), and it has been argued (29) that "chemically switchable pro-ionophore–ionophore couples can serve as models for biological "ion gates", structures in which ion transport is initiated by an external stimulus."[3]

Phenceptin: design[4]

To convert the concept of Fig. 1 into a biomimetic model of the phenytoin receptor required a peptide containing two terminal cysteines. The number, nature, and sequence of the internal amino acids of this peptide had to be selected so that, in its reduced form, the compound would not behave as an ionophore. In its oxidized, cyclic form it was necessary that (*i*) the substance be sodium-selective; (*ii*) the transport of sodium ions be blocked by phenytoin; (*iii*) biologically inactive phenytoin analogs, e.g., 5,5-dibenzylhydantoin, have no effect on sodium transport.

Many cyclopeptides behave as ionophores (30), but none exhibits selectivity for sodium ions. The structures of cyclodepsipeptide ionophores and their complexes are also well established (26, 31), but none of these ionophores possesses the desired redox-switching feature, or selectivity for sodium. Although both natural (32) and synthetic (33) peptides containing the cysteine–cystine couple are known, their transport properties have not been studied.

Nevertheless, the dimensions of the cavity (3 × 5 Å) of the cyclic target were clear from the properties, already discussed, of the ion-selectivity filter of the sodium channel (18). These correspond closely to the hole size observed in the crystal structures of sodium-selective *crown ether* ionophores (26, 34), whose cavities have a ca. 4.6 Å diameter. The complexation of sodium ions is accomplished, in such cases, by a trigonal-bipyramidal arrangement of five oxygens.

[3]For an alternative model of a receptor channel, see ref. 27.

[4]The authors thank Professor Morton Raban and his co-workers for helpful discussions during the formative stages of this design project, including prepublication information concerning ref. 29, and its potential implications for our own work.

TABLE 1. Dihedral angles of **5**

Residue	Angle (deg)			
	ϕ^a	ψ^b	ω^c	$\chi_1{}^d$
Cys^1	−83	−12	173	−128
Gly^2	−61	−66	173	
Pro^3	−75	−180	178	
Gly^4	79	−166	−180	
Pro^5	−68	−30	174	
Cys^6	−67	143	178	−174

[a]$N-C_\alpha$ dihedral angle.
[b]$C_\alpha-C'$ dihedral angle.
[c]$N-C'$ dihedral angle.
[d]$C_\alpha-C_\beta$ dihedral angle.

Examination of molecular models suggested that a cyclohexadepsipeptide would be the simplest system potentially containing these features (cf. ref. 35). This redefined the design problem to a structure of type **4**.

For the case of valinomycin, the best-known cyclodepsipeptide ion carrier (26, 31, 36), selection and syntheses of analogs (31, 37) have been conducted mainly by trial and error. This labour-intensive process was not feasible for us, and it was necessary to devise a theoretical strategy to screen candidate structures *prior* to synthesis. This strategy is based on molecular mechanics (38), and it has been documented fully (39).

Attention was focused, especially, on the use of proline and glycine residues. Proline residues were expected to reside on the exterior of the molecule, and enhance the lipophilicity required to maintain the ionophore in a membrane environment. In addition, the presence of this amino acid should inhibit transannular hydrogen bonding and excessive conformational lability (40). Glycine was expected to fit into a tightly turned cyclic peptide structure, promote cyclization (41), and reduce the possibility of steric interactions between amino acid side chains and a putative phenytoin binding site.

Figure 2 is the lowest energy structure calculated for the cyclohexadepsipeptide **5**, which contains a Gly-Pro-Gly-Pro sequence (42). The strategy consisted of a preliminary ECEPP search (39*b*, 43) of 200,000 structures, followed by final refinement of the 50 low energy structures identified in this search, using the peptide parameters (39*a*) of Allinger's MMP2(85) programme (44). Table 1 summarizes the dihedral angles of **5**.[5]

Inspection of Fig. 2 reveals that six carbonyl oxygens are directed towards the interior of the molecule, and that a trigonal-bipyramidal arrangement can be achieved from apical carbonyl oxygens of Cys^1 and Gly^4, whose separation is 5.56 Å, and equatorial carbonyl oxygens from three of CH_3CO, Gly^2, Pro^3, Cys^6. Three of the latter are amide carbonyls, and the fourth (Cys^6) is an ester carbonyl. The carbonyl oxygens of Gly^2, Pro^3, and Cys^6 form an equilateral triangle, and the diameter of the circumscribed circle is 3.63 Å.

In the potassium complex of valinomycin, the cation is bound

[5]Other peptides examined analogously led to low energy structures whose cavities did not contain the number and orientation of oxygen atoms considered necessary for complexation of sodium ions. For example, with Gly-Gly-Gly-Gly, Ala-Pro-Ala-Pro, and Pro-Pro-Pro-Pro as the four internal amino acids, substantial conformational changes appeared to be necessary to achieve a backbone structure analogous to **5**.

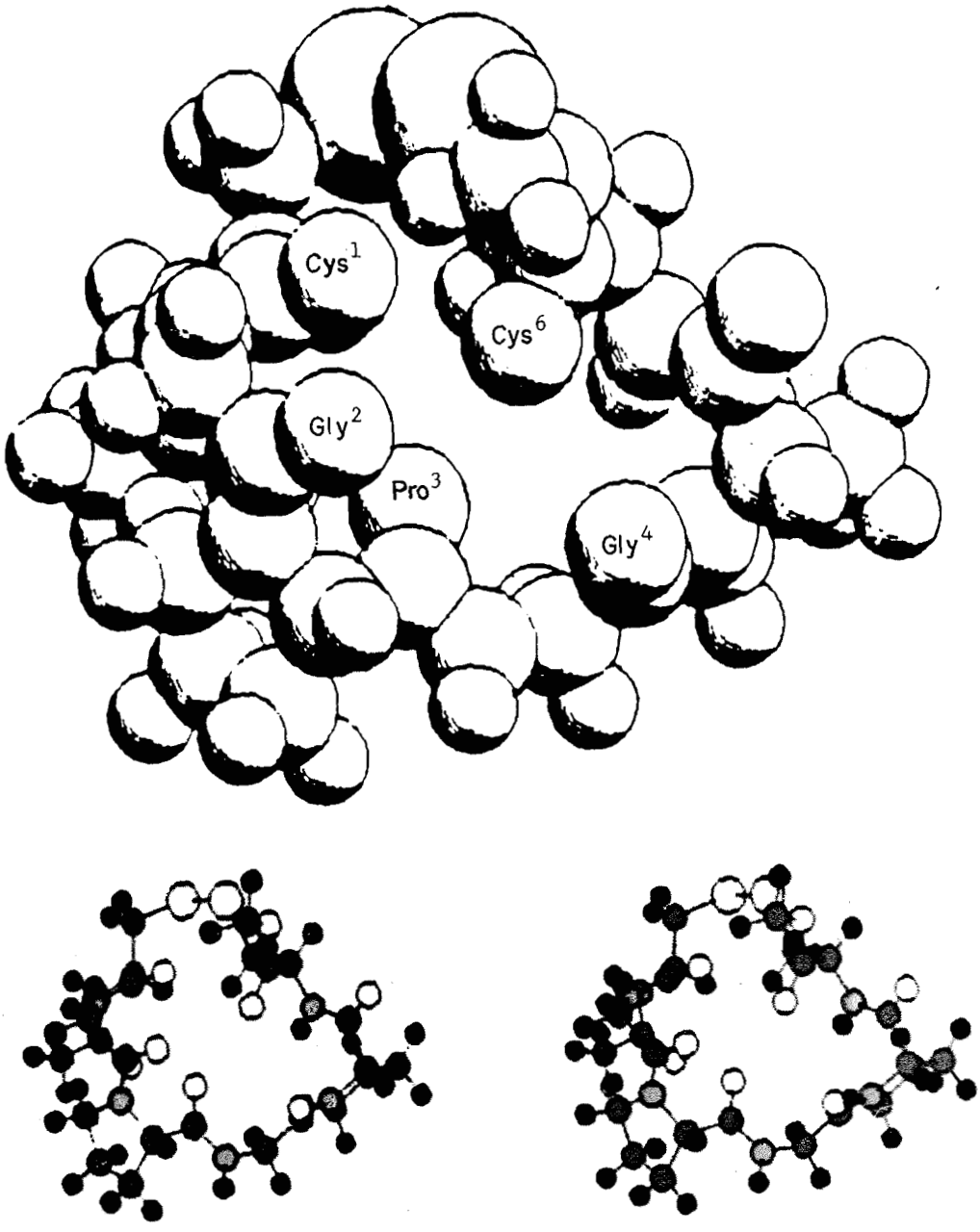


FIG. 2. The lowest energy structure calculated for the cyclohexadepsipeptide **5**. The atoms labelled Cys^1, Gly^2, ... are the carbonyl oxygens of these residues. The lower view of **5** requires a diastereoscopic viewer.

octahedrally to six ester carbonyl oxygens (26). In the potassium complex of enniatin B, three amide carbonyl and three ester carbonyl oxygens bind to the cation (26). Thus, binding to both ester and amide carbonyl oxygens is known.

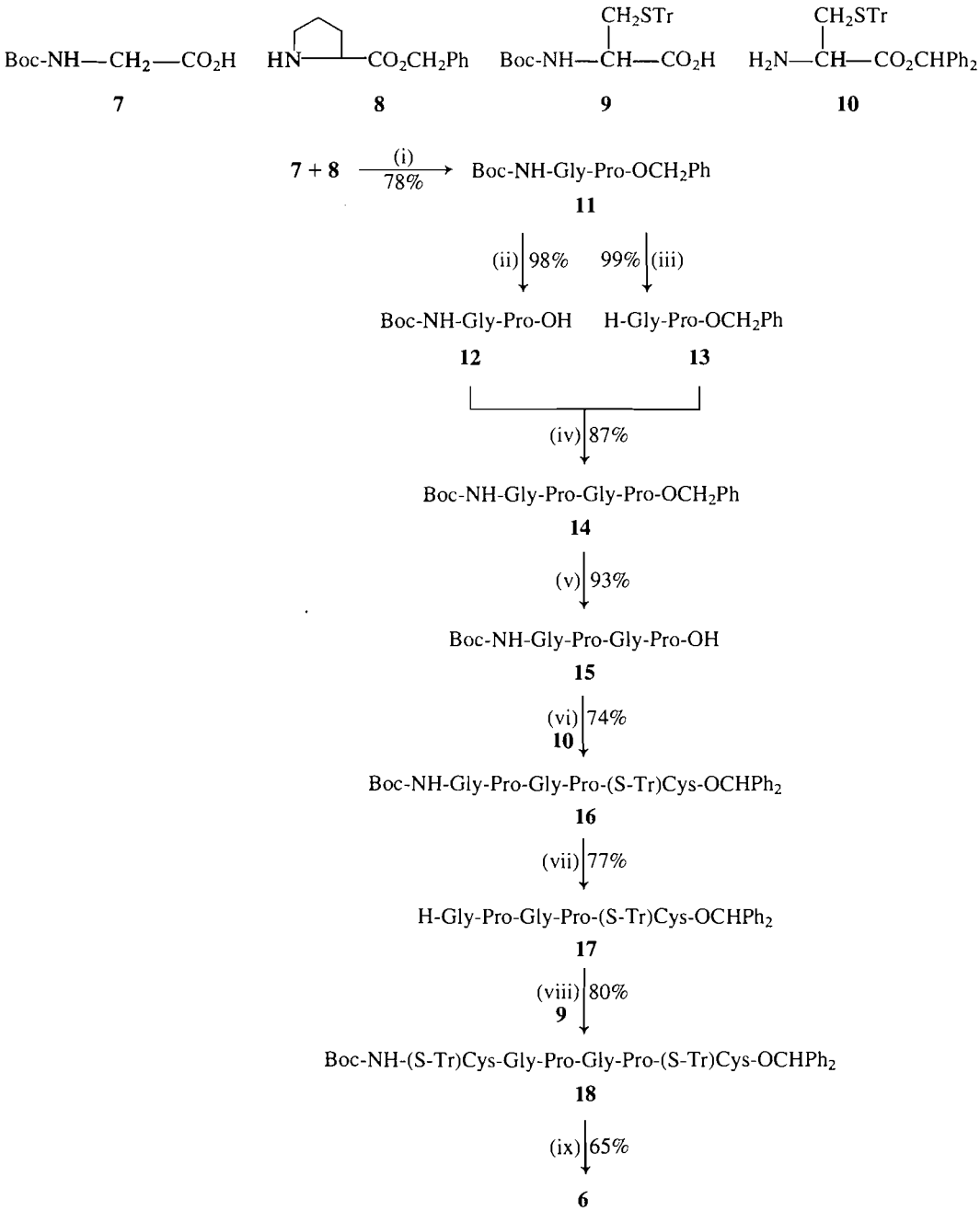
Compound **6** was selected for synthesis and experimental evaluation as a biomimetic model of the phenytoin receptor. In **6**, the replacement of CH_3CO by *t*-BuOCO was expected to reduce the basicity of this carbonyl oxygen, and promote the formation of a trigonal-bipyramidal cation complex. According to Fig. 2, the C-terminal ester methyl group lies over the carbonyl oxygens of Gly^2 and Gly^4; if one of these oxygens were to serve as a phenytoin binding site, aromatic–aromatic interactions would be promoted by the attachment of one or more phenyl substituents to the methyl carbon. The choice of benzyhydryl at this position meets this requirement, and simplifies the synthesis.

The cyclohexadepsipeptide **6** has been named "phenceptin" (*phen*ytoin re*cept*or).

Phenceptin: synthesis

The synthesis of phenceptin was carried out from Boc-glycine (**7**), the benzyl ester of L-proline (**8**), Boc-*S*-trityl-L-cysteine (**9**), and the benzhydryl ester of *S*-trityl-L-cysteine (**10**).

As summarized in Scheme 1, coupling of **7** with **8** afforded **11**, fully protected Gly-Pro. This was O-deprotected by hydro-



Reagents: (i) EEDQ; (ii) 10% Pd/C; (iii) HCl; (iv) EEDQ; (v) 10% Pd/C; (vi) EEDQ; (vii) HCO_2H; (viii) EEDQ; (ix) I_2-MeOH–pyridine

SCHEME 1

genolysis to **12**, and N-deprotected with HCl to **13**. Coupling of **12** with **13** gave **14**, fully protected Gly-Pro-Gly-Pro. Hydrogenolysis of **14** exposed the terminal carboxyl group (**15**), and coupling with **10** yielded the fully protected pentapeptide **16**, Gly-Pro-Gly-Pro-Cys. This was deprotected selectively at the N-terminus, using anhydrous formic acid (45), and the product (**17**) was coupled with **9** to form the acyclic, fully protected hexapeptide Cys-Gly-Pro-Gly-Pro-Cys (**18**). Removal of the *S*-trityl protecting groups, and cyclization to phenceptin were accomplished by treatment of **18** with iodine, in a mixture of methanol and pyridine (46).

Phenceptin was obtained in analytically pure form as an amorphous solid, which has not yet been crystallized. Although the compound is homogeneous on thin-layer chromatography in several systems, its 400 MHz ^{1}Hmr spectrum (in $CDCl_3$) is temperature dependent and suggests a 4:1 mixture of two conformations.[6] Comparison of the spectrum of phenceptin with those of the intermediates of the synthesis allowed assignment of proton resonances associated with the different residues (see Experimental).

[6]S. Wolfe and R. J. Bowers. To be published.

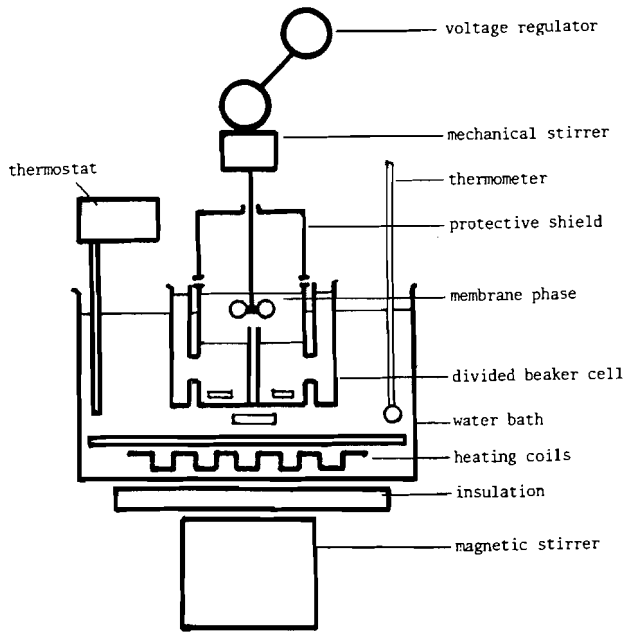


FIG. 3. Divided beaker cell for kinetic studies of cation transport through a 1-octanol membrane.

Cation transport through an *n*-octanol membrane

Most workers employ a U-tube to model the process of ion transport across a liquid membrane. In such an apparatus, water occupies each of the arms, and an organic solvent denser than water, e.g., chloroform, is placed in the base. If a salt is added to one of the arms and the organic solvent is stirred, no transport to the second arm is observed until a water-insoluble ionophore is added to the system. When an ionophore is present, the movement of salt from one arm to the other represents a biomimetic model of transport from an extracellular phase, through a membrane, to an intracellular phase.

This system did not seem biologically appropriate for an examination of the properties of phenceptin, for several reasons: (*i*) chloroform is not a proper model of a cell membrane; (*ii*) the membrane employed in a typical U-cell is too thick, averaging 8–10 cm between the two aqueous phases; (*iii*) the area of surface contact between each aqueous phase and the membrane is typically of the order of 1.15 cm^2.

The selection of 1-octanol as the membrane phase seemed more meaningful. It has been known for many years (48) that pharmacological activity can be related to octanol–water partition coefficients.

This choice necessitated a modification of the usual apparatus, because 1-octanol is less dense than water. Figure 3 shows the "divided beaker" transport cell devised for this purpose. This apparatus minimizes the separation of the intracellular and extracellular aqueous phases, and increases the area of surface contact between the membrane phase and each aqueous phase to 39.3 cm^2. All phases are stirred continuously to minimize effects of concentration gradients upon solute distribution; a series of voltage regulators control the stirring devices, and promote experimental reproducibility. All kinetic measurements were performed at 37.0 ± 0.1°C.

To evaluate the capabilities of the apparatus, an initial set of experiments was performed, using as ionophores the crown ethers 12-crown-4, 15-crown-5, and 18-crown-6. In a typical run the ionophore was dissolved in the octanol layer at a concentration of 150 μM. The extracellular aqueous phase contained an inorganic salt at a concentration of 20 mM, and the intracellular aqueous phase consisted of redistilled water.

The transport rate was determined through periodic sampling of the intracellular phase, and measurement of the concentration of the anion that migrates concomitantly with the cation to preserve electrical neutrality. With thiocyanate as the counter-anion, 12-crown-4 prefers Li^+ over Na^+, 18-crown-6 prefers K^+ over Na^+, and 15-crown-5 is selective for Na^+ (cf. refs. 26 and 31).

The behaviour of 15-crown-5 was studied more fully. The role of the counter-anion was examined using sodium chloride, sodium thiocyanate, and sodium picrate. This was found to be picrate ≈ thiocyanate ≫ chloride. Evidently the weakly hydrated picrate and thiocyanate anions are transported more rapidly than the small, spherical, strongly hydrated chloride anion. This trend is well established for chloroform membranes (49).

Chloride is obviously more appropriate than picrate for use in a biomimetic process, but this choice would have led to inconveniently slow transport rates. The problem might, in principle, be overcome by measurement of junction potentials (50) in place of transport rates, but it was considered that this was not essential at this stage. Thiocyanate was selected as a compromise, and all subsequent work was performed with this anion.

Further studies demonstrated that the transport of sodium thiocyanate is first order in the concentration of ionophore, as is observed in a U-cell with a chloroform membrane (51), and second order in the concentration of sodium thiocyanate (data not shown); this has also been observed with a chloroform membrane (25). Thus, replacement of the standard chloroform membrane with a biologically more appropriate 1-octanol membrane does not appear to alter the mechanism of the transport process when 15-crown-5 is employed as the membrane carrier. It can be concluded that the divided beaker cell is suitable for experimental studies with phenceptin.

Phenceptin is a redox-switched sodium selective ionophore with specific phenytoin binding capability

Figure 4 compares the transport behaviour of phenceptin and 15-crown-5. Under the conditions [NaSCN] = 20 mM, [Ionophore] = 150 μM, the transport rates are 0.347 μM/h with phenceptin, and 0.435 μM/h with 15-crown-5.

Reduction of phenceptin with dithiothreitol gave the acyclic compound **19**. Neither this peptide nor the phenceptin precursor **18** transported sodium ions at a measurable rate.

Replacement of NaSCN by KSCN led to the transport of potassium ions at a rate of 0.204 μM/h (Fig. 4). The selectivity for sodium ions is, therefore, 1.7. This compares to the Na^+/K^+ selectivity of 5.9 found with 15-crown-5, and the Na^+/K^+ selectivity of 12.5 observed in a functioning sodium channel (16).

These experiments demonstrate phenceptin to be a redox-switched sodium selective ionophore, as required for our biomimetic model of the sodium channel.

The effect of phenytoin on the behaviour of various membrane carriers could now be examined. Preliminary experiments were performed with the 15-crown-5/NaSCN and 18-crown-6/NaSCN systems. For these studies, the cell was operated initially for 4 h, to ensure that sodium transport was occurring. Then 1.0 mol-equiv. of phenytoin was injected into the extracellular phase. There was no change in the transport rate. The same result was obtained in subsequent experiments in which 0.5,

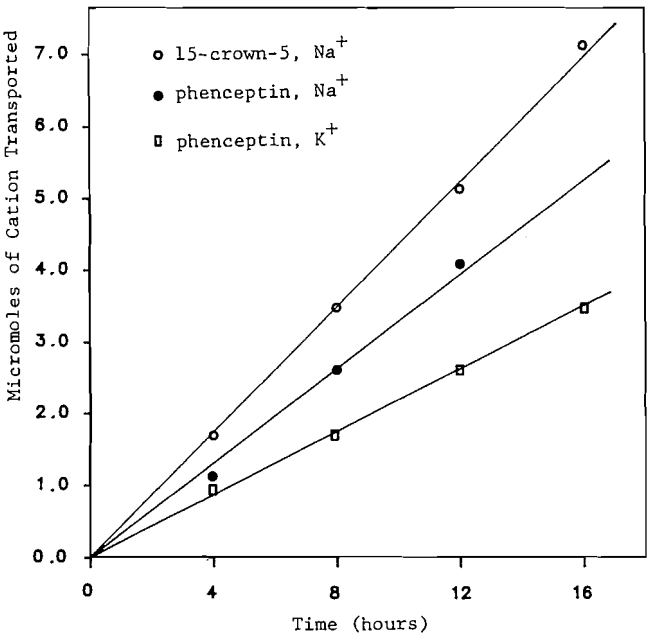


FIG. 4. Transport of sodium thiocyanate by 15-crown-5 (open circles) and by phenceptin (closed circles), and transport of potassium thiocyanate by phenceptin (squares). All data refer to experiments at 37.0°C, with cation and ionophore concentrations of 20 mM and 150 μM, respectively.

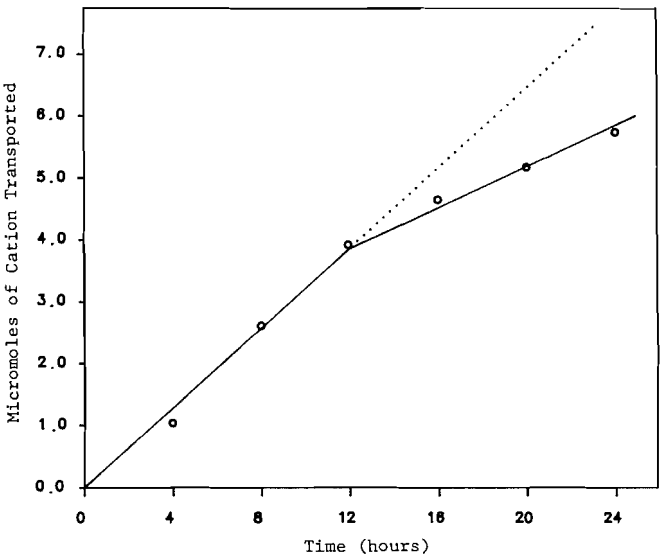


FIG. 5. Effect of addition of 5,5-diphenylhydantoin (one molar-equivalent) upon sodium ion transport by phenceptin. The hydantoin was added after 12 h, using the conditions of Fig. 4.

2.0, and 1000 mol-equiv. of phenytoin were injected after 4 h. It is clear that phenytoin has no effect on sodium ion transport by crown ether ionophores.

An entirely different result is obtained when these experiments are repeated with the phenceptin/NaSCN system. Figure 5 shows the effect of an injection of *1 mol-equiv.* of phenytoin after 12 h of sodium transport under the usual conditions. There is an immediate reduction in the rate of transport, which then continues at 40% of the original rate (60% inhibition).

However, when the *biologically inactive* 5,5-dibenzylhydantoin is substituted for phenytoin in such an experiment (Fig. 6), there is a very slight *increase* in the transport rate.

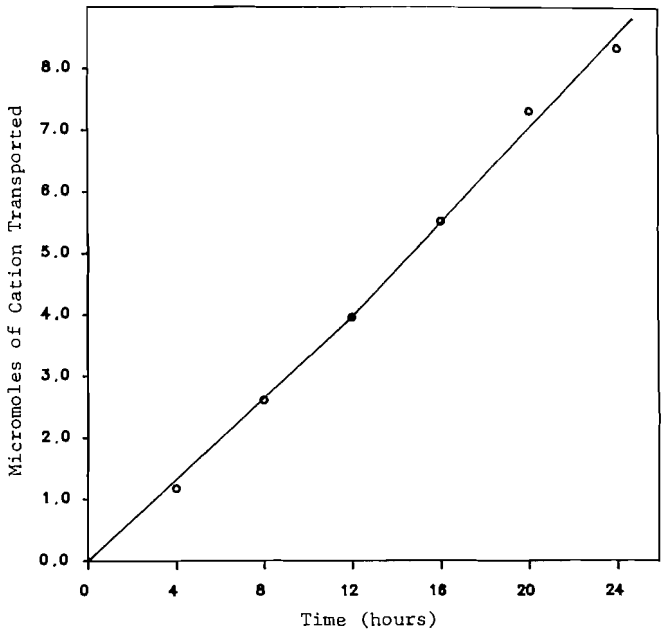


FIG. 6. Effect of addition of 5,5-dibenzylhydantoin (one molar-equivalent) upon sodium ion transport by phenceptin. The hydantoin was added after 12 h, using the conditions of Fig. 4.

Phenceptin is, therefore, a valid biomimetic model of a sodium channel phenytoin receptor.

Nature of the interaction between phenytoin and phenceptin

The inhibition of sodium ion transport by phenceptin was found to be linear in the concentration of added phenytoin, up to a 1:1 [phenytoin]/[phenceptin] ratio. Further increases in the phenytoin concentration did not increase the inhibition beyond the 60% observed with the 1:1 mixture. These observations suggested that the inhibition of sodium ion transport is achieved through the formation of a 1:1 complex.

The structure of this complex was studied by 400 MHz ^{1}Hmr at 37°C, using *n*-C_8D_{17}-OH as the solvent. Figure 7A shows the aromatic region of phenceptin in this solvent; addition of one mol-equiv. of phenytoin (Fig. 7B) leads to a significant change in the chemical shift differences of the aromatic protons of phenceptin. An aromatic–aromatic interaction (53) is suggested by these changes, but the data do not allow the nature of this interaction to be specified with certainty.

The effect of phenytoin upon the methylene protons of Gly^4 of phenceptin is more interesting, and can be interpreted. Figure 8A shows this region of the spectrum for a solution of phenceptin in *n*-C_8D_{17}-OH; Fig. 8B is the spectrum obtained immediately after addition of 1 mol-equiv. of phenytoin; Fig. 8C is the spectrum that results when the solution of 8B is shaken overnight with D_2O.

The glycine methylene protons are anisochronous because of the asymmetric environment, but coupling to the adjacent N—H is not observed in the hydroxylic solvent as a result of rapid exchange with the O—H protons. Addition of phenytoin must lead to the formation of a fairly strong complex with the glycyl amide hydrogen, because coupling of N—H to adjacent methylene protons is now observed. That this is the correct explanation of Fig. 8B is seen by the restoration of the original spectrum (Fig. 8C) following exchange of N—H with D_2O.

These observations can be understood by reference to the calculated phenceptin structure of Fig. 2. Only the N—H and

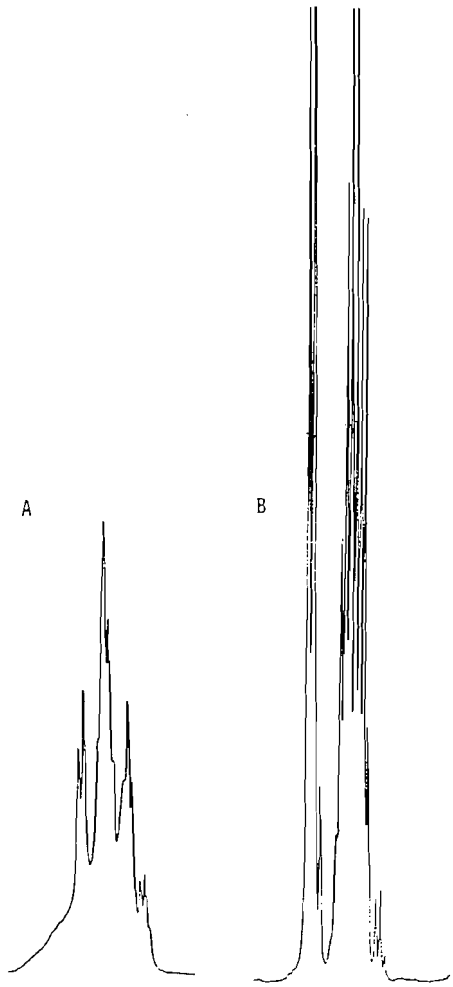


FIG. 7. (A) Aromatic region of the ^{1}Hmr spectrum of phenceptin in n-C_8D_{17}-OH solvent; (B) after addition of phenytoin (1 mol-equiv.).

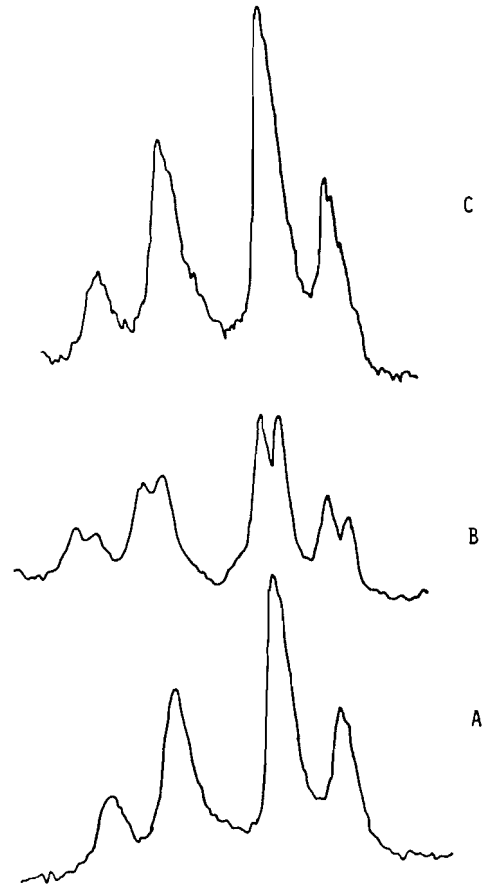


FIG. 8. (A) Glycine region of the ^{1}Hmr spectrum of phenceptin in n-C_8D_{17}-OH solvent; (B) after addition of phenytoin (1 mol-equiv.); (C) after shaking the solution of B overnight with D_2O.

C=O of Gly^4 possess orientations capable of complexation to an amide bond of phenytoin. This is shown schematically in Fig. 9, which provides an explanation for the inhibition of sodium ion transport by phenytoin, if the carbonyl oxygen of Gly^4 is also a cation binding site. The experimental and theoretical studies suggested by Fig. 9 have been completed, and will be published separately (47).

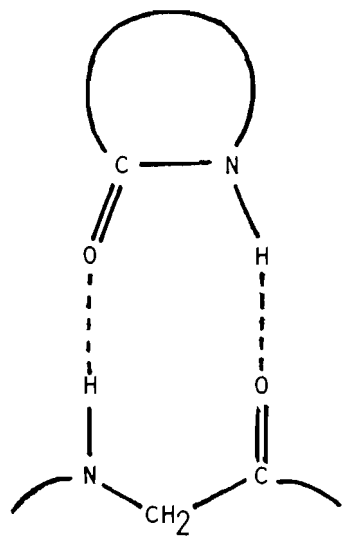


FIG. 9. Proposed interaction between Gly^4 of phenceptin and phenytoin.

Experimental

A. Syntheses

General

All solvents (Fisher or Anachemia, reagent grade) were purified before use, by standard methods (55–57). Several reagents were also purified before use. 2-*tert*-Butoxycarbonyloxyimino-2-phenylacetonitrile (BOC-ON) (Aldrich) was recrystallized from warm methanol. 1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (Aldrich, 97% pure) was recrystallized from methylene chloride – ethyl ether. Diphenylhydantoin (Aldrich, Gold Label) was recrystallized twice from ethanol. Boc-glycine (Sigma) and L-proline benzyl ester hydrochloride (Sigma) were used as received.

Proton nmr spectra of hydantoins were obtained at 60 MHz on Varian T60 or EM360 spectrometers. Proton nmr spectra of peptides were recorded at 400 MHz on a Bruker AM400 spectrometer. Chemical shifts are reported in δ values relative to tetramethylsilane or 2,2-dimethyl-2-sila-pentane-5-sulfonate as internal standards. Infrared (ir) spectra were taken on a Perkin–Elmer 180 spectrometer. Absorption spectroscopy readings were made on a Bausch and Lomb Spectronic 20 spectrophotometer. Melting points were determined on a Meltemp apparatus and are uncorrected. Thin-layer chromatography (tlc) was performed on precoated Brinkmann silica gel 60F-254 plates with aluminium backing; spots were visualized with ninhydrin, palladium chloride, iodine vapour, or ultraviolet light.

Hydantoins

*3-Ethyl-5-phenylhydantoin (**2**)*: L-Phenylglycine (Aldrich, 1.97 g, 0.013 mol) was dissolved in water (6.0 mL) containing potassium hydroxide (Fisher, 0.91 g, 0.016 mol), and ethyl isocyanate (Aldrich, 1.07 g, 0.015 mol) was added dropwise with stirring, during 3 h. The mixture was stirred overnight, filtered, and the filtrate acidified to pH 1.0. The *N*-ethylhydantoic acid was collected, dried, and cyclized by refluxing for 3 h with 6 N HCl (10.0 mL). The product crystallized upon cooling and was recrystallized from ethanol, 1.98 g (74%); mp 138°C; ir (Nujol): 1710, 1760 cm^{-1}; ^{1}Hmr ($CDCl_3$): 1.22 (3H, t), 3.60 (2H, q), 5.03 (1H, s), 6.80 (1H, s), 7.37 (5H, m); tlc: R_f 0.56 (methylene chloride: acetone: acetic acid 100.0: 100.0: 0.5), R_f 0.61 (ethanol: acetic acid 20:1), R_f 0.84 (methylene chloride: methanol 5:2).

*3-Ethyl-5-benzylhydantoin (**3**)*: The synthesis was carried out as for **2**, except that L-phenylalanine (Sigma, 2.15 g, 0.013 mol) was used in place of phenylglycine. The product was recrystallized twice from ethanol to give 2.13 g (75%), mp 145°C; ir (Nujol): 1708, 1758 cm^{-1}; ^{1}Hmr ($CDCl_3$): 0.90 (3H, t), 3.02 (2H, q), 3.30 (2H, q), 4.19 (1H, t), 5.90 (1H, s), 7.37 (5H, m); tlc: R_f 0.57 (methylene chloride: acetone: acetic acid 100.0: 100.0: 0.5), R_f 0.63 (ethanol: acetic acid 20:1), R_f 0.85 (methylene chloride: methanol 5:2).

5,5-Dibenzylhydantoin: 1,3-Diphenyl-2-propanone (Aldrich, 4.10 g, 0.02 mol), potassium cyanide (Fisher, 2.5 g, 0.039 mol), and ammonium carbonate (Fisher, 7.4 g, 0.077 mol) were mixed with 50% aqueous ethanol (80.0 mL) and heated at 60°C for 15 h, to give a clear yellow solution. A slush of crystals formed rapidly when this solution was cooled. This crude product was dissolved in 5% sodium hydroxide (50.0 mL), filtered, treated with decolourizing carbon, and reprecipitated by the addition of an excess of concentrated hydrochloric acid. The product was recrystallized twice from 60% aqueous ethanol to give 3.92 g (70%) of material, mp 304–306°C, (lit. (58) mp 305°C); ir (Nujol): 1705, 1720, 1754 cm^{-1}; ^{1}Hmr (CD_3OD): 2.94 (2H, d, 15 Hz), 3.23 (2H, d, 15 Hz), 7.24 (10H, m); tlc: R_f 0.66 (methylene chloride: acetone: acetic acid 100.0: 100.0: 0.5), R_f 0.69 (ethanol: acetic acid 20:1), R_f 0.83 (methylene chloride: methanol 5:2).

Synthesis of phenceptin

*N-Boc-S-trityl-L-cysteine dicyclohexylamine salt (**9**)*: *S*-Trityl-L-cysteine (8.74 g, 24.1 mmol) was suspended in dichloromethane (50.0 mL) and dicyclohexylamine (Aldrich, 5.3 mL, 26.5 mmol) was added, followed by BOC-ON (6.48 g, 26.4 mmol). The solution was stirred at room temperature for 24 h. Removal of the solvent under reduced pressure yielded a yellow foam, to which was added anhydrous ether (200 mL). The product crystallized after storage at −20°C for 15 h, and it was collected, dried, and recrystallized from absolute methanol. 13.0 g, mp 215–216°C, (lit. (46) mp 211–212°C).

*S-Trityl-L-cysteine benzhydryl ester (**10**)*: A mixture of benzophenone (Aldrich, 25.2 g, 0.138 mol), hydrazine hydrate (BDH, 10.0 g, 0.2 mol), and absolute ethanol (15 mL) was refluxed for 10 h. Benzophenone hydrazone was recrystallized from 95% ethanol to give long pale brown needles: 22 g (80%), mp 96–98°C (lit. (59) mp 98°C). A solution of benzophenone hydrazone (5.8 g, 30 mmol), anhydrous magnesium sulfate (Anachemia, 3.0 g), and methylene chloride (60.0 mL) was stirred rapidly in an ice-bath. To this was added activated manganese dioxide (Aldrich, 9.2 g) in one portion. Stirring was continued for 2 h at 0°C and for 1 h at room temperature. Removal of the solvent gave a dark purple oil, which, upon storage at −16°C, afforded red needles of diphenyldiazomethane: 5.2 g (88%), mp 33–34°C (lit. (60) mp 35°C).

S-Trityl-L-cysteine (11.0 g, 30.3 mmol), diphenyldiazomethane 11.74 g, 60.6 mmol), methanol (100 mL), and methylene chloride (150 mL) were mixed, yielding a suspension, which was stirred at room temperature. After 12 h a pink solution had formed. Evaporation of the solvent gave an oil, which was dissolved in a minimum of acetone and treated with *p*-toluenesulfonic acid (Eastman, 5.76 g, 30.3 mmol); The acetone was evaporated, and subsequent trituration with hexane produced an amorphous solid, 21.33 g (98%); $[\alpha_D]$ 70.7 (*c* 1.35, methanol); tlc (free base); R_f 0.78 (methylene chloride: ethyl acetate 9:1). The compound gave a single spot, which was visualized with ninhydrin and with palladium chloride spray.

*N-Boc-glycyl-L-proline benzyl ester (**11**)*: A solution of *N*-Boc-glycine (8.76 g, 50 mmol) and EEDQ (Aldrich, 13.6 g, 55 mmol) in methylene chloride (120 mL) was stirred for 5 min. Then L-proline benzyl ester hydrochloride (12.08 g, 50 mmol), triethylamine (Anachemia, 5.16 g, 51 mmol), and additional methylene chloride (40 mL) were added. Stirring was continued at room temperature for 20 h. The reaction mixture was washed successively with 10% aqueous potassium bisulfate (5 × 160 mL), saturated brine (1 × 160 mL), saturated sodium bicarbonate (3 × 160 mL), and brine (1 × 160 mL), dried over anhydrous magnesium sulfate, and evaporated to give a viscous oil. This was dissolved in warm ethyl acetate (15 mL), and hexane was added to the cloud-point (100 mL). After overnight storage at 4°C, the product crystallized, and was recrystallized twice from ethyl acetate – hexane to give colourless needles, 14.24 g (78%), mp 77–78°C (lit. (61) mp 76–77°C); ^{1}Hmr ($CDCl_3$): 1.47 (9H, s, *t*-Bu), 2.02 (3H, m, Pro β- + γ-CH_2), 2.20 (1H, m, Pro β- + γ-CH_2), 3.47 (1H, m, Pro δ-CH_2), 3.60 (1H, m, Pro δ-CH_2), 3.92 (1H, dd, 7, 18 Hz, Gly CH_2), 4.02 (1H, dd, 7, 18 Hz, Gly CH_2), 4.59 (1H, q, 5.0, 7.5 Hz, Pro α-H), 5.44 (1H, br s, urethane N-H), 7.37 (5H, d, aromatic), tlc: R_f 0.43 (methylene chloride: ethyl acetate, 9:1), R_f 0.86 (methylene chloride: methanol 5:2), one spot, ninhydrin negative.

*N-Boc-glycyl-L-proline (**12**)*: *N*-Boc-glycyl-L-proline benzyl ester (14.0 g, 38.7 mmol) was dissolved in methanol (300 mL), and 10% palladium on carbon (Aldrich, 1.01 g) was added under nitrogen. The reaction vessel was purged three times with nitrogen and three times with hydrogen, and the mixture was then stirred for 4 h under hydrogen at atmospheric pressure. The catalyst was removed by filtration through diatomaceous earth (Fisher, Celite), and the filtrate was evaporated to a viscous oil. After trituration with ethyl acetate – ethyl ether (50 mL ethyl acetate, 50 mL ethyl ether), a white crystalline solid formed, 9.75 g (98%), mp 145–146°C; ^{1}Hmr ($CDCl_3$): 1.45 (9H, s, Boc), 2.08 (3H, m, Pro β- + γ-CH_2), 2.26 (1H, m, Pro β- + γ-CH_2), 3.48 (1H, m, Pro δ-CH_2), 3.58 (1H, m, Pro δ-CH_2), 3.94 (1H, q, 5, 20 Hz, Gly CH_2), 4.08 (1H, q, 6, 20 Hz, Gly CH_2), 4.60 (1H, q, 6, 8 Hz, Pro α-H), 5.51 (1H, br, urethane N-H), 9.13 (1H, br s, CO_2H); tlc: R_f 0.41 (methylene chloride: methanol 5:2), one spot, ninhydrin negative. The compound became ninydrin positive after pretreatment with formic acid.

*Glycyl-L-proline benzyl ester hydrochloride (**13**)*: *N*-Boc-glycyl-L-proline benzyl ester (13.91 g, 38.4 mmol) was dissolved in dry ethyl acetate (360 mL). The solution was cooled to 0°C in an ice-bath, and anhydrous hydrogen chloride gas (Matheson Gas Products) was bubbled into the solution for 25 min. Stirring was continued at 0°C for 1 h, and then at room temperature for an additional 30 min. The solvent was then removed at 26°C to yield a white foam, which was crystallized from ethanol (20 mL) and ether (200 mL), 11.21 g (100%), mp 108–113°C; ^{1}Hmr ($CDCl_3$): 1.90 (3H, br, Pro β- + γ-CH_2), 2.12 (1H, br, Pro β- + γ-CH_2), 3.54 (2H, br, Pro δ-CH_2), 4.03 (1H, d, 17.5 Hz, gly CH_2), 4.17 (1H, d, 17.5 Hz, Gly CH_2), 4.60 (1H, d, 7 Hz, Pro α-H), 5.11 (1H, d, 14 Hz, CH_2Ph), 5.18 (1H, d, 14 Hz, CH_2Ph), 7.35 (5H, d, Ph), 8.36 (3H, br, NH_3^+); tlc: R_f 0.46 (methylene chloride: methanol 5:2). The compound gave one spot which was ninhydrin positive.

*N-Boc-glycyl-L-prolyl-glycyl-L-proline benzyl ester (**14**)*: A solution of *N*-Boc-glycyl-L-proline (9.71 g, 38 mmol) and EEDQ (Aldrich, 10.32 g, 42 mmol) in methylene chloride (250 mL) was stirred at room temperature for 5 min, and glycyl-L-proline benzyl ester hydrochloride (11.10 g, 38 mmol) and triethylamine (BDH, 3.84 g, 38 mmol) were then added. The resulting solution was stirred at room temperature for 20 h and then washed successively with 10% aqueous potassium bisulfate (3 × 250 mL), saturated brine (2 × 250 mL), saturated sodium bicarbonate (3 × 250 mL), saturated brine (1 × 250 mL), dried over anhydrous sodium sulfate, and evaporated to a white amorphous solid. This was crystallized from ethyl acetate – hexane, and then recrystallized from acetone (25 mL) and hexane (50 mL), 16.23 g (87%), mp 162–164°C (lit. (42) mp 162–163°C); ^{1}Hmr ($CDCl_3$): 1.47 (9H, s, Boc), 2.04 (4H, m, Pro CH_2), 2.20 (4H, m, Pro CH_2), 3.46 (2H, m, Pro δ-CH_2), 3.60 (2H, m, Pro δ-CH_2), 3.90–4.16 (4H, m, glycine CH_2), 4.60 (2H, m, Pro α-H), 5.16 (1H, d, 12 Hz, benzyl), 5.22 (1H, d, 12 Hz, benzyl), 5.49 (1H, br, urethane N-H), 7.21 (1H,

br, NH), 7.38 (5H, br d, aromatic), tlc: R_f 0.38 (methylene chloride: acetone: acetic acid 100.0: 100.0: 0.5), R_f 0.81 (methylene chloride: methanol 5:2), one spot, ninhydrin negative.

N-Boc-glycyl-L-prolyl-glycyl-L-proline (15): *N*-Boc-glycyl-L-prolyl-glycyl-L-proline benzyl ester (5.0 g, 9.6 mmol) was dissolved in methanol (350 mL), and 10% palladium on carbon (Alfa, 800 mg) was added under nitrogen. The reaction vessel was purged three times with nitrogen and three times with hydrogen, and the mixture was then stirred vigorously for 5 h under hydrogen at atmospheric pressure. The catalyst was removed by filtration through diatomaceous earth (Fisher, Celite) and the filtrate was evaporated to dryness under reduced pressure to give a white foam, which crystallized from ethyl acetate. Three recrystallizations from ethyl acetate gave 3.83 g (93%), mp 191–193°C; $[\alpha_D]$ −135° (*c* 1.5, methanol); ^{1}Hmr ($CDCl_3$): 1.46 (9H, s, *tert*-butyl), 1.94–2.32 (8H, m, Pro β- + γ-CH_2), 3.45–3.62 (4H, m, Pro δ-CH_2), 3.69–4.26 (4H, m, overlapping Gly CH_2), 4.52 (1H, m, Pro α-H), 4.60 (1H, m, Pro α-H), 5.60 (1H, br, urethane NH), 7.51 (1H, br, NH). *Anal.* calcd. for $C_{19}H_{30}N_4O_7$: C 53.46, H 7.03, N 13.13; found: C 53.54, H 7.31, N 12.95.

N-Boc-glycyl-L-prolyl-glycyl-L-prolyl-S-trityl-L-cysteine benzhydryl ester (16): A solution of *N*-Boc-glycyl-L-prolyl-glycyl-L-proline (7.0 g, 16.63 mmol) and EEDQ (Aldrich, 4.52 g, 18.3 mmol) in methanol (200 mL) was stirred for 5 min, and *S*-trityl-L-cysteine benzhydryl ester *p*-toluenesulfonate (11.67 g, 16.63 mmol) and triethylamine (1.68 g, 16.63 mmol) were then added. The solution was stirred for 44 h, and then evaporated to an oily residue. This was dissolved in ethyl acetate (170 mL) and washed successively with 10% potassium bisulfate (5 × 170 mL), saturated brine (1 × 170 mL), saturated sodium bicarbonate (3 × 170 mL), and brine (1 × 170 mL), dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was converted to a foam by addition of methylene chloride (50 mL) and removal of the solvent, followed by repetition of this process twice more. This foam crystallized from ethyl acetate (26 mL) and hexane (130 mL), (11.75 g (75%), mp 110–120°C; $[\alpha_D]$ −55.8° (*c* 1.25, methanol); ^{1}Hmr ($CDCl_3$): 1.44 (9H, s, *tert*-butyl), 1.92 (6H, m, Pro β- and γ-CH_2), 2.21 (2H, m, Pro β- and γ-CH_2), 2.66 (2H, m, Cys CH_2), 3.30–3.52 (4H, m, Pro δ-CH_2), 3.83–4.16 (4H, m, Gly CH_2), 4.47 (1H, m, Cys α-H), 4.54 (2H, m, Pro α-H), 5.45 (1H, br, urethane NH), 6.80 (1H, s, benzhydryl), 7.12 (1H, d, 12 Hz, NH), 7.28 (25H, m, aromatic); tlc: R_f 0.51 (methylene chloride: acetone: acetic acid 100.0: 100.0: 0.5), R_f 0.81 (methylene chloride: methanol 5:2). The compound gave a single spot, which was ninhydrin negative but readily visualized with palladium chloride spray.

Glycyl-L-prolyl-glycyl-L-prolyl-S-trityl-L-cysteine benzhydryl ester (17): *N*-Boc-glycyl-L-prolyl-glycyl-L-prolyl-*S*-trityl-L-cysteine benzhydryl ester (2.0 g, 2.13 mmol) was dissolved in formic acid (BDH, 100%, AnalR grade, 200 mL) that had been precooled to 10°C. The resulting solution was stirred at 10.0 ± 0.1°C for 25 min and then frozen using liquid nitrogen. The formic acid was quickly removed by freeze-drying to leave an amorphous white solid. This was dissolved in ethyl acetate, and the solution was washed with saturated sodium bicarbonate (3 × 100 mL), dried over anhydrous sodium sulfate, and evaporated. Two treatments with methylene chloride, followed by reevaporation, yielded a white foam, 1.36 g (77%); $[\alpha_D]$ −61.9° (*c* 1.1, methanol); ^{1}Hmr ($CDCl_3$): 1.63 (2H, br, NH_2), 1.91 (6H, m, Pro β- + γ-CH_2), 2.22 (2H, m, Pro β- + γ-CH_2), 2.60 (1H, q, 5, 12 Hz, Cys CH_2), 2.69 (1H, q, 8, 12 Hz, Cys CH_2), 3.28 (2H, m, Pro δ-CH_2), 3.38 (1H, d, 15 Hz, Gly CH_2), 3.40 (1H, d, 15 Hz, Gly CH_2), 3.50 (2H, m, Pro δ-CH_2), 3.92 (1H, q, 5, 15 Hz, Gly CH_2), 4.08 (1H, q, 5, 15 Hz, Gly CH_2), 4.44 (1H, m, Cys α-H), 4.54 (2H, d, 7 Hz, Pro α-H), 6.78 (1H, s, benzhydryl), 7.21 (1H, d, 7 Hz, N-H), 7.35–7.50 (26H, aromatic + N-H); tlc: R_f 0.77 (methyl ethyl ketone: acetic acid: water 4:1:1). The compound gave a single spot and was ninhydrin positive.

N-Boc-S-trityl-L-cysteinyl-glycyl-L-prolyl-glycyl-L-prolyl-S-trityl-L-cysteine benzhydryl ester (18): *N*-Boc-*S*-trityl-L-cysteine dicyclohexylammonium salt (1.05 g, 1.63 mmol) was partitioned between ethyl acetate (50 mL) and 10% citric acid (50 mL). The organic phase was separated, washed with 10% citric acid (2 × 30 mL) and brine (1 × 30 mL), dried over anhydrous sodium sulfate, and evaporated to yield 780 mg of material. This was dissolved in ethyl acetate (15 mL), and EEDQ (Aldrich, 0.443 g, 1.77 mmol) was added. The solution was stirred for 10 min, and a solution of glycyl-L-prolyl-glycyl-L-prolyl-*S*-trityl-L-cysteine benzhydryl ester (1.36 g, 1.63 mmol), in ethyl acetate (25 mL), was added. Stirring was continued for 20 h, and the cloudy reaction mixture was then filtered. The filtrate was washed successively with 10% potassium bisulfate (4 × 100 mL), brine (1 × 100 mL), saturated sodium bicarbonate (3 × 100 mL), brine (1 × 100 mL), dried over anhydrous sodium sulfate, and evaporated. Two treatments with methylene chloride yielded a white foam, 895 mg (80%); ^{1}Hmr ($CDCl_3$): 1.40 (9H, s, *tert*-butyl), 1.84 (3H, m, Pro β- and γ-CH_2), 1.95 (3H, m, Pro β- and γ-CH_2), 2.13 (1H, m, Pro β- and γ-CH_2), 2.22 (1H, m, Pro β- and γ-CH_2) 2.50–2.71 (4H, overlapping AB of ABX, Cys CH_2), 3.34 (2H, m, Pro δ-CH_2), 3.50 (2H, m, Pro δ-CH_2), 3.82–4.10 (4H, overlapping Gly CH_2), 4.43 (1H, d, Cys α-H), 4.48 (1H, d, Pro α-H), 4.53 (1H, d, Pro α-H), 4.87 (1H, d, Cys α-H), 4.98 (1H, br, urethane NH), 6.77 (1H, s, benzhydryl), 6.94 (2H, br, NH), 7.08 (1H, d, 6 Hz, NH), 7.17–7.35 (30 H, m, aromatic), 7.40 (10 H, d, aromatic); tlc: R_f 0.89 (dichloromethane: methanol 5:2). The compound gave one spot, which was ninhydrin negative.

Phenceptin (N-Boc-L-cystinyl-glycyl-L-prolyl-glycyl-L-prolyl-L-cysteine benzhydryl ester): *N*-Boc-*S*-trityl-L-cysteinyl-glycyl-L-prolyl-glycyl-L-prolyl-*S*-trityl-L-cysteine benzhydryl ester (2.5 g, 2.27 mmol) was dissolved in a mixture of methanol (500 mL) and methylene chloride (500 mL), and the solution was added dropwise, with stirring, to an ice-cold solution prepared from iodine (1.15 g, 2 equiv.) and pyridine (2 mL, 10 equiv.), in methanol (1 L). When the addition was complete (4.5 h), the ice-bath was removed and stirring was continued for 2 h, by which time tlc (methylene chloride – acetone, 1:1) showed that the starting material had been consumed. The methanol solvent was removed under reduced pressure to give a dark brown oil. This was dissolved in methylene chloride (500 mL), and the solution was washed successively with 10% potassium bisulfate (1 × 400 mL), 10% sodium bisulfate (2 × 300 mL), and water (3 × 300 mL), dried over anhydrous sodium sulfate, and evaporated. The white foam was triturated with hexane (3 × 50 ML) and filtered to give 1.811 g of material, which was chromatographed on silica gel (35 g) using acetone as eluant and a flow rate of 5 mL/min, yielding 1.18 g (65%) of phenceptin. The analytical sample was prepared by repetition of the column chromatography. $[\alpha_D]$ −1.29 (*c* 1.15, methanol); ^{1}Hmr ($CDCl_3$, 273 K, major conformation): 2.01 (6H, m, Pro β- + γ-CH_2), 2.16 (2H, m, Pro β- + γ-CH_2), 2.72 (2H, q, 4, 16 Hz, Cys CH_2), 3.08 (2H, q, 7, 16 Hz, Cys CH_2), 3.19 (2H, br, CH_2 of Gly^2), 3.47 (2H, m, Pro δ-CH_2), 3.59 (2H, m, Pro δ-CH_2) 3.92 (1H, q, 4, 18 Hz, CH of Gly^4), 4.09 (1H, q, 4, 18 Hz, CH of Gly^4), 4.42 (1H, m, Cys α-H), 4.53 (1H, m, Cys α-H), 4.66 (1H, m, Pro α-H), 4.94 (1H, m, Pro α-H), 6.20 (1H, d, 9 Hz, urethane N-H), 6.93 (1H, s, benzhydryl), 7.14 (2H, d, 7 Hz, N-H), 7.35 (11H, m, Ar + N-H); tlc: R_f 0.65 (methylene chloride: methanol 5:2), R_f 0.87 (methyl ethyl ketone: acetic acid: water 4:1:1). The peptide gave a single spot on tlc, which was not visualized with ninhydrin but became ninhydrin positive after pretreatment with formic acid. *Anal.* calcd. for $C_{38}H_{48}N_6O_9S_2 \cdot H_2O$: C 56.01, H 6.18, N 10.31, S 7.85; found: C 56.18, H 6.07, N 10.13, S 8.61.

B. Transport studies

General

The divided beaker cell was sealed with a protective shield, to minimize loss of solvent, and immersed in a water bath maintained at 37.0 ± 0.1°C, using a Haake temperature controller. The extracellular phase contained a salt (20 mM) in water (80.0 mL). The membrane phase contained the ionophore (150 μmol/L) in 1-octanol (50.0 mL). The intracellular phase was water (80.0 mL). All phases were stirred simultaneously at constant, reproducible rates. Periodic sampling of the intracellular phase allowed determination of transport rates. Experiments were repeated several times to ensure reproducibility. Blank runs were always used as controls. At no time did the divided beaker cell "leak" ions into the intracellular phase in the absence of an ionophore.

Analytical methods

Transport rates were quantified by determination of the concentration of the counter-anion in the intracellular phase. For studies involving thiocyanate as the counter-anion, the anion concentration was determined spectrophotometrically at 480 nm. Aliquots (0.50 mL) of the intracellular phase were mixed with 0.005 M ferric chloride (5.00 mL), which was made 0.18 M in perchloric acid and the thiocyanate concentration was determined from a Beer's Law plot. A new calibration curve was determined prior to each kinetic run.

When picrate was the counter-anion, the extracellular phase contained picric acid (50 mM) and HEPES buffer (10 mM) at pH 7.0. The appearance of the yellow picrate anion in the intracellular phase was determined spectrophotometrically at 355 nm.

When chloride was the counter-anion, the concentration was determined indirectly by the Volhard method (54).

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