THE UPTAKE OF NORADRENALINE BY HUMAN RED BLOOD CELLS AND GHOSTS

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by

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ABBREVIATIONS

General

CNS	central nervous system
CPT	cold pressor test
Hb	haemoglobin
HPLC-ED	high performance liquid chromatography with electrochemical detection
pH	$-\log_{10}[H^+]$
Ki	inhibition constant
Km	affinity constant
pKa	$-\log_{10}$ acid dissociation constant
Q_{10}	temperature coefficient
Vmax	maximal velocity (e.g. of transport)
$[\ ]_{i/o}$	concentration (intracellular/extracellular)

Biochemicals

cAMP	adenosine 3':5' cyclic monophosphate
ATP	adenosine 5'-triphosphate
DCCD	dicyclohexylcarbodiimide
DHB	dihydroxybenzoic acid
DIDS	4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid
DOPA	3,4-dihydroxyphenylalanine
EDTA	ethylenediaminetetraacetic acid
5-HT	5-hydroxytryptamine
IAA	iodoacetic acid
MeOH	methanol
$MeSO_4$	methylsulphate
NA	noradrenaline
NMDG	N-methyl-D-glucamine
NMN	normetadrenaline
PCA	perchloric acid

PCMBS	p-chloromercuribenzene sulphonic acid
VMA	vanilylmandelic acid

Biological buffers

HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
MOPS	3-(N-morpholino)propanesulphonic acid
PIPES	Piperazine-N,N'-bis(2-ethanesulphonic acid)
POPSO	Piperazine-N,N'-bis (2-hydroxypropanesulphonic acid)
TES	(N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; 2-([2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino) ethanesulphonic acid)
Tris	(tris(hydroxymethyl)aminomethane)

Enzymes

AChE	acetylcholinesterase
COMT	catechol-O-methyl transferase
G3PD	glyceraldehyde-3-phosphate dehydrogenase
MAO	monoamine oxidase

INTRODUCTION

The aims of this thesis are twofold. The literature regarding NA accumulation by human red blood cells is sparse and sometimes contradictory. One objective is therefore, to attempt to clarify the mechanism by which NA enters red cells and the factors which might affect this process. These results are presented in Chapters 1-4. In addition, the action of NA as a potential regulator of red cell cation transport is considered in detail in Chapter 5.

The fact that biological membranes are permeable to NA has been recognised for many years. Prior to discussing the literature relevant to this phenomenon, however, it is necessary to consider a brief summary of the characteristics of the various mechanisms which exist at the membrane level to permit the movement of a solute from one environment (e.g. outside the cell) to another (e.g. inside the cell). This discussion is of importance because, as stated above, there has been some controversy as to the precise method by which the human red cell itself handles NA.

SIMPLE DIFFUSION

The random Brownian movements of molecules or ions in solution cause a solute, over a period of time, to disperse from areas of high concentration until the solution has become homogeneous. This is the process of simple, or passive, diffusion which can also occur between compartments separated by an artificial or biological membrane. In the latter case the close packing and hydrophobic nature of the chains in the centre of the membrane add a constraint on the type of molecule that can diffuse into the cell at any significant rate. For instance, charged and very large molecules may be almost entirely excluded unless a specific transport process for them exists. It is also recognised that even for small molecules, such as H_2O and MeOH, diffusion across biological membranes only occurs at 10^{-3} - 10^{-2} times the rate that is observed across an aqueous barrier. Diffusion is thus

not a rapid process.

Diffusion across a biological barrier obeys Fick's first law, which states that the flux (J) of substrate per unit area through the membrane depends only upon the diffusion coefficient of the molecule (D) and its concentration gradient across the membrane (dc/dx).

FICK'S LAW: $J = -DA\ dc/dx$

where dc = concentration difference across the membrane
A = area of membrane
x = thickness of membrane

It should be noted that, while D accounts for the different rates of diffusion of different compounds, these differential rates depend partly on the nature of the membrane. D must clearly, therefore, include membrane specific factors and is not solely a property of each molecule.

In this equation no term is included for the binding of substrate to the membrane since discrete binding sites are not involved. Consequently, passive diffusion does not saturate and the rate of movement of substrate is thus a simple function of concentration, except at extremely high substrate concentrations.

It is considered probable that diffusion may proceed via two mechanisms, either by the so called "lattice model", or through aqueous pores.

The Lattice Model

In this model diffusion occurs by the movement of the permeant molecule into a "vacancy" which arises by the lateral movement of membrane components on one side of the phospholipid bilayer. As a corresponding "vacancy" occurs on the other side of the bilayer, the molecule is able to diffuse into this gap and hence traverse the membrane. The principle energy barrier to this form of movement is the activation energy required to move the

molecule from the aqueous phase into the apolar environment of the phospholipid bilayer. Thus, permeability may in some cases be loosely correlated with the hydrophobicity of the diffusing species (see, for example, Collander, 1949). The best correlation of permeability is in fact observed if expressed as the number of hydrogen bonds that must be broken as the substrate passes from aqueous solvent into the bilayer (Houslay & Stanley, 1984).

The Aqueous Pore Model

It has been calculated that some molecules, for instance H_2O and MeOH, have greater permeabilities than would be predicted solely on the basis of the lattice model. Such observations point to the existence of aqueous pores for the transport of these compounds. In the human red cell the diameter of these pores, calculated from the rate of H_2O movement across the membrane, would be in the region of 0.7 nm. It is also possible that these substances pass in a non-specific way through the pores of transport proteins designed to mediate the transport of other larger molecules.

Evidence in support of the aqueous pore model comes from the observation that biological membranes are also, in general, more permeable than synthetic phospholipid vesicles, suggesting that some of the diffusion properties of membranes are a feature of the protein component rather than just the phospholipids.

FACILITATED DIFFUSION

Many compounds are transported more rapidly than would be predicted on the basis of diffusion through the membrane. A common example is D-glucose which, at a concentration of 1 mM, is transported approximately 10^4 times faster at 25°C than would be expected on the grounds of its lipid solubility or number of hydrogen bonds that form in aqueous solution. D-glucose transport is not ATP-dependent, however, and will only proceed as long as a concentration gradient persists. Uptake is therefore neither by means of simple diffusion nor by a directly energy consuming process. It is now recognised that the movement of D-glucose, and

many other compounds, across the red cell membrane occurs by the process of facilitated diffusion.

An important characteristic of facilitated, as opposed to simple, diffusion is the ability to saturate the transport system at high substrate concentrations, and in some cases to distinguish between optical isomers of the same compound. L-glucose is not absorbed by red cells. This provides a clear and experimentally testable distinction from passive diffusion, and furthermore suggests the involvement of an enzyme-like protein with a substrate binding site.

The facilitated transport (J) of a substrate across the membrane may be described by the Michaelis-Menten equation:

$$J = \frac{S\ Vmax}{Km + S}$$

Where, S = substrate concentration
Vmax = maximum velocity of transport
Km = S at Vmax/2

Transport does not necessarily proceed only in one direction at a given time, however. The net flux of substrate, J_{net}, is therefore given by the composite equation:

$$J_{net} = J_{in} - J_{out}$$

Where, $J_{in} = (S_{out}\ Vmax_{in}) / (Km_{in} + S_{out})$

$$J_{out} = (S_{in}\ Vmax_{out}) / (Km_{out} + S_{in})$$

Furthermore, $Vmax_{out}$ and $Vmax_{in}$, and Km_{in} and Km_{out} may or may not be identical, thus implying that facilitated diffusion may be a concentrative process. It is interesting to note that for the transport of D-glucose across the human red cell membrane, the net flux of substrate is some 85 times slower than the rate of exchange. This demonstration in itself is an important criterion

for distinguishing between facilitated diffusion and simple diffusion. In other facilitated transport systems the net flux may actually become so small that the system may be regarded simply as an exchange reaction. An example is the carnitine-acylcarnitine exchange transporter of the inner mitochondrial membrane (Ramsay & Tubbs, 1975).

Some of the properties of simple and facilitated diffusion are illustrated in Table 1. As can be seen, another of the typical characteristics exhibited by facilitated diffusion is the phenomenon of counter-transport, which arises as a consequence of the reversible nature of the process. Counter-transport may occur when more than one substrate binds to the same transport protein. For example, when red cells are equilibrated with a suitable non-metabolisable sugar, and then a high concentration of glucose is added outside the cells, the glucose analogue will be transported out of the cell against a concentration gradient. The energy for this process comes from the coupled diffusion of glucose and there is no requirement for ATP.

Classical models of facilitated diffusion involved either a shuttle of carriers between the two surfaces of the membrane, or tumbling molecules which alternately revealed a substrate binding site at each surface. As Singer (1977) discusses, however, whilst such moving carrier models might aptly describe the kinetics of facilitated transport, they are unlikely to exist in vivo since the required movement of large hydrophilic regions of protein through the apolar core of the membrane is not thermodynamically favourable.

Direct evidence against these early models comes from early measurements of the orientation of transport proteins in the membrane. The models would predict a random insertion of the carrier protein in the membrane, while it has been satisfactorily demonstrated that proteins such as Band 3 (which mediates red cell anion movements) are all aligned in the same direction. In addition, cross-linking of these molecules does not necessarily adversely affect their function. The properties of the Band 3 protein are reviewed by Knauf (1979).

The current concept of a transport protein is that of the 'gated pore'. It is proposed that the protein can exist in two states which are interconvertible by a conformational change. One of the properties of a multimeric protein is that a relatively low input of energy can give rise to a large rearrangement of the subunits and it is, therefore, quite conceivable that the energy of substrate binding should be adequate to achieve this. In addition, although the substrate may bind to the same site from each side of the membrane, the topography of the surrounding protein of the gated pore may differ considerably inside and out. Thus, the observation of different binding properties on either side of the membrane strongly supports this type of model, and is less easily incorporated into the 'shuttle' or 'tumbling' schemes.

A consequence of the gated pore model is that molecules smaller than the substrate might be expected to be able to penetrate the open pore and be transported as a result of a conformational change, even if they have little or no affinity for the substrate binding site. When the permeability to 4- and 5-carbon polyols was investigated in human red cells, it was found that a significant fraction of the permeability was inhibited by glucose, suggesting a shared transport system. The fact that the smaller molecule was transported more rapidly however, argues against transport by competition with the glucose binding site and suggests that the rate limiting step of transport is the diffusion of the compound into the postulated pore of the glucose transport protein. Calculations of the pore volume from the rate of transport give rise to a hypothetical pore diameter of 0.8 nm, which is in good agreement with the size of the glucose molecule (0.72 nm). It remains the case however, that direct experimental tests for the gated pore are not easy to devise.

Schematic models of the proposed mechanisms of facilitated diffusion are illustrated in Figure 1.

TABLE 1

GENERAL CHARACTERISTICS OF MEMBRANE TRANSPORT PATHWAYS

DIFFUSION	FACILITATED DIFFUSION	ACTIVE TRANSPORT
Net flux ceases at electrochemical equilibrium	Net flux ceases at electrochemical equilibrium	Can achieve transport against electrochemical gradient
No energy coupling	May be indirectly coupled to electrochemical energy	Directly coupled to energy supply
Low specificity	High specificity	High specificity
No counter-transport	May display counter-transport	Reversible under extreme conditions
Transport remains linearly related to concentration of substrate over a wide range	Saturates at high substrate concentrations, obeying Michaelis-Menten kinetics	Saturates at high substrate concentrations, obeying Michaelis-Menten kinetics

Figure 1

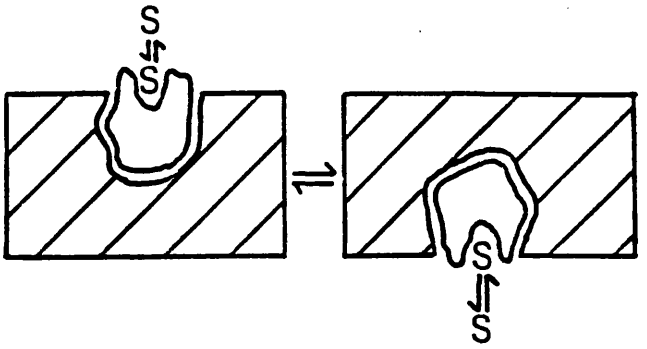
Models of membrane transport proteins

The diagram is adapted from Singer (1977). Each of the three models represents possible mechanisms by which facilitated transport may proceed. They may be respectively described as:

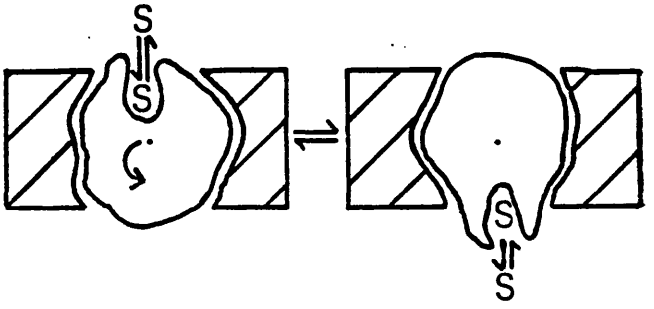
a)The reciprocating carrier
b)The rotating carrier
c)The gated pore

The hatched regions represent the lipid bilayer, while 'S' denotes the transported substrate. Models a) and b) both account for the kinetics of facilitated diffusion, but are unlike the structure of known transport proteins. The movements of models a) and b) are also less energetically favoured than c), as they require transport of bulky hydrophilic moieties from one side of the membrane to the other. The gated pore model does not require movements of protein across the membrane following a conformational change.

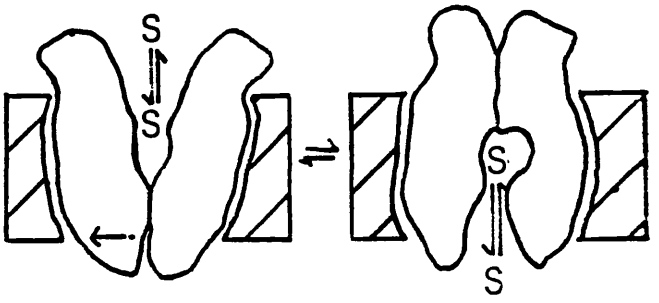
a)



b)



c)



ACTIVE TRANSPORT

During active transport, the movement of substrate across the membrane is directly coupled to an energy source, usually ATP. As a result, cells are able to accumulate specific substances against considerable concentration gradients (for example, up to 10^4-fold for Ca^{2+}-ATPase). Membrane proteins mediating active transport are large structures, often forming multimers, and have large portions of polypeptide chain anchored in the aqueous medium to either side of the lipid bilayer. This structure also favours a gated pore type of mechanism although the architecture may be of a more complicated nature than those structures believed to mediate facilitated diffusion. One obvious modification may be the addition of covalently attached polypeptides with associated ionophoric or ATPase activity.

During each catalytic cycle the transport protein hydrolyses an ATP molecule and as a result becomes phosphorylated. The introduction of a highly charged phosphate group may then reasonably drive the conformational change of a gated pore.

A striking feature of active transport proteins is their ability to produce vectorial transport - generally speaking, the direction of movement of substrate is strictly defined. This may result in part from the fact that ATP is usually only significantly present on one side of the membrane, and strictly speaking even active transport is reversible. Under extreme and unphysiological conditions some reverse flux of substrate may be observed, accompanied by the formation of ATP, (e.g. Garrahan & Glynn, 1967). Such events are unusual, but importantly, the tight coupling between transport and ATP hydrolysis is still maintained.

THE DISCOVERY OF NORADRENALINE TRANSPORT

1.The identification of the transmitter released by sympathetic nerves. As early as 1901, Langley reported that the effects produced by stimulation of the sympathetic nerves were closely mimicked by the application of extracts of the adrenal glands.

Subsequently, Takamine (1901) isolated a pharmacologically active compound from the adrenal glands of sheep and oxen. He named it adrenaline. Elliot (1905) was able to demonstrate that the actions of adrenaline upon smooth muscle were similar to those resulting from sympathetic stimulation, and suggested that this novel compound might be the active factor released at the periphery as the result of sympathetic nervous system activity. Five years later however, Barger and Dale (1910) concluded that adrenaline could not itself satisfactorily assume this role. Indeed, they hypothesised that the action of several other catechol-containing compounds could more closely account for the effects observed.

The first demonstration of neurohumoral transmission was achieved over a decade later by Loewi (1921), and in the same year Cannon and Uridil (1921) also demonstrated the release of an adrenaline-like substance to the liver subsequent to stimulation of the sympathetic nervous system. The precise nature of the neurotransmitter substance remained obscure, however, for the next 25 years, during which period three theories became popular:

1) That adrenaline was the transmitter (e.g. Gaddum & Kwiatkorski, 1939).

2) That adrenaline, or a closely similar compound, was liberated but modified at the target organ to produce one, or both, of the active substances sympathin E and sympathin I (e.g. Cannon & Rosenblueth, 1933).

3) That adrenaline, once liberated, caused the excitatory effects documented, whilst a degradation product, named adrenoxin, was responsible for the inhibitory effects observed.

The existence and actions of NA became increasingly certain in the period 1946-1950. Initially it was demonstrated that significant amounts of this compound were present in splenic extracts and that such extracts became greatly depleted of amine following surgical sympathectomy (von Euler, 1946a,b). Tullar (1948) was able to synthesise (±) NA and in addition split the racemic compound into its two optical isomers. Von Euler (1950) went on to show that NA in living tissues was present as the (-) or l form. At about the same time, Peart (1949) demonstrated the

release of NA from the spleen following stimulation of the splenic nerve supply. Many of the above findings were soon duplicated by various other workers in the field.

2. The demonstration that peripheral tissues accumulate exogenous NA. Although the compound was not discovered until a later date, evidence was obtained as early as the 1930s that NA, as well as being released as a result of sympathetic nerve stimulation, was also accumulated from the circulation by a variety of tissues. Burn (1932), studying the perfused hind limb of the dog, found in his experiments that tyramine and ephedrine exhibited only a small portion of the constrictor action normally attributed to them. If, however, adrenaline was added to the circulation the other two agents were greatly enhanced. It was also noted that tyramine and ephedrine had no appreciable constrictor action if the post-ganglionic sympathetic fibres had degenerated. The latter work was performed on a cat perfused fore-limb preparation. The conclusion Burn drew was that "there is a store of adrenaline in the neighbourhood of the endings ready to be liberated when the sympathetic impulse arrives." It was suggested that during the course of the preparation of the hind limb, the store became seriously depleted such that injections of tyramine and ephedrine were ineffective. The subsequent addition of adrenaline to the circulation enabled these stores to replenish themselves, thus restoring the actions of the constrictor drugs.

In the 1940s and 1950s Raab, Nickerson and their colleagues made the important discovery that increases in tissue catecholamine content occurred as a result of administration of large doses of NA and adrenaline (Raab, 1943; Nickerson, Berghout & Hammerstrom, 1950; Raab & Gigee, 1955). Following this work the new availability of tritium-labelled compounds of high specific activity made it possible to examine with greater accuracy the disposition of injected quantities of catecholamine in doses within the physiological range. After a bolus injection of ^{3}H-adrenaline in mice, Axelrod, Weil-Malherbe & Tomchick (1959) were able to show that the compound disappeared in two distinct

phases. Immediately following injection a rapid (about 5 minutes) period of removal ensued such that about 70% of the amine was eliminated. This was mainly by O-methylation. The remaining 30% disappeared over a slower time-course during which labelled amine could be detected in heart, spleen, lung and kidney. In 1961, Whitby, Axelrod & Weil-Malherbe focussed their attention upon the fate of ^{3}H-NA after infusion into mice. After about 6 hours, approximately 20% of ^{3}H-NA appeared bound to the tissues of the mice. This and other work prompted Stromblaad and Nickerson (1961) to suggest that:

"It is possible that specific uptake is a major factor in terminating the action of injected or endogenously released catecholamines. It is reasonable to assume that this mechanism would be relatively more effective in disposing of norepinephrine released locally by sympathetic nerve endings, in close proximity to the storage sites, than of circulating catecholamines."

NEURONAL AND EXTRANEURONAL UPTAKE OF NA

Although the work presented in this thesis is intended to probe further the mechanism of and factors affecting NA uptake by human red blood cells, the majority of work pertaining to NA transport has been performed upon other tissues. Since, in some cases, the results to be presented seek to draw some distinction between the pathways of NA movement in red cells and other tissues, there follows a short summary of the mechanisms by which other tissues may acquire catecholamines.

The most extensively studied uptake systems have been those responsible for the accumulation of NA by adrenergic neurons and in various peripheral effector tissues. Neuronal NA uptake is mediated by a high affinity transport system (Km = 0.2 - 0.4 uM) situated in the axonal membrane of adrenergic neurons. Uptake is highly dependent upon the presence of extracellular sodium and is stereochemically selective in most tissues and species for the (-) isomer. The NA carrier binding sites are not entirely specific for NA however, and a variety of other compounds may act as alternative substrates. Neuronal NA uptake is potently inhibited

by cocaine, by various sympathomimetic amines and by tricyclic anti-depressant drugs such as imipramine and amitriptyline. Neuronal NA uptake is frequently referred to as 'Uptake I'.

Operating alongside Uptake I in various smooth muscle and glandular tissues is a second NA transport system, named 'Uptake II'. Uptake II differs from Uptake I in having a much lower affinity for NA (Km = approximately 250 μM), a lesser sodium dependency and an inability to distinguish between the (+) and (-) optical isomers. Other compounds such as adrenaline may also be transported by the Uptake II mechanism, and indeed have higher affinities for the carrier than NA itself. Despite the low affinity for NA however, Uptake II is found to a greater extent in most peripheral organs than Uptake I with the result that both transporters may handle approximately equal amounts of amine. Amines taken up by Uptake II are normally rapidly degraded by the enzymes MAO and COMT, although, in tissues exposed to very high catecholamine concentrations, the rate of influx may exceed the catabolic capacity of the tissue. Unchanged amine will, accordingly, accumulate in the extraneuronal sites. Uptake II is potently inhibited by O-methylated catecholamine metabolites such as metadrenaline, by several haloalkylamines (e.g. phenoxybenzamine) and by various steroids (e.g. β-oestradiol).

The existence of two amine transport mechanisms was originally proposed by Lindmar and Muscholl (1964). Similar conclusions were also reached by Carlsson, Hillarp and Waldeck (1963).

THE ROLE OF UPTAKE IN THE INACTIVATION OF NA

The neuronal uptake of NA following its release at the adrenergic nerve terminals is an important mechanism in the inactivation of the transmitter. The exact extent of re-uptake of amine is not easy to define, although it would seem likely that in organs with a dense sympathetic terminal innervation, in which release sites are situated relatively close to target sites, that the recapture mechanism should be more efficient than in tissues such as arterial smooth muscle, in which there is only a sparse

sympathetic innervation. In examples such as cardiac tissues and vas deferens, which fall into the former 'densely innervated' category, the overflow of NA (and metabolites) may increase some 3-4 fold when tissues are exposed to cocaine. this would suggest that in the region of 70-80% of the released NA is normally reabsorbed at source; only a small amount of the total liberated may remain free to enter the circulation and interact with blood cells. It should be noted, however, that similar effects caused by phenoxybenzamine and other α-adrenoceptor antagonists increase the sympathetic overflow of NA not by blocking Uptake I, but by blocking a negative feedback mechanism in the adrenergic nerve terminal whereby released NA acts upon presynaptic α-adrenoceptors to inhibit additional transmitter release. Furthermore, as previously stated, phenoxybenzamine is also a potent inhibitor of Uptake II and thus inhibits NA being accumulated and metabolised postsynaptically. This action also tends to increase the amount of unchanged NA appearing in overflow type experiments.

From studies on the overflow of NA and its metabolites from stimulated tissues after exposure to inhibitors of Uptake I and Uptake II, it appears that the two mechanisms constitute alternative routes for terminating the actions of neurally released NA. Therefore, if Uptake I is inhibited (e.g. by cocaine), a larger proportion of NA is diverted to the 'Uptake II - followed by metabolism' mechanism. The balance between the two mechanisms is of importance as it implies that Uptake II offers an alternative mechanism for transmitter inactivation under conditions in which neuronal recapture is inadequate. Such conditions might occur in densely innervated tissues in which the sympathetic innervation is fired repetitively at high frequencies, or in less well innervated and active tissues where Uptake II sites are comparatively more common. It is also likely that Uptake II sites are important not merely as a backup mechanism for Uptake I, but also as a means of rapid inactivation of catecholamines released into the circulation by the adrenal medulla. Adrenaline, for instance, is taken up by Uptake II sites with greater affinity than NA. Uptake II sites are present in great abundance in the

smooth muscle cells of small blood vessels and are thus ideally situated as one means of removal of circulating catecholamines during their passage through peripheral vascular beds.

CATECHOLAMINE UPTAKE BY BLOOD CELLS

It is clearly apparent from the preceding summary that mechanisms exist in order to facilitate the removal of circulating catecholamines from the bloodstream. It is therefore not surprising to note that blood cells themselves are able to both accumulate and metabolise plasma borne NA and related compounds.

An early indication that blood cells might accumulate catecholamines was presented by Bain, Gaunt and Suffolk (1937), who worked on tissues obtained from the cat. These authors noted that adrenaline was reversibly associated with the cells and could be recovered by suspension of equilibrated cells in adrenaline-free plasma, serum or Locke's solution. Up to 80% of the original adrenaline present within the cells could be recovered by making an "equilibrium mixture", although it was not clear whether the remaining irrecoverable 20% represented destruction of adrenaline, or some irreversible binding phenomenon. The results of such early studies should, of course, be treated with some caution, as the only assay for adrenaline available to these workers was a crude measurement of blood pressure elevation performed in spinal animals.

In more recent years, investigators have focussed their attention more closely upon two specific cell types: the platelet and the red blood cell.

NA uptake by platelets

Weil-Malherbe and Bone (1958) studied the distribution of both NA and adrenaline between platelets and plasma. They observed that the platelet-bound proportion of one amine correlated closely to that of the other and it was calculated that the concentration of catecholamine attained intracellularly could rise as high as 125 times greater than that in plasma. It was noted that not all of this catecholamine was bound, as lysis of platelets by freeze-

thaw treatment or by exposure to a surface active agent resulted in partial release. By 1969 Abrams and Solomon were able to conclude that the uptake of NA by human platelets resembled to some extent that which had already been described in the adrenergic neuron. Using (±) ^{14}C-NA (2.1 μM) as a marker, uptake was shown to be concentrative, resulting in a distribution ratio of 5.1 : 1, and markedly temperature dependent. NA uptake was also inhibited by a variety of metabolic poisons (e.g. cyanide and dinitrophenol) and diminished by ouabain. Substitution of Li for Na in the extracellular medium also reduced uptake. Drugs shown to interfere with NA uptake by the neuron also had a similar effect on the platelet. Uptake of ^{14}C-NA was impaired by 5-HT, tryptamine, tyramine, guanethidine and a variety of anti-histamines. The authors pointed out however, that the release of NA from the platelet did not necessarily reflect the action of a similar stimulus on the sympathetic nervous system - while the two situations were similar, certain discrepancies did arise. Born and Smith (1970) extended these observations as a result of the measurement of ^{3}H-adrenaline uptake. It was noted that only about half of the radioactivity within platelets after a 5 hour incubation remained as intact adrenaline, whilst the remainder was identified as an acidic metabolite from which adrenaline could be recovered following acid hydrolysis. The rate of uptake was linearly related to substrate concentration up to at least 10 μM and concentration ratios (intracellular : extracellular) of up to 12:1 were recorded. The formation of metabolite was inhibited by tropolone, an inhibitor of COMT activity (Belleau & Burba, 1963) but not by MAO inhibitors, suggesting that only one pathway of metabolic degradation was operative. On the basis of a number of experiments the authors suggested that human platelets may store adrenaline both in the cytosol and also in intracellular organelles, from which, like 5-HT, it may be specifically released. Evidence thus exists in support of the platelet as a site for the storage and degradation of catecholamines.

A question that arises at this stage is in regard to the significance of such a mechanism for whole body catecholamine

metabolism. In other words, although it may be readily demonstrated that platelets have the ability to accumulate and degrade catecholamines, is this process of any measurable physiological significance? One possible manner of addressing this problem would be to study the transport of NA by platelets obtained from subjects with well defined abnormalities of catecholamine metabolism, which may, for instance, manifest themselves as elevated plasma NA levels. Elevated plasma NA may occur as a consequence of a variety of factors, which may include either (or both) impaired uptake by blood cells, or elevated return of non-metabolised catecholamine from the blood cell to the plasma. Mattiasson and Hood (1982) studied the handling of NA by platelets of subjects related to patients with essential hypertension who had shown elevated circulating NA levels. They discovered that the initial efflux rates of NA in these individuals were significantly higher than those measured in age-matched controls. Although this observation is not proof that platelets may in any way actively regulate plasma catecholamines, it is consistent with such a theory. Another possible reason for the uptake of NA by blood cells would be to act as reservoirs, releasing NA as and when required to the plasma. This possibility will be considered later.

Catecholamine uptake by human red blood cells

The literature concerning the uptake of NA by red blood cells is less easily interpreted than that concerning platelets.

The first detailed investigation of NA accumulation by human red cells was performed by Schanker, Nafpliotis and Johnson (1961). These authors studied amine uptake at 37°C. in the presence of between 1-4 mM concentrations of various drugs, and measured it by direct estimation of the relevant compound within the cell at various time intervals. It was suggested on the basis of the data obtained that NA and other related compounds (e.g. 5-HT and adrenaline) entered the red cell by diffusion through the lipid membrane. Therefore, those compounds with the greater lipid solubilities were incorporated more rapidly within the cell. It

was, however, noted that the relationship between rate of uptake and ethyl acetate/water partition coefficient (which was employed as an index of lipid solubility) was not precisely defined. To explain the concentrative nature of accumulation observed, it was postulated that compounds were able to bind to some component of the intracellular milieu, most probably haemoglobin.

Two further pieces of evidence were presented to support the hypothesis that uptake of these compounds was mediated by simple diffusion. First, an increase in [5-HT] from 0.5 to 5.0 mM did not affect either the time course or degree of equilibration of compound. Therefore the fraction of drug entering the cells in a given time was independent of the concentration - the degree of uptake was directly proportional to the concentration gradient in accordance with the law of simple diffusion. Secondly, accumulation was also shown to be pH-dependent such that uptake was favoured by elevated extracellular pH levels which would confer increased lipophilic character upon the substrate molecule and hence facilitate its movement across the lipid membrane. While the conclusions drawn by the authors are valid, a problem with this type of approach is, however, that no steps were taken to account for either oxidative decay of substrate, or for intracellular metabolism or efflux of substrate. Such considerations are likely to be important as NA accumulation was measured after periods of up to 8 hours. During this period it is clearly possible that considerable amounts of amine become catabolised, and hence any estimate of uptake is likely to be an underestimate. The true rates of uptake for these compounds may, therefore, not relate quite so simply to the ethyl acetate/water partition coefficients measured, and may even exceed the rate at which simple diffusion could operate. As stated earlier, elevated rates of uptake are sometimes more easily explained in terms of facilitated or active transport, rather than simple diffusion.

Roston (1966, 1967) extended the findings of Schanker, Nafpliotis and Johnson (1961) by measuring the rates of uptake of tritiated adrenaline and NA into human red cells. Due to the availability and ease of detection of isotopes, Roston was able to

use smaller, more physiological quantities, of catecholamine. In comparison to the study of Schanker, Nafpliotis and Johnson (1961), the ratios of intracellular to extracellular amine measured after 30 minutes of incubation at 37°C were much higher. Roston (1967) therefore suggested that such a rapid entry of NA into red cells may be an active process accounting for the uptake of small amounts of catecholamine, while diffusion might be the principle route of entry at higher concentrations. Roston also presented evidence to the effect that catecholamines were not adsorbed on to the walls of red cells, under the experimental conditions described.

The mechanism of entry of NA and other amines into human red cells was studied in more detail in a report published at approximately the same time by Born, Day and Stockbridge (1967). In agreement with Schanker, Nafpliotis and Johnson, these authors also concluded that (-)NA was taken up by red cells at rates proportional to the concentration of amine in the extracellular medium in accordance with a simple diffusion process. The highest concentration considered in this later paper, however, was only 16 ug ml^{-1}, considerably less than the concentrations used by the previous authors. The possibility therefore still exists that the uptake of NA could be saturable, but was studied in this paper at substrate concentrations substantially less than those required to maximally activate a postulated carrier mechanism. Two observations made by Born, Day and Stockbridge (1967), suggest that this latter possibility should not be discounted. First, while (-)NA was accumulated by red cells, (+)NA was not taken up. The uptake process was therefore able to distinguish between the two optical isomers, which would be hard to explain in terms of simple diffusion. Additionally, uptake was almost entirely inhibited by a reduction in temperature to 2°. Such a dependence upon temperature, whilst not eliminating the possibility that uptake might be at least in part mediated by passive diffusion, is more characteristic of either facilitated or active transport. Born, Day and Stockbridge (1967) also tested the hypothesis presented by Roston (1967) that red cells act as a site for the

enzymatic degradation of catecholamines. This was achieved by simultaneous measurements of amine accumulation using i) bioassay, and ii) radioactively labelled molecules.

The results indicated that after 1 hour at 37°C, approximately 10% of each amine taken up had become inactivated, and the authors therefore proposed that, while red cells may act as a site for the gradual metabolism of catecholamines, the speed of this process would be such that they would not contribute significantly to the rapid termination of the pharmacological actions of the amines in vivo. Due to the fact that the measured rates of release of amine were slower than the corresponding rates of uptake, however, it was again suggested that catecholamines probably became bound within the red cell which may therefore act as a reservoir for plasma amines.

Danon and Sapira (1972) also studied the accumulation of catecholamines by human red cells using radio-labelled tracers. These authors suggested that cells were able to accumulate both NA and adrenaline against their concentration gradients, so as to reach an equilibrium distribution ratio of intracellular : extracellular concentration, of about 2:1 after 2 hours. Uptake was again shown to be temperature dependent, while in direct comparison to Born, Day and Stockbridge (1967), these authors produced a kinetic analysis in favour of carrier-mediated saturable uptake. The value for half-saturation of the postulated carrier by NA was quoted as 4.54 mM, and it is thus immediately obvious that the ranges of [NA] previously studied would not have caused transport to become maximally active. The maximum rate of uptake of NA quoted by Danon and Sapira was 2.67 mM per 2 hours. At the same time as NA uptake proceeded, these authors also noted increasing concentrations of intracellular catechol metabolites. The metabolites detected were all identified as 3-O-methyl derivatives, and appeared as the result of a process which obeyed first order kinetics. Analysis indicated that the apparent Km for transformation of ^{14}C-NA to ^{14}C-normetadrenaline was 8.97 X 10^{-5}M, while the Vmax for the process was 1.38 X 10^{-5}M per 2 hours. Thus it seems apparent that the maximum rate of catabolism may not be

able to keep pace with the rate of uptake at high substrate concentrations, a factor which will be considered at greater length in Chapter 1 of this thesis.

Having potentially identified a carrier mechanism, the authors then wished to ascertain whether the process was in any way similar to either Uptake I, Uptake II, or the system described in the platelet, which is currently considered to be a reliable model for the study of the 5-HT containing neurons of the CNS (Da Prada, Cesura, Launay & Richards, 1988). To this end the effects of a variety of drugs known to interfere with the latter phenomena were studied in the red cell, but no similarities could be detected. The mechanism of uptake of NA by human red cells would thus seem to be distinct from those employed in these other named tissues.

Further evidence relating to the mechanism of NA accumulation by human red cells was revealed by Bluth and Banaschak (1976). In this instance it was proposed that uptake proceeded by a two phase reaction. In a rapid initial phase it was suggested that NA became adsorbed to the cell walls. Binding was not assessed directly, however, but as the decrease in medium amine concentration following suspension of red cells. Subsequently, NA was proposed to diffuse in a slower manner across the membrane and into the cell. The nature of the binding sites for NA could not be elucidated by the authors, but neither phase of the reaction was affected by either α or β-receptor blockers. It could be shown, however, that 'binding' was a pH-dependent phenomenon in the same manner as the pH-dependence of uptake described by Schanker, Nafpliotis and Johnson (1961). Considering the observations both of Schanker *et al.* and Roston (1967) that red cell membranes do not bind NA, the results presented by Bluth and Banaschak are surprising. It is possibly the case that the results may be explained in terms of two phases of uptake - a rapid phase followed by a slower phase - although it remains unclear as to why this observation was not previously made.

In a subsequent paper, Banaschak and Bluth (1978), focussed their attention upon the mechanism of their postulated second

phase of uptake. Uptake was once more described as a pH-dependent phenomenon, sensitive to temperature, and capable of accumulating NA within the cell against a concentration gradient. Once again, NA was proposed to bind reversibly to haemoglobin, with a calculated binding stoichiometry of 1:1. Unlike previous authors, Banaschak and Bluth (1978) suggested that the purpose of red cell NA accumulation might be to transport catecholamines in the bloodstream: for example, adrenaline from the adrenal gland to the peripheral target organs. In this process conditions would apply under which the molecule, by virtue of binding to haemoglobin, would be protected from oxidative degradation. This hypothesis does not, however, take into account how NA would avoid degradation at the hands of COMT. Neither is it readily obvious how the red cells would be able to efficiently discharge the correct quantity of amine upon arrival at some designated target tissue.

More easily reconciled with the other publications in the field is the work of Blakeley and Nicol (1978). Although the experiments described in this paper utilised rabbit red cells, certain similarities clearly exist between the NA handling of these and human red cells. It was shown that rabbit red cells also break down NA, after uptake, by virtue of intracellular COMT activity, and also that the uptake was temperature sensitive. Uptake also exhibited saturation kinetics and was partially stereospecific: the (-) isomer entered the cells twice as fast as the (+) isomer, a situation similar to that described by Born, Day and Stockbridge (1967). Kinetic analysis of the data indicated that the Km for (-)NA uptake was 6.6 $\pm$ 0.4 mM, and for (+)NA was 11.3 $\pm$ 0.6 mM. In other words, the reduced uptake of the (+) isomer was due to a decreased affinity for transport. The maximum rates of accumulation of each species were closely similar: for (-)NA 3.5 $\pm$ 0.4 X 10^{-7} mol min^{-1} ml^{-1}, and for (+)NA 3.6 $\pm$ 0.2 X 10^{-7} mol min^{-1} ml^{-1}. While Vmax, as estimated by Blakeley and Nicol, is considerably greater than that quoted by Danon and Sapira (1972), the values for Km agree closely between the two tissues and studies.

Blakeley and Nicol also measured the uptake of 5-HT and, interestingly, also commented upon the fact that this compound and NA entered the red cell at rates which were proportional to their lipid solubility. This observation is essentially the same as that made by Schanker, Nafpliotis and Johnson (1961), who chose to interpret the information solely in terms of passive diffusion. It is apparent from Blakeley and Nicol, however, that this conclusion is not necessarily correct.

In addition to the above, Blakeley and Nicol also demonstrated that 5-HT and NA competed for uptake, and furthermore exhibited counter-transport: the downhill gradient of one amine was able to energise uphill transport of the other in the opposite direction. The evidence presented in this paper would therefore suggest the existence of a shared facilitated diffusion system in rabbit red cells for both NA and 5-HT (and possibly other compounds). Active transport was eliminated on the grounds that metabolic inhibitors were without effect on amine transport.

With regard to the binding of NA by red cells, Blakeley and Nicol could also find no evidence for adsorption to the plasma membrane, casting further doubt on the conclusions of Bluth and Banaschak (1976). Dialysis experiments did provide evidence for the binding of intracellular amine by haemoglobin, however, and a further mechanism by which concentrative uptake could also be achieved was discussed: ion-trapping. Blakeley and Nicol argued that if uptake is proportional to lipid solubility then it would seem likely that the less ionised, and therefore more lipophilic, forms of amine would be those which are favoured for transport. As a consequence of crossing the red cell membrane, however, the transported molecule enters an environment which is conventionally approximately 0.2 pH units more acidic. Hence, in the case of NA, the molecule becomes more positively charged and thus less favoured for transport: the molecule is trapped within the cell. Since the work on rabbit red cells was published it has also been repeatedly demonstrated that rat red cells are also able to accumulate and metabolise NA (e.g. Alexander, Velasquez & Vlachakis, 1981; Yoneda, Alexander & Vlachakis, 1983).

Shortly after Blakeley and Nicol reported their findings, a similar study was published but this time focussing once more upon human red cells (McQuitty & Nicol, 1979). The results also closely agreed with those of Blakeley and Nicol. (-) NA was shown to enter the cell so as to achieve a cell/medium concentration ratio of 1.79 $\pm$ 0.12 : 1 after 4 hours, while 5-HT entered more rapidly and appeared more avidly bound (ratio 3.47 $\pm$ 0.33 : 1 after only 1 hour). Cooling to $0^{o}C$ completely inhibited the entry of both compounds. (-) NA entry followed Michaelis-Menten kinetics, and the Km and Vmax were respectively, 2.1 mM and 1 X 10^{-7} mol $min^{-1}ml$ red $cells^{-1}$.

THE SCOPE OF THE PRESENT WORK

The more recent of the evidence cited above would tend to support the hypothesis that NA enters human red cells by some carrier mediated phenomenon, although the evidence is not conclusive. The aim of these studies therefore, was two-fold:

1. To probe further the precise mechanism by which NA crosses the membrane.

2. To assess in more detail the factors (e.g. pH, presence and absence of specific ions and other amines etc.) which may affect this transport.

In an attempt to clarify the situation, experiments have been performed to measure NA uptake in both intact red cells and red cell ghosts. One advantage of working with ghosts is that intracellular components can be eliminated during haemolysis and, if required, replaced with ingredients specifically chosen by the investigator. By removal of much of the red cell haemoglobin content, and the incorporation of a compound designed to specifically inhibit COMT, it is therefore possible to vastly reduce intracellular binding and metabolism of amine. Initial rate uptake studies under such conditions should more closely represent events solely at the membrane level, and hence aid in solving the inconsistencies present in the literature.

Despite the comparatively slow rates of NA degradation measured by Born, Day and Stockbridge (1967), the importance of

catecholamine metabolism in determining red cell uptake was stressed by Atuk, Turner, Carey and Ayers (1986), who observed a marked stimulation of NA accumulation by red cells from patients suffering from catecholamine-secreting tumours (phaeochromocytoma). De Champlain, Bouvier, Cleroux and Farley, (1984), also observed elevated intracellular NA concentrations in red cells obtained from hypertensives, as compared to controls, which mirrored plasma amine levels.

In addition to the study of NA transport, the final chapter in this thesis focusses attention upon the interactions of NA with the various cation transport mechanisms of the human red cell. It has been widely demonstrated that NA may affect sodium handling in a variety of systems (e.g. Watlington, 1968; Berthon, Capiod & Claret, 1985), and it is also suspected that cation handling is abnormal in certain clinical conditions in which catecholamine metabolism is altered. The work presented attempts to discern whether there is any causal link between the two factors.

METHODS

BLOOD

Experiments were performed using both fresh human blood and bank blood. Fresh blood was generally obtained from male volunteers, to minimise any possible effects of circulating hormone fluctuations, and was drawn into evacuated vessels containing Li-heparin anticoagulant (Becton Dickinson UK. Ltd., Cowley, Oxfordshire).

For experiments in which fresh blood was not used, blood was kindly supplied by the National Blood Transfusion Service, Regional Headquarters, Sheffield, via the Department of Haematology, Leicester Royal Infirmary. Blood was stored in acid-citrate-dextrose anticoagulant at $4^{o}C$ and was routinely used within two weeks of collection.

FLUX MEASUREMENTS

UNIDIRECTIONAL INFLUX DETERMINATIONS

Cells

Whole blood was centrifuged at $4^{o}C$ and plasma and buffy coat removed to leave the red blood cells. Red cells from fresh blood were subsequently washed at least twice in isotonic buffered saline, pH 7.4 at $4^{o}C$, and at least once in flux incubation medium. Red cells obtained from bank blood were generally washed in 100 mM NaCl, 10 mM Tris-MOPS (pH 7.4), to remove osmotically fragile cells, and also preincubated for 1-2 hours at $37^{o}C$ in an isotonic medium containing glucose (10mM), to restore intracellular ATP levels. Red cells were finally resuspended in medium at approximately 5% haematocrit for influx determinations, with reagents added as appropriate to the experiment.

Flux incubations were commenced by the addition of a small volume of pre-warmed radioactive tracer (^{3}H, ^{14}C, ^{86}Rb or ^{22}Na) to a final activity of 0.3 - 1.5 $\mu Ci\ ml^{-1}$. Fluxes were routinely measured in triplicate over 15-30 minute incubation periods, during which uptake of radioactivity remained linear with time.

^{22}Na uptake was measured in the presence of ouabain ($10^{-4}M$), and thus no correction for backflux was necessary (Brand & Whittam, 1984).

After the required interval, uptake was stopped by decanting each cell suspension into ice-cold microcentrifuge tubes, followed by rapid centrifugation (15 seconds, 11,600g), using an MSE Microcentaur centrifuge (Fisons Scientific Apparatus, Loughborough, UK.). It was important to sediment cells as quickly as possible because complete temperature equilibration, and hence effective termination of flux, may take up to three minutes (Young & Ellory, 1982). A sample of supernatant was saved and the remainder aspirated and discarded. The cell pellet was then rapidly washed thrice in 10 volumes of ice-cold isotonic buffered $MgCl_2$ (107 mM $MgCl_2$, 10 mM Tris-MOPS, pH 7.4 at 4°C).

Cell pellets containing ^{22}Na were counted without further treatment. Pellets containing primarily β-emitting radiation were haemolysed in 7 mM NH_4OH after which known volumes were deproteinised with 25% (v/v) of 15% (w/v) trichloroacetic acid. Precipitated protein was sedimented by centrifugation (5 minutes, 11,600g), and aliquots of the supernatant counted either after addition to a suitable scintillant (e.g. for ^{3}H and ^{14}C), or alone by Cerenkov emission (^{86}Rb). The scintillant used was Fisofluor I (Fisons Scientific Apparatus, Loughborough, UK.). Pellets containing γ-radiation were counted using a Packard "Autogamma" 5650 counter (Packard, Pangbourne, Berkshire, UK.). Samples emitting β-radiation were quantified using a Packard Model 2450 β-counter.

Samples of the original radioactive supernatant were treated in essentially the same way as cells, after appropriate dilution.

Data were corrected for chemical quenching where necessary. Quantification of fluxes was achieved by the measurement of oxyhaemoglobin optical densities of cell aliquots at 540nm on a Pye Unicam SPL-550 UV/VIS spectrophotometer (Pye Unicam Ltd., Cambridge, UK.).

Ghosts

Influx in ghosts was measured essentially as for cells, except that it was not possible to quantify data by Hb measurement. Instead, ghost pellets washed free of external radioactivity were diluted in a commercially available sterile isotonic solution (Isoton, Coulter Electronics) and counted on a model Fn Coulter Counter (Coulter Electronics, Luton, Bedfordshire). Results were expressed per 10^{10} ghosts. The Coulter Counter was attached to a mean cell volume computer (model MCV/HCT), which enabled further information to be obtained with regard to the size of the ghosts.

NET UPTAKE EXPERIMENTS

The net uptake of Na, K and NA by red cells was also measured. Cells were treated and suspended as for tracer uptake studies, but in the absence of isotope. Each incubation was commenced by the addition of washed cells to pre-warmed media and allowed to proceed for 2-3 hours at 37°C.

Na and K movements

Intracellular Na and K concentrations were measured by flame photometry using an internal Li^+ standard, after cells had been washed 4 times in ice-cold isotonic buffered $MgCl_2$, then haemolysed in 7mM NH_4OH.

NA movements

Intracellular NA concentration was measured by HPLC-ED, after cells had been washed 4 times in ice-cold isotonic buffered saline, haemolysed and deproteinised using 33% (v/v) of 1 M PCA. Analysis was performed on PCA extracts, which were frozen and stored at -70°C until assays could be carried out.

UNIDIRECTIONAL ^{22}Na EFFLUX MEASUREMENTS

These were by a modification of the method of Priestland and Whittam (1968). Washed red cells were suspended at about 50% haematocrit in flux medium, in the presence of approximately

5 μCi ml^{-1} ^{22}Na. The suspension was then incubated for 3 hours at 37°C. A second identical incubation was carried out simultaneously in the absence of isotope, to provide samples of cells for intracellular Na and K determinations. At the end of this loading period the ^{22}Na-containing cells were separated from the radioactive medium by centrifugation. These cells were then washed in ice-cold isotonic medium until extracellular radioactivity was effectively zero.

The flux incubation was started by adding ^{22}Na-loaded red cells, to a final haematocrit of about 4%, to pre-warmed vessels containing flux medium at 37°C. Effluxes were measured in triplicate in the presence and absence of plasma (33% v/v), ouabain ($10^{-4}M$) and bumetanide ($10^{-4}M$).

Samples of whole suspension were taken at the beginning and end of each flux incubation to allow determination of cell counts per minute.

As it is known that the rate of appearance of extracellular counts is not linear during the first 15 minutes of an incubation of this type (e.g. see Brand, 1981), samples of suspension for estimation of appearance of this extracellular radioactivity were taken at 10 minute intervals between 15 and 55 minutes after the start of the flux incubation. The increase in appearance of extracellular counts during this period was used to calculate the efflux rate constant. Each sample was centrifuged and 200 μl aliquots of supernatant taken for counting.

Calculation of ^{22}Na efflux

Let N_t^c = cell counts per minute at time t

and N_t^s = supernatant counts per minute at time t

$$N_t^c = N_o^c \quad e^{-kt}$$

It follows that: $$\frac{N_t^c}{N_o^c} = e^{-kt}$$

and, as $N_t^c = N_o^c - N_t^s$

therefore, $\frac{N_o^c - N_t^s}{N_o^c} = e^{-kt}$

or, $\ln [1 - (N_t^s / N_o^c)] = -kt$

where, k = sodium efflux rate constant.

Values of k were calculated, and an efflux rate constant determined by linear regression of k against time. Efflux rate constants were expressed in units of $hour^{-1}$. The absolute values of ^{22}Na efflux were calculated as the product of efflux rate constants and $[Na]_i$.

CHOICE OF NA ISOTOPE

Under most circumstances tritiated compounds are less stable than their ^{14}C-labelled counterparts. The possibility of artefactual isotope effects should therefore be considered, as even trace amounts of impurities or degradation products may give rise to spurious results. This is illustrated by the following example, described by Christensen (1975).

Christensen considered the case of a labelled substrate of 99% radiological purity, ordinarily an acceptable commercial standard. He also presupposed that the remaining 1% of isotope was of a form accumulated or fixed by cells more rapidly and completely than the substrate itself, and by an independent process. It is possible that such impurities may be generated through radioactive disintegration during storage of the preparation. Let it be assumed, for the purposes of calculation, that the experiment is performed under such conditions that during the brief period of observation, the transport mechanism brings the substrate to the same apparent concentration per unit weight within the cell as in the solution - i.e. to a distribution ratio of 1. The following calculations would therefore hold:

DENSITY OF CELL SUSPENSION (g 100ml^{-1})	RADIOISOTOPE TAKEN UP (% TOTAL)			
	Fixation of impurity	Transport of substrate	Total uptake	Apparent distribution ratio
0.01	1	0.01	1.01	101
0.1	1	0.1	1.1	11
1.0	1	1.0	2	2
10.0	1	10.0	11	1.1

The calculation clearly shows the erroneous conclusions which may be reached if radio-isotope purity is not of the highest standard. Only in the last case does the observed uptake represent predominantly the compound under study.

For the majority of experiments described in this thesis ^{3}H-NA was used as a tracer. Table 2 shows that care must be exercised if isotope is stored for prolonged periods of time under conditions against those recommended by the manufacturer. ^{3}H-NA uptake was apparently 25-30% elevated compared to NA influx measured with freshly purchased ^{14}C-NA, if the tritiated isotope was stored at -20°C. This increase presumably represents the accumulation of tritiated degradation products. Storage under liquid nitrogen, however, seemed to adequately protect ^{3}H-NA from decomposition, such that uptake of ^{3}H-NA (2-3 months old) and fresh ^{14}C-NA were indistinguishable.

CHOICE OF BUFFER

The adequacy of a particular biological buffer depends not only upon the range of pH values within which it is effective, but also upon the manner with which it interacts with components of the system under study. Amine buffers have been widely, and rather indiscriminately, used for many years in a variety of systems, in spite of the accumulated experimental evidence which suggests that some of them are far from biochemically inert. For example, Tris(hydoxymethyl)aminomethane, (Tris) has been shown, in

TABLE 2

EFFECTS OF RADIOISOTOPE DECAY ON APPARENT NA UPTAKE IN HUMAN RED CELLS AND GHOSTS

$[NA]_o$ (mM)	EXPERIMENT A		EXPERIMENT B	
	^3H uptake	^{14}C uptake	^3H uptake	^{14}C uptake
62.5	13.19	10.76	14.08	14.35
90	17.43	13.89	23.14	22.28
140	25.88	20.11	33.42	34.85

Units of flux:
Experiment A: μmol NA 10^{10}ghosts^{-1} 15 min^{-1}.

Experiment B: μmol NA ml cells^{-1} 15 min^{-1}.
All values represent the mean of three separate estimations.

EXPERIMENT A: ^3H-NA was stored for over two months at -20°C prior to use, against the recommendations of the manufacturer. This resulted in a significant increase in uptake (mean 25.6%, $p<0.0005$, paired t-test), presumably indicative of the uptake of tritiated degradation products. ^{14}C-NA was utilised immediately upon receipt.

EXPERIMENT B: ^3H-NA was stored under liquid nitrogen for up to two months prior to use. ^3H-NA uptake is indistinguishable from that measured with fresh ^{14}C-NA, presumably indicating that such conditions are suitable for the storage of the tritiated compound.

Uptake of ^3H-NA and ^{14}C-NA stored under liquid nitrogen were also comparable at lower concentrations of NA.

addition to other actions, to bind to the Na^+ site of the (Na + K)-ATPase (Rempeters & Schoner, 1983). HEPES and TES are also not biochemically inert (Roberts, Liron & Wong, 1985).

Accordingly, in a preliminary experiment, a series of buffers were examined in order to ascertain:

1)Their capacity to satisfactorily maintain a constant extracellular pH, and

2)Their relative effects, if any, on NA uptake.

Data are presented in Table 3.

The choice of buffer system did not appear to affect NA uptake, which was measured in most experiments in the presence of 10mM Tris-MOPS. The one exception to this rule was in experiments which necessitated the presence of plasma or HCO_3^- salts. In these cases, pH was maintained by gassing with an appropriate O_2/CO_2 mixture.

STABILITY OF NA IN AQUEOUS SOLUTION

NA is unstable in the presence of oxidising agents, and decays rapidly in aqueous solution at room temperature and physiological pH. Plasma may retard this effect at physiological NA concentrations, by virtue of the high-affinity low capacity binding properties of serum proteins (Powis, 1975; Danon & Sapira, 1972): see Table 4. However, the presence of plasma may also create difficulties for the maintenance of a stable medium pH, unless constant gassing is maintained (see previous section).

To prevent oxidation of NA a suitable reducing agent, L-ascorbic acid, was routinely added to experimental media. The efficiency of L-ascorbic acid was monitored both directly, by HPLC, and by its ability to prevent the appearance of red oxidation products, which may be measured spectrophotometrically at 485nm (Cantley, Ferguson & Kustin, 1978). Results are described in Table 5.

Consequently, experiments were carried out at one of two L-ascorbic acid concentrations. For kinetic studies in which [NA] ranged from 1 - 250mM, L-ascorbic acid was used at 5mM, whilst in

TABLE 3

EFFECTS OF DIFFERENT BUFFER SYSTEMS ON ^{3}H-NA UPTAKE BY HUMAN RED CELLS

BUFFER	NA UPTAKE	MEDIUM pH
10mM TRIS-MOPS	54.65	7.34
10mM HEPES-TRIS	54.92	7.37
10mM PIPES-TRIS	55.43	7.43
10mM POPSO-TRIS	51.32	7.37
10mM PIPES-NaOH	59.74	7.48
10mM POPSO-NaOH	49.31	7.39

Units of uptake: pmol NA ml cells^{-1} 15 min^{-1}.

Uptake was measured from media containing (mM): NaCl 145, KCl 5, glucose 10, L-ascorbic acid 0.1 and buffer, as described in the table, pH 7.4 at 37^{o}C.

NA was present at a final concentration of 350 nM.

After 15 minutes incubation, radioactive cell suspensions were sampled into ice cold tubes and treated as described in Methods.

Parallel incubations were also performed in the absence of isotope. These suspensions were also centrifuged after 15 minute incubations at 37^{o}C, and the cell pellet discarded. The supernatants were used to measure extracellular pH.

All buffers appeared to maintain medium pH close to 7.4 and there was no differential effect upon NA uptake.

TABLE 4

EFFECT OF PLASMA ON NA STABILITY DURING INCUBATION AT 37°C

BUFFERED SALINE			PLASMA		
Incubation time	[NA] (nM)	[NA] % original	Incubation time	[NA] (nM)	[NA] % original
0	8.35	100	0	13.40	100
30	2.64	31.6	10	14.33	106.9
60	0.91	10.9	40	14.14	105.5
180	0.54	6.5	50	13.78	102.8

[NA] was measured by HPLC-ED and values represent means of duplicate determinations.

Buffered saline comprised (mM): NaCl 150, Tris-MOPS 10, pH 7.4 at 37°C. NA was dissolved in buffered saline and diluted as required.

Incubations performed in plasma contained approximately 2% (v/v) buffered saline: the remainder was plasma.

The stabilising effect of plasma was observed to persist for periods of up to 3 hours, under the conditions described above, in two repeat experiments. Decay of adrenaline (approximately 1 nM) during this period was also negligible.

TABLE 5

THE EFFECTS OF L-ASCORBIC ACID ON NA STABILITY

1. Incubations without ascorbic acid

[NA] (mM)	ABSORBANCE CHANGE*			
	15 min	39 min	49 min	125 min
140	+0.051	+0.240	+0.369	+1.746
70	+0.024	+0.101	+0.163	+1.119
14	+0.007	+0.018	+0.029	+0.351

2. 15 min incubation in the presence of ascorbic acid

[ASCORBIC ACID]	[NA]	ABSORBANCE CHANGE*
10 μM	1 mM	+0.003
	10 μM	0.000
	10 nM	0.000
1 mM	140 mM	0.000
	70 mM	-0.001
	14 mM	-0.002

All incubations were performed in (mM): NaCl 150, Tris-MOPS 10, pH 7.4 at 37°C.

*Absorbance change represents the change in absorbance units, measured at 485nm, corresponding to the appearance of NA oxidation products, (Cantley, Ferguson & Kustin, 1978).

other experiments ([NA] < 1 mM), the compound was incorporated at a concentration of 0.1 mM.

CATECHOLAMINE ANALYSIS

Preparation of samples

Samples were prepared for HPLC by a modification of the method described by Anton and Sayre (1962), as detailed by Walker (1987).

Known volumes of thawed samples were suitably diluted, then added to aliquots of dihydroxybenzylamine (DHB, 15 pmol 100 μl^{-1} in 10^{-4}M PCA). DHB was incorporated into each assay as an internal standard to allow for variations in the efficiency of extraction. Recovery was usually in the range 60-70%. Ice cold deionised water was added to this mixture to a final volume of about 2 ml, and the pH adjusted to 8.0 (± 0.1) using 50 mM K_2-EDTA, 1 M Tris-HCl, pH 8.6. An excess (30-40 mg) of acid-washed alumina (Al_2O_3) was then added to each vial, which was then capped, inverted and constantly mixed for 15 minutes to allow adsorption of catecholamines.

After the mixing had stopped, the Al_2O_3 quickly settled and the supernatant was aspirated and discarded. The Al_2O_3 was then rapidly washed thrice in excess ice cold deionised water. After the final wash, catecholamines were eluted by regularly agitating the alumina for 15 minutes in the presence of 0.25 ml PCA (10^{-4}M). The PCA extracts were kept on ice prior to injection on to the HPLC column.

Chromatography

Aliquots (100 µl) of PCA extracts were injected on to an "Ultrasphere" reverse phase ion pair column (25cm length, 4.6mm internal diameter; Anachem Ltd., Luton, UK.). Approximate retention times, at a flow rate of 0.9 ml min^{-1}, were 8 minutes (NA), 11 minutes (adrenaline) and 16 minutes (DHB). Catecholamines were quantified by the current required to oxidise them at a glassy carbon working electrode, (BAS inc., West Lafayette, U.S.A.), as compared to standards containing precisely known amounts of amine.

The mobile phase comprised (mM): Na acetate (80), NaOH (60), K_2-EDTA (1), 1-octanesulphonic acid (0.5) and citrate-acetate (30), pH 5.2, in 10% (v/v) HPLC grade methanol, (Fisons Scientific Apparatus, Loughborough, UK.). The mobile phase was frequently degassed and remade at regular intervals, and was circulated through the HPLC system by a single piston pump with a 5 ml head (Gilson 302 metering pump, Anachem Ltd., Luton, UK.).

GHOSTS

INTRODUCTION

Red blood cell ghosts are produced essentially by three steps: hypotonic haemolysis, followed by reversal to isosmotic conditions, which is in turn followed by a resealing period. Haemolysis enables the experimenter to substantially remove undesired intracellular components by dilution into an excess of hypotonic medium. At the reversal stage, a variety of specific ions, metabolic inhibitors or biochemical messengers may be incorporated within the leaky membrane sacs, whilst resealing restores the permeability of the ghost near to that of the original cell, thus effectively trapping those compounds added at reversal.

The ghost population formed after hypotonic haemolysis of red blood cells is inhomogeneous and may be conveniently split into 3 categories: types I, II and III (as described by Hoffman, 1962).

Type I ghosts reseal immediately after haemolysis and for this reason will not incorporate substances added at any later stage. Furthermore, when isotonicity is restored (reversal), these ghosts shrink, thereby raising the concentration of the intracellular constituents in an uncontrolled fashion. Type II ghosts only reseal after reversal and incubation at 37°C, while type III ghosts never regain the low cation permeability of the parent red cells. It is therefore the type II ghosts which have proven of most use to investigators in the field of membrane transport, and the preparative protocols which follow were specifically designed to increase the yield of these ghosts at the

expense of types I and III.

Control of two crucial factors facilitates a high percentage yield of type II ghosts, and these are temperature and pH. Haemolysis at 0°C followed by resealing at 37°C maximises the yield of type II ghosts compared to type I (Bodemann & Passow, 1972), while increasing the acidity of the haemolysis medium favours the production of type II at the expense of type III (Lepke & Passow, 1972). The optimum pH for haemolysis is 6.

Ghosts were prepared by two methods:

Method One

This method is based upon that described by Richards and Eisner (1982). Red blood cells were washed by repeated suspension followed by centrifugation (2000g, 10 min, 4°C; MSE Chilspin Centrifuge, Fisons Scientific Apparatus, Loughborough, UK.) in ice cold buffered isotonic saline (150mM NaCl, 10mM Tris-MOPS pH 7.4 at 4°C). After the final wash, the cells were loosely packed and the supernatant removed. Packed cells were drawn into a syringe and haemolysed by addition to approximately 40 volumes of constantly stirred ice cold hypotonic medium which comprised (mM): NaCl (2), KCl (5), $MgCl_2$ (4), Tris-MOPS (8), pH 6.5. Vigorous stirring was maintained for 5 minutes, after which the ghost membranes were collected by centrifugation (18,000g, 15 min, 4°C; Sorvall RC5B, DuPont (UK.) Ltd., Stevenage, Hertfordshire). A small volume of 3 M salt solution was then added to each aliquot of membranes, which were then left on ice for two minutes. The increased tonicity causes the leaky ghosts to shrink, thereby increasing the concentration of trapped haemoglobin and favouring its efflux (Bjerrum, 1979).

The haemolysis procedure was subsequently repeated twice, in order to further deplete the red cell membranes of the small amounts of residual haemoglobin. After the final haemolysis, the pH of the suspension was adjusted to 7.2 such that the intra-ghost pH would be similar in magnitude to that measured in cells. The suspension was then centrifuged (18,000g, 10 min, 4°C), and after recovery of the ghosts, a small amount of 3 M salt solution, containing 3 mM tropolone, an inhibitor of catechol-O-

methyltransferase activity (Belleau & Burba, 1963), was added to restore tonicity. The isotonic ghosts were then resealed by gentle agitation for 60 minutes at 37°C. After resealing, the ghosts were suspended at 50% haematocrit in isotonic buffered saline prior to use. Results showed that ghosts could be stored at 50% haematocrit at 4°C for up to 24 hours without affecting their transport activity.

Method two

This method is based upon that described by Bodemann and Passow (1972). Washed, loosely packed red cells were pipetted into 15 volumes of ice cold 4 mM $MgSO_4$ under constant stirring, which was maintained for a further 5 minutes. Reversal was then achieved by the addition of a known quantity of a 3M salt solution. After reversal, mixing was maintained for a further 5 minutes. The suspension was then incubated at 37°C for 30-60 minutes, prior to centrifugation (18,000g, 10 min, 4°C). The resealed ghosts were washed 2-3 times in isotonic buffered saline, to eliminate residual extracellular haemoglobin, and then layered on top of a cushion of 43% (w/v) sucrose, containing 25 mM NaCl, 25 mM Tris-HCl pH 7.2, for ultracentrifugation (75,000g, 60 min, 4°C; Centrikon ultracentrifuge T2055, MSE, Crawley, Sussex, UK.). After one hour two bands of ghosts were clearly apparent, that at the base of each tube comprising types I and III, with a band of type II ghosts floating at the top of the sucrose cushion (see Bodemann & Passow, 1972). The type II ghosts were aspirated away and washed several times in isotonic buffered saline prior to storage or flux measurements.

Centrifugation of ghosts, prepared by method one, over a sucrose cushion produced just one large band near the top of the tube, confirming that the protocol is selective for type II ghosts.

CHARACTERISATION OF GHOSTS

The ghosts utilised in the majority of experiments in this thesis were prepared by method one (adapted from Richards & Eisner, 1982). The following section, therefore, deals almost exclusively with this preparation, unless otherwise stated in the text.

Sidedness of ghosts

The orientation of ghosts was assessed using two marker enzymes:

1. Acetylcholinesterase (AChE): outer surface marker, and
2. Glyceraldehyde-3-phosphate dehydrogenase (G3PD): inner surface marker.

For each enzyme the appearance of reaction product was followed spectrophotometrically at the appropriate wavelength. AChE activity was estimated by a modification of the method described by Ellman, Courtney, Andres and Featherstone (1961). G3PD activity was measured by the method of Cori, Slein and Cori (1948).

Two assays per sample per enzyme were performed, namely:

1. A control incubation, using untreated ghosts, and
2. An incubation in the presence of 0.1% (v/v) Triton X100.

Each assay was performed in duplicate. In the presence of Triton X100 the ghosts become permeabilised such that substrate is allowed free access to both sides of the membrane. Hence, an estimate of "total" (condition 2) and "outside only" (condition 1) enzyme activity may be made. The results of these assays, which were routinely carried out for initial ghost preparations, are described in Table 6.

As can be seen, a comparison of the data with values from the literature indicates that that the ghosts obtained represent a fairly typical right-side-out population, and the small differences which occur may be easily explained. First, it could be argued that permeabilising ghosts would not be expected to reduce any measured enzyme activity: this should either remain unchanged, or rise as additional reactive moieties are exposed.

TABLE 6

SIDEDNESS OF GHOST PREPARATIONS

RED CELL OUTER MEMBRANE MARKER: ACETYLCHOLINESTERASE (n=34)

	Experimental observations	Literature values*
% OF SITES ACCESSIBLE TO SUBSTRATE (Control)	100%	100%
% OF SITES ACCESSIBLE TO SUBSTRATE (Triton X100 present)	92.2 ± 1.8%	95.6%

RED CELL INNER MEMBRANE MARKER: GLYCERALDEHYDE-3-P DEHYDROGENASE (n=31)

	Experimental observations	Literature values*
% OF SITES ACCESSIBLE TO SUBSTRATE (Control)	16.9 ± 0.8%	10.1%
% OF SITES ACCESSIBLE TO SUBSTRATE (Triton X100 present)	100%	100%

All data are mean ± standard error of mean.

Acetylcholinesterase activity was followed as an increase in absorbance at 412nm and room temperature, caused by the liberation of free thiol groups (Ellman, Courtney, Andres & Featherstone, 1961).

Glyceraldehyde-3-phosphate dehydrogenase activity was followed as an increase in absorbance at 340nm and room temperature, following the reduction of NAD (Cori, Slein & Cori, 1948).

*Literature values were obtained from Steck and Kant (1974).

Triton X100, however, reduces AChE activity by between 5-8%, in both literature sources and results obtained here. Such small reductions may represent the destruction of small areas of membrane by Triton X100 such that a minor number of AChE receptor sites are solubilised and lost. The use of a less severe detergent, e.g. saponin, may have been preferable. Alternatively, Triton X100 may slightly inhibit the enzyme reaction being followed. The result would also be consistent with Triton X100 causing a small percentage of ghosts to evert, assuming the inside-out orientation, although this would seem unlikely to occur.

In the case of G3PD, only 16.9 ± 0.8% of the maximal activity was observed in the absence of Triton X100. This figure is about 7% greater than the data quoted in the literature (Steck & Kant, 1974), which may imply that the ghost preparations utilised in these studies contained larger than usual amounts of fragmented membrane. This may be a consequence of the protocol which demands repeated haemolysis and washing, rather than a single, potentially less destructive haemolysis step. Since such membrane fragments do not appreciably bind ^{3}H-NA (see Chapter 1), and may be discriminated against on grounds of size when coulter counting, they should not cause experimental inaccuracies to arise during influx measurements. It seems possible however, that a small percentage of ghosts may reseal in the inside-out configuration.

Haemoglobin content

Samples of intact red cells and ghosts were taken, packed and haemolysed by osmotic shock in a known volume of dilute ammonia solution. Internal haemoglobin was then measured spectrophotometrically as oxyhaemoglobin at 540nm. The percentage of haemoglobin retained by the ghosts was then expressed as a percentage of the original red cell haemoglobin, allowing for the different numbers of ghosts and cells packed per unit volume (see Ghost volume, below). In 18 experiments, the mean residual haemoglobin per ghost was 3.93 ± 0.21% (range 2.49 - 5.46%).

Although noticeable quantities of haemoglobin were lost upon each haemolysis step, haemolysis was never repeated more than a third time during ghost preparation, in an effort to maintain membrane integrity and to reduce the tendency towards fragmentation.

Ghost volume

The mean volume of red cell ghosts was 63 ± 2 fl (range 49 - 77 fl), as compared to 94 ± 3 fl (range 83 - 121 fl) for intact red cells (n=12 paired observations). In general, larger ghosts were obtained from larger cells (see Figure 2). The original cell volume was roughly proportional to the length of storage of blood cells, so that larger ghosts were normally derived from blood stored in the bank for the greater periods of time. (Blood was stored for periods of up to 2 weeks). It is noted that the mean ghost volumes are considerably less than those reported by various authors using one step haemolysis protocols. For instance, Funder and Wieth (1976) produced ghosts with a mean volume of 94 ± 3 fl (range 88 - 110 fl), a range of values closely similar to those obtained here for intact red cells. It is also noted, however, that Bjerrum (1979), a major proponent of the repeated haemolysis ghost preparation method also obtained shrunken ghosts with a mean volume of 62 ± 4 fl (range 55 - 87 fl). An important determinant of ghost volume might therefore appear to be the number of times that haemolysis is undergone. The homogeneity of ghosts prepared by this method, as compared to red cells, is illustrated in Figure 3.

As would be expected, since ghosts are smaller than cells, the former may be more tightly packed than the latter. The mean number of cells per millilitre was estimated, by Coulter counting, as 1.01 ± 0.03 X 10^{10}, compared to value of 1.19 ± 0.02 X 10^{10} for ghosts.

Yield and permeability of ghosts

The method used to prepare ghosts was chosen so as to maximise the yield of cation-tight (type II) ghosts at the expense of types I and III. It was therefore considered important to check

whether or not this objective was achieved. A rough estimate of the yield of type II ghosts was achieved using the following rationale:

$$\frac{[Na] \text{ per unit volume of ghosts}}{[Na] \text{ per unit volume of resealing suspension}} \times 100 = \% \text{ type II ghosts}$$

Ghost $[Na]_i$ was measured by flame photometry after repeated washing in a Na-free solution.

In two preliminary experiments, the mean apparent yield of type II ghosts, calculated from the above equation was 81.6% (range 78.1 - 85.1%), presumably indicating a low level of contamination by type III ghosts which are not tight to cations, and would therefore lose their Na_i during the washing procedure. It was assumed that type I ghosts were satisfactorily manually eliminated as a dense pellet at an earlier stage of the preparation.

A further two experiments of a similar nature were also undertaken to investigate the effects of tropolone upon ghost permeability. When present in the haemolysing medium, tropolone caused some reduction in the ability of ghosts to retain Na_i such that the apparent yield of type II ghosts fell to 54.9% (range 51.0 - 58.8%). Although these latter values compare favourably with the 57% type II yields obtained by Bodemann and Passow (1972), it was noted that these authors felt it necessary to further purify their preparation using a density gradient centrifugation technique. In order to try and obviate this step it was considered preferable to incorporate tropolone at the onset of reversal rather than at the instant of haemolysis. When this order was followed, the presence of tropolone did not appear to render the ghost population more leaky to cations, as assessed by ^{86}Rb uptake.

CONDITION	^{86}Rb UPTAKE (μmol ml ghosts^{-1} h^{-1})
Control	2.03
1.5mM tropolone	1.94
6.0mM tropolone	1.99

Figure 2

The relationship between cell size and ghost size.

The volumes of samples of washed red cells were estimated using a model Fn Coulter Counter plus MCV/HCT attachment (Coulter Electronics, Luton, Bedfordshire). Ghosts were then prepared from the same red cell suspensions, using the method described Richards and Eisner (1982), see text.
The volume of these ghosts was subsequently measured. The plotted line was calculated by linear regression analysis of the data ($r=0.82$, $n=11$).

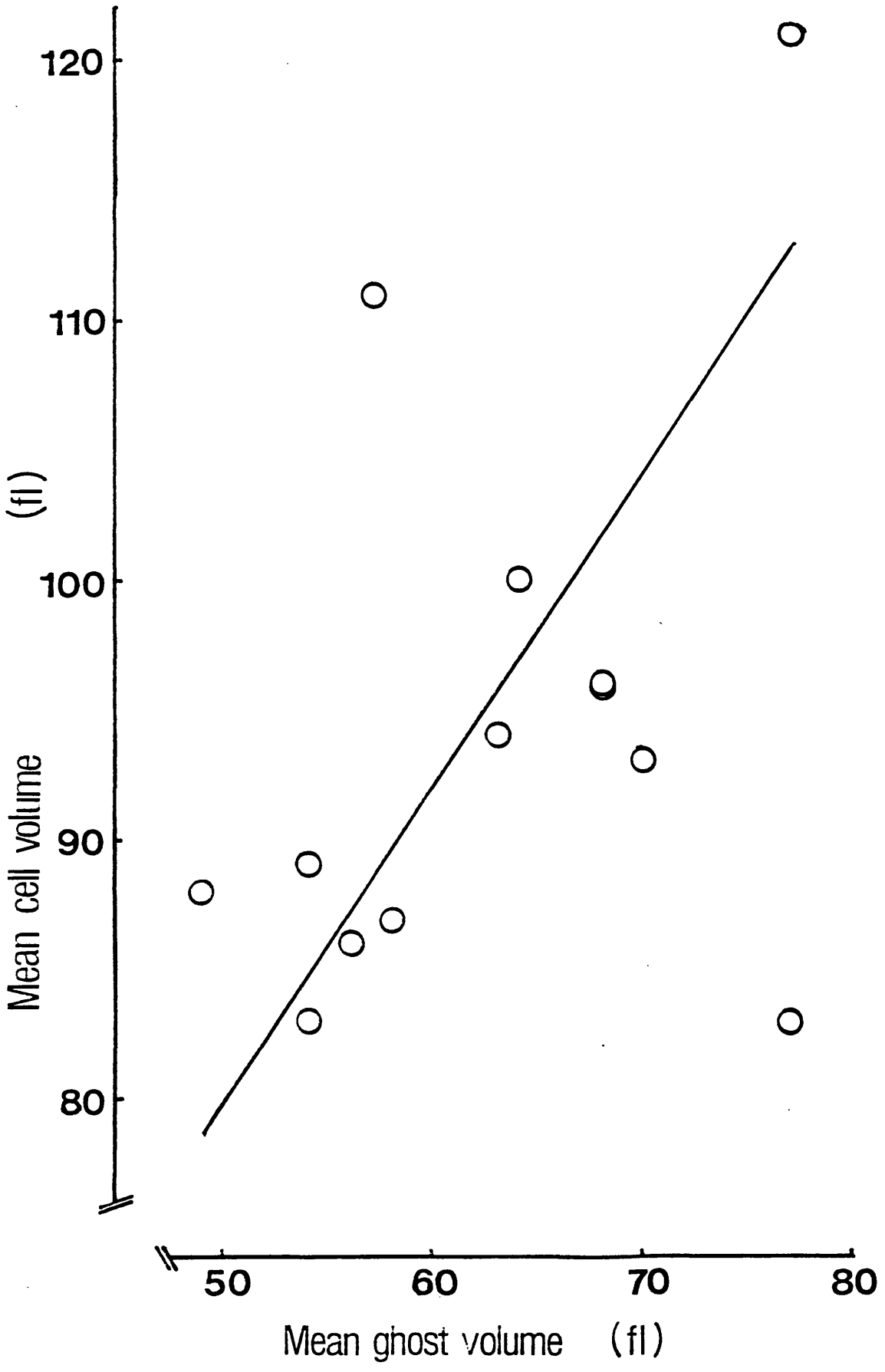
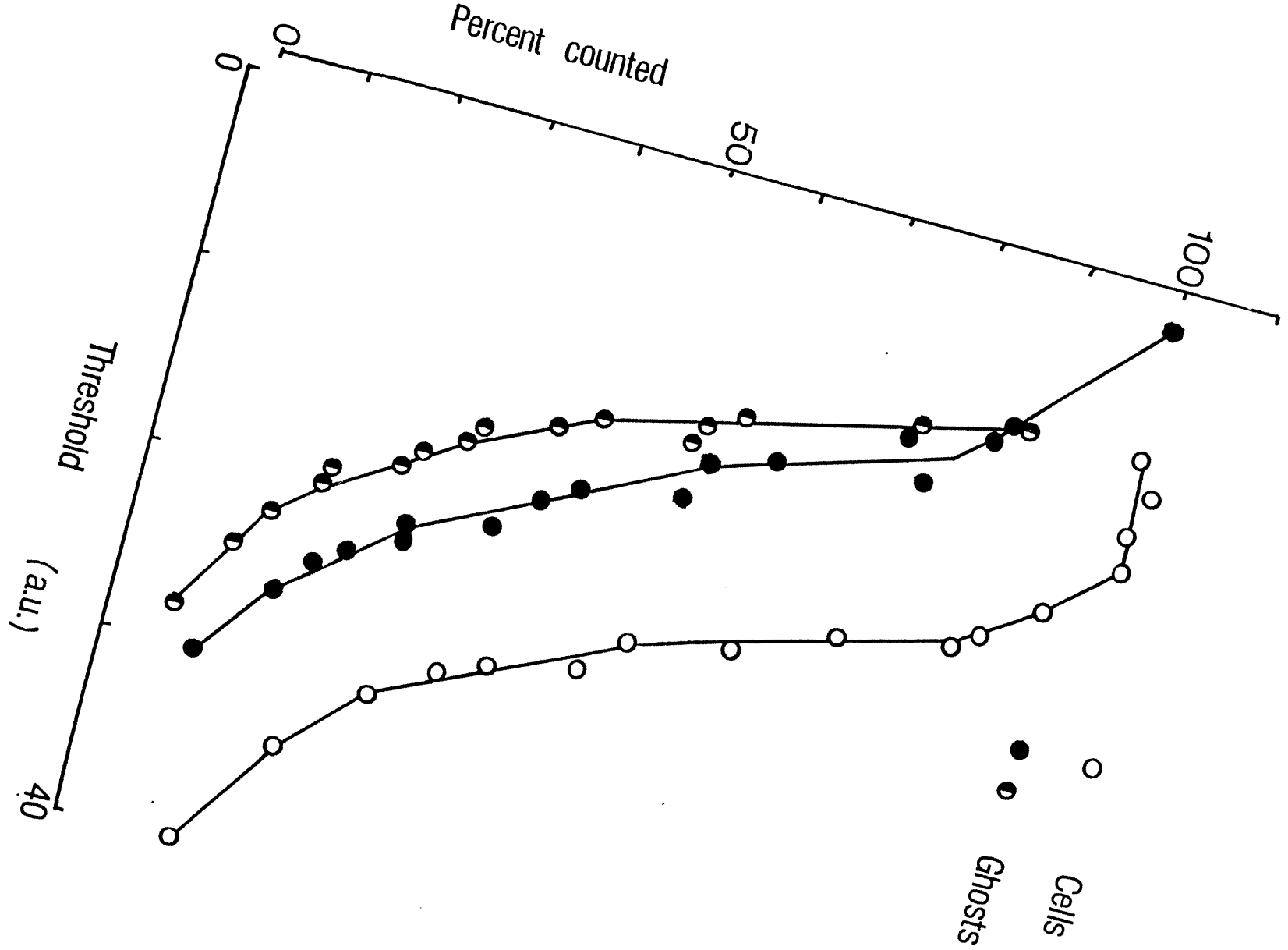

120
110
100
90
80
(fl)
Mean cell volume
50
60
70
80
Mean ghost volume (fl)

Figure 3

The size distribution of red cells and ghosts

This figure illustrates the heterogeneity of populations of intact red cells (open symbols) and ghosts prepared by the methods described by Richards and Eisner (1982) (closed symbols), and Bodemann and Passow (1972) (half-open symbols). The data was obtained using a model Fn Coulter Counter. The threshold control was initially set close to zero and a reading taken. This reading was assumed to represent the total number of cells or ghosts present in the suspension. Further readings were then made at higher threshold values (expressed in arbitrary units). By increasing the threshold, the smaller particles are gradually eliminated from the counted sample, such that the total number of particles recorded falls. Each value was plotted as a percentage of the total number of particles originally noted. The similar slopes of the three lines indicate that populations of cells and ghosts are heterogeneous, with respect to size, to approximately the same extent. The shift to the left of the two plots obtained from ghosts indicates, as previously described, (Figure 2), that mean ghost volume is somewhat less than mean cell volume. Additionally, the rapid decline in the numbers of ghosts measured between threshold values of 0 and 10 may represent the presence of membrane fragments. The possibility of such debris existing, and the implications of this for transport studies, are discussed elsewhere in the text.


0
Percent counted
50
100
0
Threshold
(a.u.)
40
Ghosts
Cells

Furthermore, as previously stated, when ghosts prepared by this method were spun over a sucrose density gradient, following the methodology of Bodemann and Passow (1972), the whole preparation was observed to remain in the band favoured by type II. The evidence would therefore tend to be consistent with the fact that the ghosts produced are as tight as possible to cations. The crucial question to pose, however, concerns the permeability of the ghosts to NA. This is considered in detail in Chapter 1.

CATION LOADING OF RED CELLS

Introduction

For experiments in which it is desired to measure fluxes in cells of varying internal cation composition, and where it is also not convenient to prepare ghosts, the polyene antibiotic nystatin is of considerable use. Nystatin interacts reversibly with cholesterol containing membranes to form pores of sufficient size to enable monovalent, but not divalent, ions to pass through (e.g. Andreoli & Monahan, 1968). Hence, under suitable conditions, cells suspended in a high-Na medium containing nystatin equilibrate to leave cells with a high internal Na concentration, at the expense of intracellular K.

The major advantage of treating red cells with nystatin, as opposed to other cation loading procedures, for example the PCMBS protocol of Garrahan and Rega (1967), is that it is a very rapid manoeuvre. The half time for K-Li exchange, for example, is less than one minute under the conditions to be described (Cass & Dalmark, 1973), and haemolysis is negligible.

Nystatin treatment of red blood cells

The following method is based upon that outlined by Cavieres (1982). Fresh human red cells were washed twice and loosely packed in a loading medium which contained (mM): NaCl + KCl (150), Na_2HPO_4 (0.5), pH 6.7 at room temperature, and sucrose (27). Sucrose was added to the medium to inhibit the colloid osmotic swelling of the cells while they were leaky to monovalent ions. Nystatin (5mg ml^{-1}) was dissolved in methanol, and the solution

cleared by centrifugation. This methanolic solution was then diluted one hundred fold in loading medium, to give a final ionophore concentration of 50 $\mu g\ ml^{-1}$. Cells were then added to approximately 4% haematocrit and allowed to equilibrate for 30 minutes at room temperature in the dark. The suspension was regularly gently mixed, by inversion, throughout this period.

The equilibrated suspension was then centrifuged and washed five times in 30 volumes of nystatin-free loading medium. A further four washes were subsequently performed using flux incubation medium, prior to experimental manipulations and characterisation. During these latter washes pH was gradually restored to 7.4.

Characterisation of nystatin treated cells

Intracellular electrolyte concentration

It proved possible to produce cells which were essentially Na- or K-free, as desired. Typically, equilibration of cells at 4% haematocrit with 50 $\mu g\ ml^{-1}$ nystatin in 150mM XCl yielded a final $[X]_i$ of about 110 mmol l cells^{-1}(X = Na or K). The data is shown in Table 7.

The fact that the sum total of intracellular electrolytes is greater in both sets of treated, as compared to fresh, cells, implies that the treated cells showed some increase in volume. Assuming negligible haemolysis occurred during nystatin treatment (no extracellular haemoglobin could be observed during the course of any experiment), this inference is also confirmed by the packed cell optical densities which were all reduced after exposure of cells to ionophore. A reduced packed cell optical density measurement, under conditions of negligible haemoglobin loss, represents haemoglobin "dilution" by increased cell water. Steps were taken to limit cell swelling by varying the sucrose concentration in the loading medium so as to more precisely balance the colloid osmotic pressure exerted by the intracellular constituents. Extra washes with nystatin-free loading medium were also performed in case residual ionophore was causing cell volume perturbation, but this course of action merely led to increased

cell fragility (see next section). Slightly swollen cells were therefore used for the flux determinations.

Fragility of nystatin treated cells

In a series of preliminary experiments, fresh and nystatin treated cells were exposed to progressively larger osmotic shocks in order to determine their fragility. The results are illustrated in Figure 4.

It can be seen that the nystatin treated cells are markedly more prone to hypotonic haemolysis at any given $[NaCl]_o$ than the fresh population, and that this increased fragility is at least in part a function of the number of washes each batch received (up to a maximum of 12 in some cases). The fresh cells were not exposed to nystatin and were not washed in total more than 4 times - during the preliminary stages of the experiment. Another factor which may contribute to elevated fragility in nystatin treated cells is the unphysiological intracellular ion composition. Brand (1981) also observed increased fragility of red cells, after being treated with PCMBS, when one cation (e.g. Na) was raised to high intracellular levels at the expense of another (e.g. K).

The nystatin treated cells were, however, apparently viable over the periods of time and under the osmotic conditions in which the experiments were performed.

Cell metabolism

It was important to check that fresh cells treated with nystatin remained metabolically active. This was investigated by studying the ouabain-sensitive extrusion of Na and gain of K (which requires ATP) by "high Na" cells. To this end net changes in ion concentration were measured by flame photometry after cells had been incubated for one hour at 37°C in a medium containing (mM): choline Cl (145), KCl (5), NaCl(1) (to enable ouabain binding to proceed).

In three experiments, cells with a mean initial $[Na]_i$ of 97.0 ± 4.0 mmol l^{-1} lost between 3.4 and 12.4 mmol Na 1 $cells^{-1}$ $hour^{-1}$ (between 3.5 and 12.8% of their initial content). This data

TABLE 7

CHARACTERISATION OF NYSTATIN TREATED RED CELLS

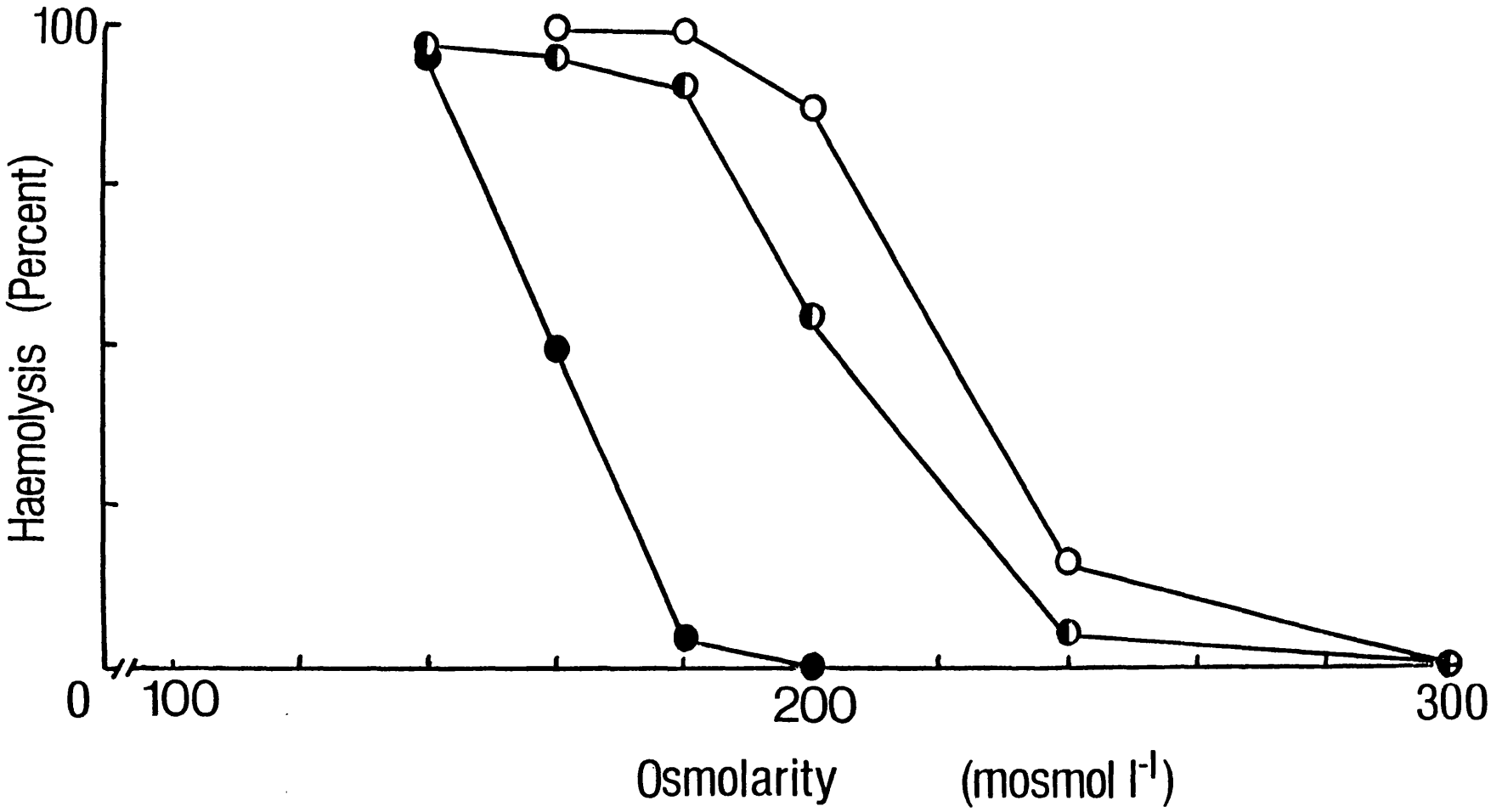
	FRESH CELLS				NYSTATIN TREATED CELLS							
					"High Na" cells				"High K" cells			
	Na_i	K_i	Na_i+K_i	PC OD	Na_i	K_i	Na_i+K_i	PC OD	Na_i	K_i	Na_i+K_i	PC OD
1	6.23	92.1	98.3	295	112.4	1.5	113.9	266	1.4	104.4	105.8	257
2	5.57	101.7	107.3	299	112.7	0.5	113.2	234	1.5	102.3	103.8	260
3	6.23	94.3	100.5	277	109.3	0.6	109.9	246	1.4	105.8	107.2	253
4	7.07	94.6	101.7	303	113.4	0.6	114.0	238	5.5	97.3	102.8	274
5	7.72	94.6	102.3	314	136.9	0.8	137.7	308				
6	10.15	90.0	100.2	305	105.0	1.4	106.4	247				
Mean	7.16	94.6	101.7	299	115.0	0.9	115.9	257	2.5	102.5	104.9	261
(SEM)	(0.67)	(1.6)	(1.3)	(5)	(4.6)	(0.2)	(4.5)	(11)	(1.0)	(1.9)	(1.0)	(5)

Nystatin treated red cells were prepared as described in text. For "high Na" cells, loading medium [NaCl] = 150 mM. For "high K" cells, loading medium [KCl] = 150 mM.
All electrolyte values are means of triplicate determinations and are expressed in units of mmol l cells^{-1}.
Packed cell optical densities (PC OD), are means of duplicate spectrophotometric estimations of oxyhaemoglobin, following suitable dilutions in 7 mM NH_4OH, at 540 nm.

Figure 4

The effect of nystatin on red cell fragility

Red cells were suspended at approximately 15% haematocrit in isotonic buffered saline. Cells were either freshly washed and untreated (closed symbols, n=3), treated with nystatin as described in text (half-open symbols, n=2), or treated with nystatin and washed an extra three times in nystatin free media (open symbols, n=2). Aliquots (100 µl) of these suspensions were taken and added to test tubes containing 9.9 ml of NaCl solutions of varying osmolarity (140-300 mosmoles litre^{-1}). The tubes were vigorously mixed, then centrifuged to sediment red cells and membrane fragments. The optical densities of the resultant supernatants were then measured at 540nm and plotted as a percentage of a standard (100% haemolysis) which was prepared from 100 µl cell suspension haemolysed in 9.9 ml of 7 mM NH_4OH.


100
Haemolysis (Percent)
0
100
200
300
Osmolarity (mosmol l-1)

is consistent with that of Cavieres (1982), who quotes that cells were loaded with 80 mmol Na l^{-1}, of which approximately 10% (6.4 mmol l cells^{-1}) was lost over the same period of time. In high Na cells, changes in $[K]_i$ were smaller and hence more difficult to accurately measure, but a mean increase of 1.68 ± 1.13 mmol K l cells^{-1} hour^{-1} was recorded. The original mean $[K]_i$ was 7.45 ± 3.04 mmol l cells^{-1}.

Of these changes in intracellular cation concentration, certain proportions were mediated via the Na pump - in the presence of ouabain (10^{-4}M) both Na loss and K gain were reduced. The mean ouabain-sensitive Na extrusion was 3.03 ± 0.33 mmol l cells^{-1} hour^{-1}, while the mean ouabain-sensitive K uptake was 2.46 ± 0.23 mmol l cells^{-1} hour^{-1} (see Table 8).

It is therefore apparent that nystatin treated cells retain the ability to actively transport Na and K. Na pump stoichiometry is also maintained close to the expected value of 3Na : 2K (for example see Kaplan, 1985).

Passive permeability

The passive permeability of nystatin treated cells to cations was estimated by measuring ^{22}Na influx in the presence of ouabain (10^{-4}M) and bumetanide (10^{-4}M). ^{22}Na uptake was measured in both "high Na" (n=8) and "high K" (n=4) cells, suspended in a buffered medium containing 145 mM NaCl and 5 mM KCl.

Mean ^{22}Na uptake into "high Na" cells was 11.92 ± 2.75 mmol l cells^{-1} hour^{-1} (range 2.52 - 25.15 mmol l cells^{-1} hour^{-1}), as compared to 10.28 ± 2.94 mmol l cells^{-1} hour^{-1} in "high K" cells (range 6.17 - 18.69 mmol l cells^{-1} hour^{-1}). Therefore, a large downhill electrochemical Na gradient did not give rise to an increase in ^{22}Na influx. The likely explanation for this observation is the leakiness of the cells, even after the end of the washing process. The mean results for ^{22}Na uptake quoted above are approximately 5 times the normally expected values for human red cells (see, for example, Brand & Whittam, 1984).

TABLE 8

NET TRANSPORT OF Na AND K BY NYSTATIN TREATED RED CELLS

	$[Na]_i$ (mmol 1 cells^{-1})				$[K]_i$ (mmol 1 cells^{-1})			
	t_0	t_{60}	t_{60} + ouabain (10^{-4}M)	ouabain-sensitive movement	t_0	t_{60}	t_{60} + ouabain (10^{-4}M)	ouabain-sensitive movement
1	93.71	81.35	83.79	-2.44	11.01	10.44	8.32	+2.12
2	92.43	89.02	92.59	-3.57	9.94	12.60	9.70	+2.90
3	104.96	96.71	99.79	-3.08	1.40	4.35	1.98	+2.37
Mean	97.03	89.03	92.06	-3.03	7.45	9.13	6.67	+2.46
(SEM)	(3.98)	(4.43)	(4.63)	(0.03)	(3.04)	(2.47)	(2.38)	(0.23)

All values are means of triplicate estimations.
Incubations were performed at 37°C in medium containing (mM): choline chloride 145, KCl 5, NaCl 1, Tris-MOPS 10, pH 7.4.
- values of net movement represent net efflux, + values net influx.

COLD PRESSOR TESTS AND NA INFUSION PROTOCOLS

Male volunteers were recruited with the approval of the local ethical committee and gave signed consent prior to the start of each experiment.

Cold pressor tests

Subjects were asked to assume a supine position for the duration of the cold pressor test (CPT), during which blood pressure was measured at regular intervals. Prior to CPT an initial peripheral venous blood sample was obtained for plasma catecholamine estimation, and two further samples (A and B) were later taken via an indwelling cannula. Samples A and B were used for plasma catecholamine determination, intracellular Na and K estimation, and ^{22}Na efflux measurement by the method previously described.

Sample A (pre-CPT) was taken approximately 20 minutes after the initial sample, once the subject's blood pressure had reached a stable level. After a further 20 minutes, the subject was instructed to immerse the hand of the free arm in an ice-water mixture, and blood sample B (post-CPT) was obtained at the height of the consequent blood pressure elevation, which normally occurred within one minute.

NA infusions

As for CPT, subjects remained supine throughout the duration of NA infusions which were performed in the presence of a medically qualified individual. Volunteers were cannulated and infused with 0.9% (w/v) saline (25 drops/minute) during an initial period of 20-60 minutes to allow the blood pressure to reach a stable level. Shortly before the end of this period, an initial blood sample was taken for plasma catecholamine determination, intracellular electrolyte estimation and ^{22}Na efflux measurements, as for CPT. Each subject was then infused with NA (50 ng kg body weight^{-1} minute^{-1}) for a period of one hour. Fifty minutes after the start of this infusion a second blood sample was collected and also used for catecholamine, electrolyte and Na transport

measurements.

Subjects were subsequently infused for a further hour with an increased concentration of NA (100 ng kg body weight^{-1} minute^{-1}), or recalled to receive the higher dose at a later date. A blood sample was also taken towards the end of this period to measure the stated parameters.

Treatment of blood samples

Blood samples from CPT and NA infusions were treated in an identical manner. Blood was collected into Li-heparin tubes, and placed on ice. Samples were centrifuged within five minutes of collection, and a plasma sample removed for catecholamine determination. Samples were stored at -70^{o}C if determinations could not be performed immediately.

Red blood cells were then prepared for flux measurements as previously described.

MATERIALS

Radioactive isotopes

Radioactive isotopes were purchased from both Amersham International plc., Amersham, Buckinghamshire, UK and New England Nuclear, Boston, Mass., U.S.A. ^{3}H-NA was obtained as the hydrochloride, while ^{14}C-NA was obtained as NA-d-bitartrate. Both were supplied in aqueous solution, sealed under nitrogen. ^{22}Na was obtained as a carrier free aqueous solution. ^{14}C-guanethidine was a kind gift from CIBA-Geigy Ltd., Basle, Switzerland.

Chemicals

All chemicals were of Analar grade where possible, and unless otherwise stated were purchased from The Sigma Chemical Company, Poole, Dorset, UK. Exceptions included methyl sulphate salts (Kodak Ltd., Liverpool, UK) and acid-washed alumina (BAS, Anachem Ltd., Luton, UK). Scintillation fluid was obtained from Fisons plc., Loughborough, Leicestershire, UK. Metaraminol tartrate was a gift from Merck Sharp & Dohme, Hoddesdon, Hertfordshire, UK. and bumetanide was kindly supplied by Leo Laboratories, Princes Risborough, Buckinghamshire, UK.

CHAPTER 1

TRANSPORT OF NA BY THE RED CELL MEMBRANE

INTRODUCTION

As previously described, there exists a considerable literature to suggest that catecholamines are accumulated by human red blood cells. Amines are also known to be taken up by rabbit and rat red cells. For example, dopamine enters human red cells and binds avidly to haemoglobin (Bryon and Bischoff, 1970). L-DOPA also rapidly penetrates red cells, wherein degradation may occur. Degradation was shown to proceed to such an extent that when rats were injected with L-DOPA, red cells attained more DOPA per unit weight than any other tissue in the body (Juang, Reches & Fahn, 1983). Adrenaline has also been shown to enter red cells (for example, see Roston, 1966). The particular interest of this thesis is, however, the handling of NA by the human red cell.

It is well known that catecholamines interact at the membrane level with red cells, which have been shown to possess β-receptors (Rasmussen, Lake & Allen, 1975). These authors described an increased degree of hypotonic haemolysis in the presence of 10^{-11} - 10^{-9} M isoproterenol and adrenaline. This process was shown to be mediated via adenylate cyclase activation in the red cells of rats, but not in this way in human cells. NA accumulation has also been demonstrated by a number of authors, although there is some confusion regarding the precise mechanism of uptake. Schanker, Nafpliotis and Johnson (1961), described the penetration of red cells by NA and related compounds, for example 5-HT, in some detail. Uptake was shown to be highly pH-sensitive and, the authors concluded, probably effected by passive diffusion. Once within the cell, it was suggested that NA also bound to haemoglobin.

In a later paper, Zimon, Sheps, Hazelrig, Schurger and Owen (1966) also described uptake of NA by red cells. Interestingly, this group were able to observe quantitative differences in NA

handling in patients in whom errors in catecholamine metabolism were already known or suspected, as compared to control subjects. For instance, red cells from individuals with orthostatic hypotension exhibited a reduced avidity for NA and thus appeared to transfer less amine from blood to cells than normal. Additionally, red cells from 8 out of 10 patients with diagnosed catecholamine-secreting tumours (phaeochromocytoma) also had a reduced avidity for NA. In contrast, a recent report by Atuk, Turner, Carey and Ayers (1986) suggested that red cells obtained from people with phaeochromocytoma were able to accommodate significantly greater quantities of amine than matched controls. In either case, the reason for the altered distribution remained unknown, and might represent a balance between factors including transport, binding and metabolism of amine. The possibility clearly arises, however, that the red cell mass may be directly responsive to local and/or systemic variations in catecholamine metabolism, and thus play an important role in the regulation of peripheral NA levels.

Roston (1967) also described rapid movements of NA into red cells, and concluded that active transport might account for "small" (physiological) amounts of catecholamine uptake, while diffusion was the favoured process for larger amounts. Born, Day and Stockbridge (1967) also concluded that the concentration dependence of large amounts of NA accumulation by red cells was consistent with the kinetics of simple diffusion, although other characteristics of uptake could not be adequately explained on this basis. Again, it was postulated that significant portions of amine were bound by the cell matrix. In addition to its binding to haemoglobin, Born, Day and Stockbridge (1967) suggested that NA might be held by an ion trapping mechanism, a theory expanded upon by Blakeley and Nicol (1978). This hypothesis argues that, once accumulated inside the cell, the NA molecule acquires an additional cationic charge due to the physiological transmembrane pH gradient (pH out > pH in), and its possession of two ionisable groups with high pK_a values (see Chapter 4). It is argued that this combination of factors renders the molecule less permeant and

hence reduces the likelihood of efflux. NA transport is thus asymmetric and possibly concentrative.

Subsequently, Danon and Sapira (1972) postulated for the first time an active uptake of bulk quantities of NA by the red cell, a process which obeyed Michaelis-Menten kinetics. McQuitty and Nicol (1979) also proposed saturable uptake of NA by a process similar to that documented in rabbit red cells (Blakeley & Nicol, 1978). The evidence would, therefore, seem at the very least to cater for the possibility of specific carrier-mediated uptake of NA, linked to intracellular binding and metabolism. Binding may allow NA to be stored and transported around the body, prior to re-release, or may simply act as a substrate reservoir for the catabolic enzyme catechol-O-methyl transferase (COMT). It is therefore interesting to note the conclusions of Cuatrecasas, Tell, Sica, Parikh and Chang (1974), that: "the binding of ^{3}H-NA to intact cells and microsomes" in various tissues may not "measure catecholamine receptor interactions, but rather a membrane catechol-binding protein which may be related to the enzyme COMT. True receptors are probably scarce."

Some of the results do appear at first sight, however, to be inconsistent, and are examined in greater detail at a later stage. Clearly though, due to the discrepancy in the literature, it is important to re-examine the basic characteristics of NA uptake, with a view to discovering whether or not a membrane carrier might be integrally involved in NA transport. It is conceivable that some of the anomalies regarding the literature pertaining to NA transport may arise specifically as a consequence of the binding properties of NA and its susceptibility to metabolic degradation.

As mentioned intracellular NA is metabolised by COMT in a methylating reaction which yields normetadrenaline (NMN). NMN is subsequently exported from the red cell by a mechanism which remains to be elucidated. Red cell total NMN levels are therefore significantly higher than those found in plasma (Yoneda, Alexander, Vlachakis & Maronde, 1984) and a favourable downhill electrochemical gradient for extrusion is thus maintained. The magnitude of this gradient is approximately 50-fold in the rat.

NMN is additionally catabolised by monoamine oxidase (present only in small amounts in red cells) and aldehyde dehydrogenase to yield vanilylmandelic acid (VMA), see Figure 7. VMA is eventually excreted in the urine and may be assayed as a crude estimate of the status of total body catecholamine metabolism.

Both within the red cell and in the plasma, NMN and NA may exist in free or conjugated states, the conjugated amine being formed by binding to either sulphate or glucuronic acid, and remaining distinct from that portion associated with haemoglobin. In the red cell NMN is, however, primarily found in the free state. Consistent with the cell acting as an important site of NA inactivation is the observation, in red cells of rats, that both free and conjugated forms of NMN increase significantly and to similar extents as NA during stress. Red cell NMN levels also correlate strongly with COMT activity. In human red cells NA is primarily conjugated with sulphate while 77% of plasma NMN also exists as a sulphate complex. In rats, conjugation is primarily with glucuronic acid.

In addition to studies of ^{3}H-NA uptake by intact red cells, and in view of the existing evidence, steps were taken to attempt to find a suitable non- or slowly metabolisable NA analogue for uptake studies. NA accumulation was also measured under conditions in which both intracellular metabolism and binding were minimised in case these factors might have some bearing upon the discrepancies noted in the literature. This was achieved by utilising red cell ghosts which are comparatively free of both intracellular haemoglobin (thus reducing the internal binding capacity) and COMT activity. A specific inhibitor of this enzyme, tropolone (Belleau & Burba, 1963), was incorporated into ghosts in order to inhibit any residual traces of COMT activity. It is noted that other workers have also found advantages in studying the uptake of substrates in metabolism-free systems. One example is the use of 3-O-methyl glucose as an alternative and inert substrate in investigations of glucose transport (e.g. Brown & Sepulveda, 1985).

Guanethidine: a non-metabolisable NA analogue

Guanethidine (ismelin) may be considered representative of drugs that depress the function of post-ganglionic adrenergic nerves. Guanethidine also sensitises effector cells to catecholamines, and hence effective blockade must mean that the amount of mediator released is drastically reduced. The fact that guanethidine is only slowly metabolised is inferred from the fact that its action persists in vivo for several days after the drug has been discontinued. The half-life of guanethidine in man is 5 days (Swales, 1979).

Many of the actions and effects of guanethidine and related drugs have been reviewed by Boura and Green (1965). It is now generally accepted that guanethidine is taken up by and stored in adrenergic nerves, and thus accumulation is essential for its action. Uptake involves the same mechanism responsible for the membrane transport of NA, and the uptake and subsequent actions of guanethidine can be inhibited by sympathomimetics, phenoxybenzamine, cocaine and tricyclic antidepressants. Guanethidine apparently accumulates in and displaces NA from intraneuronal storage granules, and is itself released by nerve stimulation. Thus it fits the definition of a false transmitter, although this mechanism may not adequately explain its clinical usefulness.

^{14}C-guanethidine was obtained as a gift from Ciba-Geigy, Basle. The concentration dependence of its uptake by human red cells was measured under identical conditions to those described for NA influx into red cells and red cell ghosts.

Experiments utilising red cell ghosts

Over the last decade, increasing numbers of membrane transport investigators have focussed their attention upon the advantages offered by the study of vesicles. The preparation of vesicles allows discrete membrane sacs to be isolated, which may be largely free of unnecessary and unwanted cellular contaminants. In addition, the scientist is able to incorporate within each batch the precise constituents desired for a given experiment. In

particular, the epithelial physiologist has derived benefit from these techniques which allow, for instance, apical vesicles to be concentrated at the expense of dissimilar adjacent basolateral material. Enrichment of one cell fraction to the detriment of another is commonly assayed by the variation in activity of one or more known marker enzymes, and similar techniques may be employed to ascertain the orientation (sidedness) of vesicle population. For instance, if studying an asymmetric membrane parameter, it is important to know whether the vesicle preparation has sealed in an exclusively "right side out" or "inside out" configuration, or as a mixture of both.

The ability to ghost red blood cells has been actively exploited for many years. Straub (1953) noted that compounds to which the red cell was normally impermeable could be incorporated during haemolysis, offering potential experimental advantages. Subsequently, Hoffman, Tosteson and Whittam (1960) were able to show that red cells haemolysed by osmotic shock were able to regain the low permeability to small monovalent cations, characteristic of red cells, following restoration of isotonicity and a short period of incubation at 37°C. The ghosts described in this paper lost intracellular potassium at approximately twice the rate of freshly starved red cells. In a further report, Hoffman (1962), was able to demonstrate that the characteristics of ^{24}Na efflux from ghosts closely resembled those of intact red cells, in that active transport, exchange diffusion and passive components could all be recognised. It was noted that the ghost preparation was not, however, homogeneous with regard to its cation handling characteristics, and could be divided into three distinct categories (see Methods). Despite this latter disadvantage, Hoffman (1980) was able to go on to produce convincing evidence to link the active transport of Na, via the ouabain-sensitive Na-K pump, to a number of metabolic intermediates. (This work, although published in 1980, was performed largely in 1965).

More recently the simplification and control, of metabolism afforded by ghosts has enabled kineticists (e.g. Eisner & Richards, 1981) to probe deeper into the mechanism of Na pump

activation. Other workers, at the same time, have turned their attention to alternative pathways of cation movement. Dunham and Logue (1986), for example, have described a K-Cl cotransport mechanism in ghosts, which is, in fact, activated by the preparative techniques employed by these authors. The activation of this system is thought to depend on the oxidative state of certain membrane sulphydryl groups, which varies as a consequence of haemolysis. The result is a clear indication of the need to carefully characterise a ghost population prior to drawing conclusions from experimental data. It is evident from a number of studies that red cell and ghost membranes may not behave in an entirely identical fashion.

Other membrane transport phenomena, in addition to those involving the movement of cations, have been investigated in ghosts and in general the evidence seems to suggest that membrane carriers are able to survive the necessary haemolysis and resealing manoeuvres, provided that temperature and pH are satisfactorily controlled during preparation. It also seems to be the case in these studies, despite the work of Dunham and Logue (1986), that carriers function in a quantitatively and qualitatively similar fashion in both ghosts and red cells, although the rates of passive diffusion of certain molecules in both directions across the membrane may be enhanced.

For example, Funder and Wieth (1976) observed that chloride self-exchange was of a similar magnitude in both cells and ghosts, and possessed similar characteristics including saturation kinetics, temperature dependence and inhibition by DIDS. Jung (1971) used the assay described by Lowry, Rosebrough, Farr and Randall (1951) to demonstrate that ghosts lost some 50-75% of their original non-haemoglobin protein, which may explain their leakiness. Bjerrum (1979) demonstrated that the "major" membrane proteins were apparently retained independent of the precise nature of the preparation, and this may suggest that Jung's 50-75% might represent smaller and non-specific protein components. Further evidence that red cells tend not to lose major membrane components when ghosted comes from the work of Susanni, Ross,

Scriven and Rosendorff (1985) who demonstrated the retention of β-receptors by baboon red cells following hypotonic haemolysis.

The ghost experiments described in this chapter concentrate on two aspects of NA transport, in an attempt to extend the observations using intact cells:

1)Does the perturbation of the cell membrane during ghost preparation affect NA uptake, and, if so, what conclusions may be drawn from such a result?

and 2)Does NA transport in ghosts exhibit the same concentration dependence as that witnessed in intact red cells?

RESULTS

EXPERIMENTS UTILISING RED CELLS

Linearity of NA uptake

^{3}H-NA accumulation was measured under conditions in which uptake was shown to be linear with time. This avoided the necessity to correct for simultaneous tracer efflux (backflux) during each incubation. Flux incubation periods were also minimised, when possible, such that the build up of NA metabolites was kept to the lowest possible levels. This was in order to reduce any possible effects of metabolites on NA transport. Blakeley and Nicol (1978), working on rabbit red cells, did not detect the appearance of extracellular metabolites (e.g. NMN) until after approximately 16 minutes of incubation performed at an NA concentration of 6 μM at 37°C. Some intracellular accumulation of NMN could be observed, however, after only 4 minutes. Routinely, and unless otherwise stated, the experiments described in this thesis measured ^{3}H-NA uptake over 15 minute periods.

Influx remained linear over this period in both cells and ghosts,at the highest $[NA]_o$ levels considered (250 mM). Figure 5 depicts the relationship between and time. Data for ghosts, rather than cells, has been presented as internal [NA] should rise more rapidly in this COMT-depleted preparation, thus accentuating the tendency towards backflux. If uptake remains linear under such conditions, as it does, then it may be safely assumed that uptake at lesser concentrations, and in cells, is also linear. This was shown to be the case.

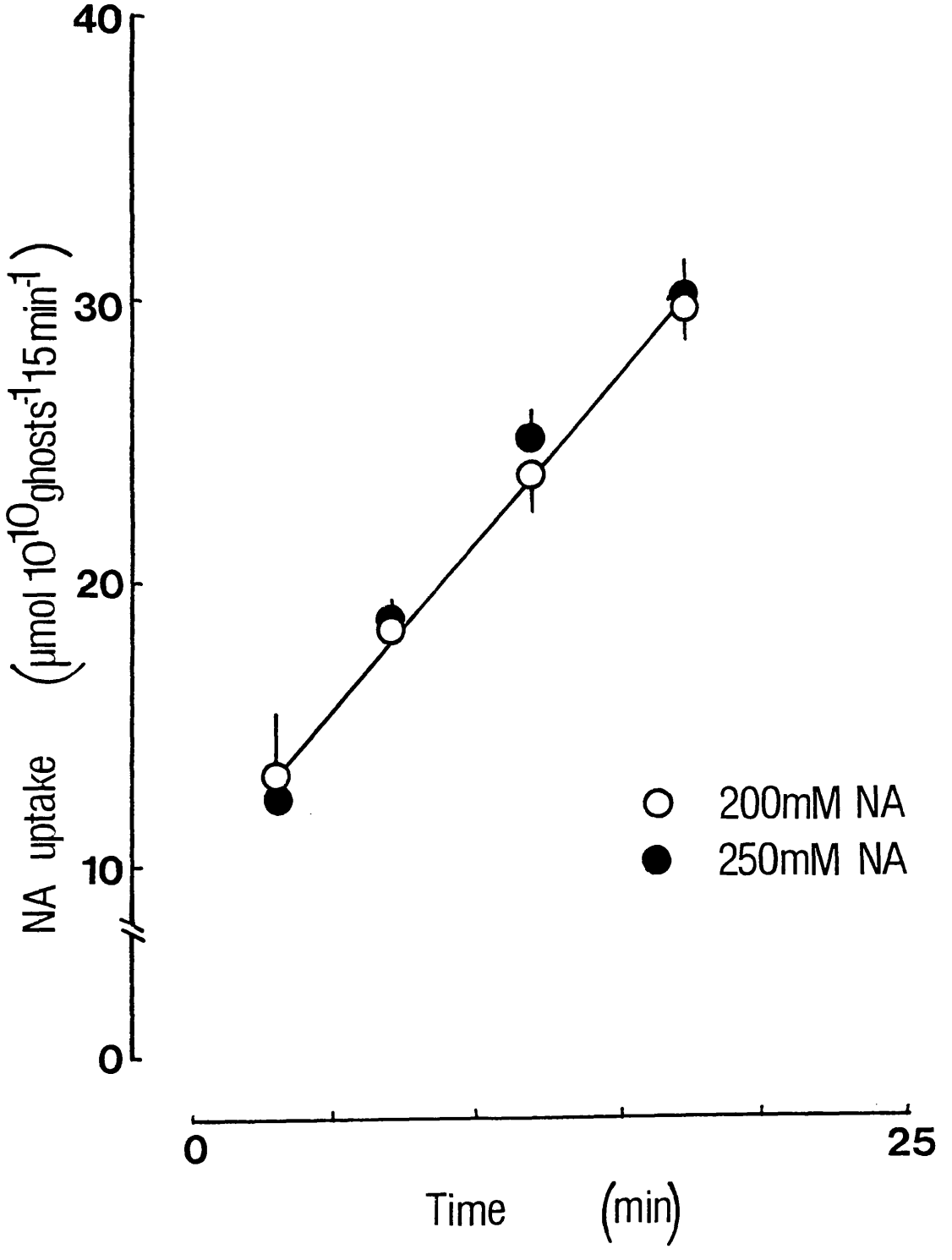
Concentration dependence of NA uptake

Typically, as extracellular substrate concentrations are increased, membrane bound carrier components are recruited so as to shuttle solute across the cell surface in increasing amounts until saturation is achieved. When saturated, no additional substrate may interact with the carrier populations and therefore a maximum velocity of uptake (Vmax) may be observed. The Vmax of a transport process is a convenient experimental parameter to measure and is good evidence for carrier mediated transport. In

Figure 5

The linearity of NA uptake

The time course of ^{3}H-NA influx into ghosts was measured from solutions containing 200 mM (open symbols) and 250 mM (closed symbols) NA. Uptake remained linear with time for periods in excess of 15 minutes, which was chosen as the standard incubation time for the majority of flux experiments in this thesis. Each point illustrated represents the mean and standard error of triplicate measurements. Where error bars are not shown, the error falls within the limits of the symbol.


40
30
20
10
0
NA uptake (µmol 10^{10} ghosts^{-1} 15 min^{-1})
200mM NA
250mM NA
0
25
Time (min)

contrast, the rate of uptake by passive diffusion is characterised by a linear dependence upon substrate concentration and is not classically described by saturation kinetics.

The mechanism by which NA enters human red cells is not clear. Born, Day and Stockbridge (1967) proposed that the accumulation of NA was consistent with simple diffusion, although uptake was not studied at concentrations in excess of about 80 μM NA_o. In this range, the rates of uptake reported agree closely with those obtained in these studies. These authors, however, also described a component of stereospecific uptake; (+) NA did not enter the red cell. It was noted that such a result indicated the possible existence of a receptor or carrier for NA in the membrane, and it is therefore conceivable that Born and co-workers were studying a carrier mechanism operating at submaximal substrate concentrations. Further circumstantial support for the existence of such a mechanism comes from the observation that the rates of entry of NA and two related compounds, histamine and 5-hydroxytryptamine, were not simply related to the concentrations of the unionised lipid-soluble molecules, as might be expected for simple diffusion.

The subsequent work of Danon and Sapira (1972) suggested that NA uptake was an "active" process, with a Vmax of 2.67 mmol 1 $cells^{-1}$ 2 $hours^{-1}$, and an apparent affinity constant (Km) of 4.54 mM. Km is defined as the concentration of substrate at which uptake is half-maximal. One problem with this study, however, is the fact that the highest substrate concentration presented to the cells, so far as can be ascertained from the text, was in the region of 300 - 400 μM, comfortably 10-fold less than Km. Interestingly though, McQuitty and Nicol (1979) also reported concentrative, temperature dependent, saturable NA uptake in red cells with an apparent Km of 2.1 mM, close to the value quoted by Danon and Sapira (1972). The authors suggested that the likely mechanism of NA accumulation was facilitated diffusion.

In order to clarify the route of entry of NA into red cells, a series of experiments were performed to study the concentration dependence of uptake. Cells were exposed to NA concentrations of

up to 250 mM and one experiment, typical of eight performed in this range, is illustrated in Figure 6. It can be clearly seen that uptake remains linearly related to concentration, an observation made both in fresh cells and those obtained from bank blood. These results are therefore, at first sight, consistent with NA entry being mediated by simple diffusion.

The effect of cell volume on NA uptake

Although the literature is not conclusive, there is a trend towards the hypothesis, in the more recent publications, that NA uptake may be mediated via facilitated diffusion. It was therefore important to verify that the results presented in the previous section were genuine. One possible source of error arises from the osmotic effects upon red cells of exposure to large amounts of amine, while no alternative compound was utilised to maintain osmotic balance. The question therefore arises: how might variations in cell volume affect NA uptake?

Two experiments were performed in order to determine the effects of cell volume on NA uptake. In these experiments uptake of a single concentration of NA was measured from media supplemented by either mannitol or sucrose such that extracellular osmolarity ranged between 220 - 500 mosmoles l^{-1}. Relative cell volume was calculated by a comparison of the numbers of packed cells in 1 ml at each osmolarity, as measured by a Model Fn Coulter counter, and the data is illustrated in Table 9.

It was apparent that NA accumulation was not dependent upon cell volume. This observation suggests that much of the uptake observed may represent binding, either to the cell surface or to some component of the interior. Possible candidates for intracellular NA binding include haemoglobin (e.g. McQuitty & Nicol, 1979), ATP, Mg (e.g. Colburn & Maas, 1965) and sulphate (e.g. Yoneda, Alexander & Vlachakis, 1983).

Alternatively, if COMT activity were to be rapidly activated by NA influx then substrate might be eliminated at sufficient speed such that the reduction in cell water and consequent concentration of intracellular amine noted at high concentrations

Figure 6

The concentration dependence of NA uptake by human red cells

^{3}H-NA uptake into red cells was measured in the presence of various concentrations of up to 250 mM extracellular substrate, as described in the text. The data represents one experiment and each point is the mean of duplicate determinations. The line fitting the data was drawn by eye.

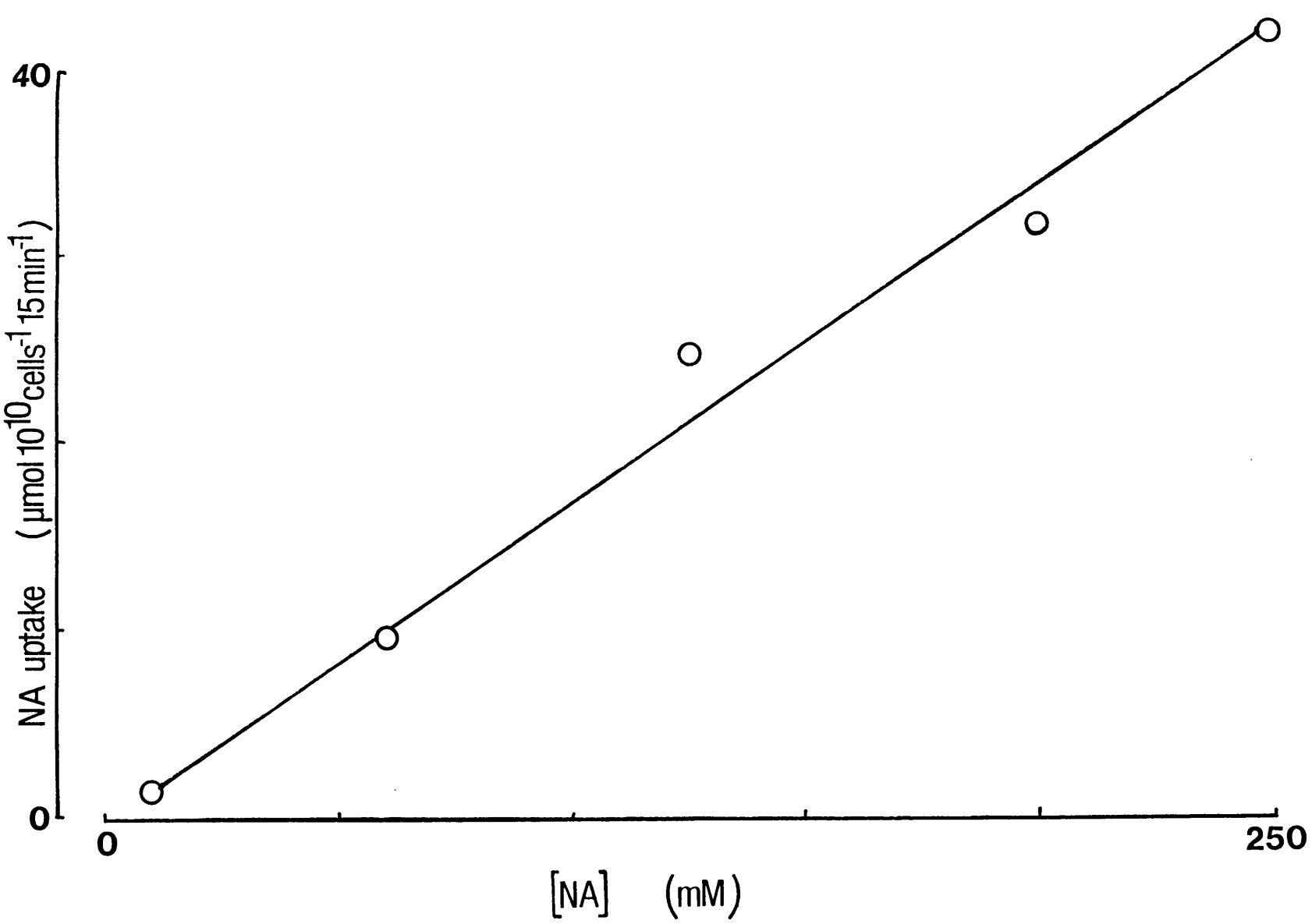

40
0
NA uptake (μmol 10^{10}cells^{-1} 15min^{-1})
0
250
[NA] (mM)

TABLE 9

THE EFFECT OF CELL VOLUME ON NA UPTAKE

Extracellular Osmolarity (mosmols l^{-1})	Experiment One			Experiment Two		
	Cells ml^{-1}	RCV	Uptake*	Cells ml^{-1}	RCV	Uptake*
220	9.16×10^9	1.17	0.159	9.53×10^9	1.11	0.156
267	9.87×10^9	1.08	0.150	1.06×10^{10}	1.00	0.147
295	1.07×10^{10}	1.00	0.141	1.12×10^{10}	0.95	0.149
337	1.07×10^{10}	1.00	0.154	1.17×10^{10}	0.91	0.155
370	1.23×10^{10}	0.87	0.138	1.26×10^{10}	0.84	0.142
500	1.35×10^{10}	0.79	0.151	1.31×10^{10}	0.81	0.145

*Uptake was measured in nmol 10^{10} $cells^{-1}$ 15 min^{-1}.

Extracellular osmolarity was varied using mannitol (Experiment one): $[NA]_o$ = 1.07 μM.
sucrose (Experiment two): $[NA]_o$ = 1.05 μM.

All values are means of three determinations.

All media contain: 100 mM NaCl, 10 mM glucose, 10 mM Tris-MOPS pH 7.4.

of mannitol or sucrose might not become rate limiting for uptake. The possible link between NA accumulation and metabolism is considered in greater detail later in this chapter.

NA binding

In an attempt to verify that the results obtained truly represent uptake, "no side" membranes were prepared and incubated with ^{3}H-NA under standard influx conditions. Membranes were prepared by a modification of the method described by Romero (1976).

Approximately 80 counts per minute were shown to associate with 1 ml of membranes, as opposed to in the region of 1000 - 2000 c.p.m. per ml red cells. Therefore, although some minimal degree of membrane binding may occur, this probably represents at greatest 5 - 10% of the total radioactivity associated with the cell. A limited degree of membrane binding is consistent with the work of Banaschak and Bluth (1978) who proposed that NA uptake into intact red cells occurred in two stages:

1) Rapid adsorption of NA to the cell membrane, followed by
2) Slower entry of amine into the cell, where binding of NA to haemoglobin took place with a stoichiometry of 1:1.

Born, Day and Stockbridge (1967) and Roston (1967) however, could detect no appreciable binding of NA to red cell membrane fragments, consistent with the observations made in this study.

Isotopic equilibration of NA

Isotopic equilibration of NA ($[NA]_o$ = 1.03 μM) was complete after 3 hours at 37^{o}C. Estimation of $[NA]_i$ from tracer data suggested that the ratio of $[NA]_i$: $[NA]_o$ reached values of unity, or slightly greater. It therefore seemed possible that uptake might be concentrative, although not as pronounced as that described by, for instance, McQuitty and Nicol (1979) ($[NA]_i$: $[NA]_o$ = 1.79 $\pm$ 0.12 : 1), or, Danon and Sapira (1972) ($[NA]_i$: $[NA]_o$ = 1.5-2.7 : 1). It is not prudent, however, to ascribe any

level of significance to these results, as it is not certain whether intracellular label represents free or bound NA, or products of metabolism. Indeed, Yoneda, Alexander and Vlachakis (1983), using a modified radio-enzymatic assay for catecholamines, concluded that red cells contained significantly less NA per unit volume than plasma. Therefore, an alternative, and in this instance preferable, method of assessing NA_i may be found in the direct measurement of amine, utilising high performance liquid chromatography with electrochemical detection (HPLC-ED).

Uptake of NA measured by HPLC-ED

Five preliminary experiments were performed in order to determine free $[NA]_i$ in red cells freshly sampled from the peripheral circulation of adult male volunteers. Plasma [NA] in these samples ranged between 1 and 3 nM, within the limits quoted for normal, resting, subjects (e.g. Pluto, Burger & Weicker, 1986). It was not, however, possible to detect any catecholamine in the PCA extracts of red cell haemolysates. Two possible explanations for this observation exist:

1) The dilution factor involved in haemolysis and subsequent precipitation with PCA (x 7.5) diluted $[NA]_i$ to levels below the limit of the technique (about 0.5 pmol ml^{-1}), (Walker, 1987).

and/or 2) Catabolism of NA is sufficiently rapid that little NA remains within the red cell at the time of sampling. The principal route of NA catabolism in the red cell is via the action of COMT, see Figure 7 (Axelrod & Cohn, 1971).

The work of Yoneda, Alexander, Vlachakis and Maronde (1984), suggests that option 1) alone might adequately account for the lack of NA_i.

In order to circumvent this problem, red cells were exposed to high (μM) concentrations of NA over periods of 3 hours at 37°C. PCA extracts of 'NA-loaded' haemolysates were subsequently analysed for resultant amine levels. it was found that to measure any appreciable amount of intracellular NA, cells had to be

exposed to in the region of 50 μM NA_o, approximately 10^4-fold in excess of the plasma levels quoted as normal in the peripheral circulation. It may therefore be assumed, if the technique is sound, that red cell COMT is comfortably able to catabolise even the largest amounts of amine presented to it *in vivo*. This hypothesis is substantiated by the affinity constant quoted for the NA-COMT complex of 2×10^{-4} M (Axelrod & Cohn, 1971). A rate limiting step in catecholamine catabolism *in vivo* may therefore, quite possibly, be the speed at which NA crosses the cell membrane.

A total of eight experiments were performed to investigate the NA_i levels achieved in media containing 100 μM NA_o. The variability in results was greater than that observed for tracer influx studies, with the resultant $[NA]_i$, after 3 hours, ranging between 60.6 and 2591.0 nmol 1 cell H_2O^{-1}. This scatter might possibly represent a variation in COMT activity between individuals, as the binding capacity of the red cells seems unlikely to vary by a factor of approximately 40 from one subject to another.

At the same time in other experiments uptake of tracer never varied more than 3-fold between subjects. In the first instance this may suggest that there is a comparatively weak link between uptake and metabolism. It should be stressed, however, that the majority of results obtained with HPLC did fall within such a 3-fold span (marked ** in Table 10), and the uppermost and lowest values may therefore be considered an extreme. Directly comparable simultaneous measurements of uptake using both tracer and HPLC techniques were not performed because it was not possible to either measure $[NA]_i$ (by HPLC) after 15 minute incubations, or measure linear components of ^{3}H-NA influx after 3 hours. It is proposed that differences in COMT activity may influence rates of amine accumulation, but that such influences become more pronounced in certain individuals with time. Therefore, there might appear to be some latency in the activation of the enzyme *in vitro*.

The mean $[NA]_i$ achieved in these experiments (see Table 10)

Figure 7

Catabolism of NA

The figure describes the various pathways of NA catabolism known to exits in vivo.
The abbreviations used are as follows:

COMPOUNDS	1	NA
	2	3,4-dihydroxyphenylglycolaldehyde
	3	3,4-dihydroxymandelic acid
	4	normetadrenaline
	5	4-hydroxy,3-methoxyphenylglycolaldehyde
	6	vanilylmandelic acid
	7	MOPEG
	8	DOPEG
ENZYMES	mao	monoamine oxidase
	comt	catechol-O-methyl transferase
	ad	aldehyde dehydrogenase
	alc d	alcohol dehydrogenase

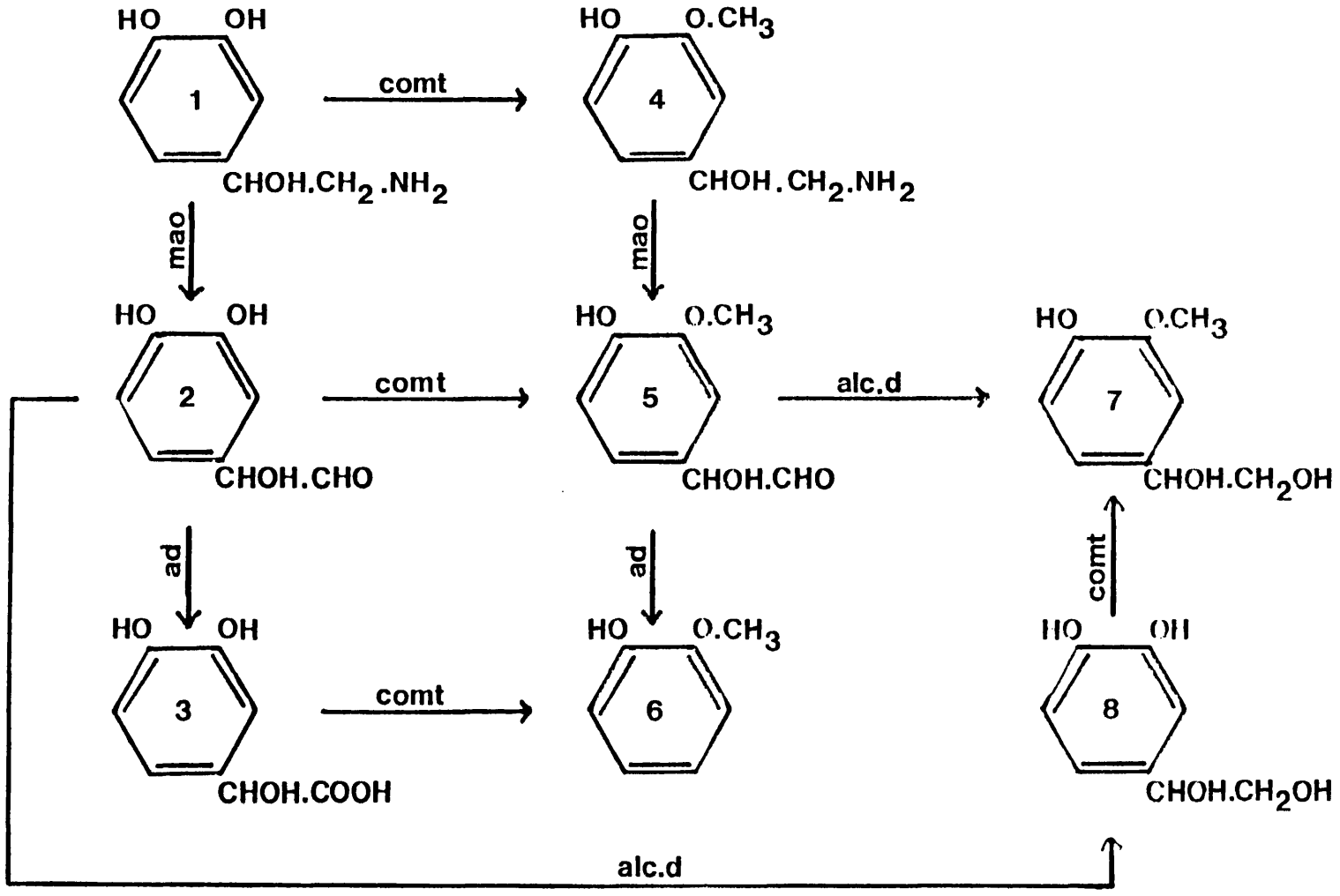

HO OH
1
comt
HO O.CH3
4
CHOH.CH2.NH2
CHOH.CH2.NH2
mao
mao
HO OH
2
comt
HO O.CH3
5
alc.d
HO O.CH3
7
CHOH.CHO
CHOH.CHO
CHOH.CH2OH
ad
ad
comt
HO OH
3
comt
HO O.CH3
6
HO OH
8
CHOH.COOH
CHOH.CH2OH
alc.d

TABLE 10

ACCUMULATION OF NA BY RED CELLS ESTIMATED USING HPLC-ED

EXPERIMENT	$[NA]_i$ (pmol ml cells^{-1})	$[NA]_i$* (pmol ml cell H_2O^{-1})
1	881.4	1203.1 **
2	44.4	60.6
3	61.0	83.3
4	1503.5	2052.3 **
5	1128.7	1540.7 **
6	199.1	271.8
7	1006.2	1373.5 **
8	1898.2	2591.0 **

Measurements were made after a 3 hour incubation period with $[NA]_o$ 100 μM.
All incubations were performed at pH 7.4.
*Assuming 1 ml red cells contains 0.7326 ml H_2O, calculated as follows:

1 kg red cells contains 666g H_2O.
1 l red cells weighs 1.10 kg.
Therefore 1 l red cells contains 666 x 1.1 kg H_2O = 732.6 g.

was approximately 1.15 μmol 1 cell H_2O^{-1}. It is noted that this value is closely comparable to the value of 1 μmol 1 $cells^{-1}$ obtained from tracer studies in which cells were exposed to 100-fold less NA_o under otherwise identical conditions. There would therefore seem to exist convincing evidence that NA_i is either catabolised or bound rapidly, and that the capacity for inactivation of amine by the red cells is considerable.

In a further six experiments, net uptake of NA was also measured in cells obtained from bank blood. Accumulated $[NA]_i$ in these cells fell within the same range of values as for fresh cells. Furthermore, the resultant $[NA]_i$ levels noted, resided at the lower end of the scale, suggesting that cold stored cells do not lose the ability to catabolise and/or store amines. The concentration and time dependence of NA uptake is illustrated in Figure 8.

Identification of metabolites

If tracer studies suggest uptake rates which are approximately 100 times greater than the amounts of amine measured by HPLC, it is reasonable to suggest that some of the balance may be comprised of products of metabolism. That this inference was reasonable was prompted by the observation that the height of at least one unidentified HPLC peak correlated closely with the height of NA in each sample in which both compounds appeared (n=31 observations, r=0.6782). This unknown was subsequently identified as 3,4-dihydroxyphenylglycol (DHPG) on the grounds that authentic samples of the same compound exhibited identical column retention characteristics.

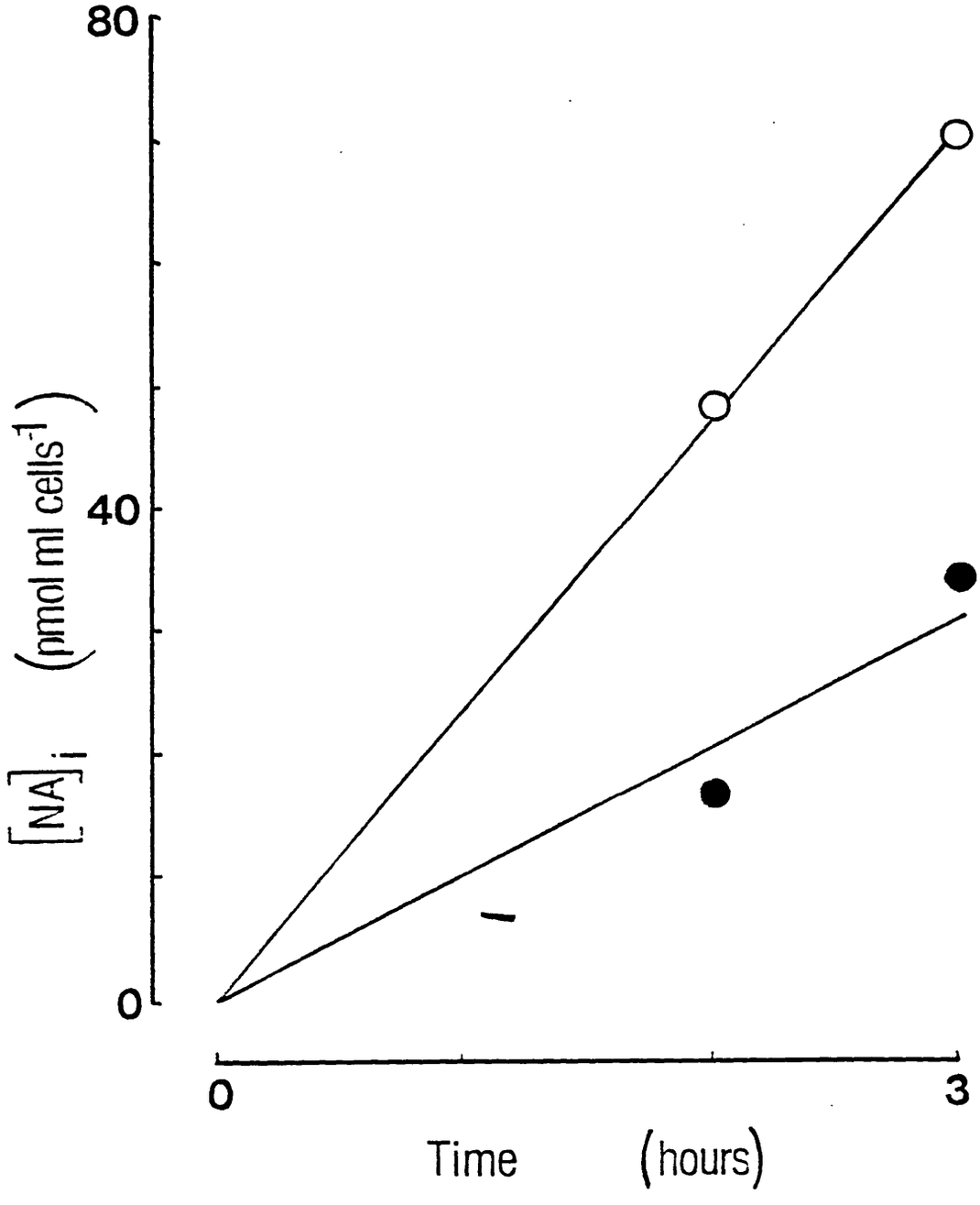
Evidence for the intracellular conversion of NA to adrenaline was also found, and another compound, probably 3,4-dihydroxymandelic acid (DOMA) also appeared on some chromatograms. DOMA eluted less frequently than DHPG, consistent with the fact that the latter is a precursor of the former (see Figure 7).

While the limitations of the HPLC system employed and the specificity of the extraction procedure for the catechol moiety prohibited large scale identification of metabolites, the

Figure 8

The time course of accumulation of NA_i

Net uptake of NA was measured by HPLC-ED, in red cells suspended in media containing 50 μM NA (closed symbols and 100 μM (open symbols). It proved impossible to detect NA within red cells washed free of extracellular amine until 2 hours after the start of the incubation. Beyond this point, levels of NA_i increased with time in a concentration dependent manner. The data represents one experiment which is typical of 2 performed. Each point is the mean of duplicate measurements.


80
40
0
$[NA]_i$ (pmol ml cells^{-1})
0
3
Time (hours)

information obtained provided indisputable evidence for intracellular catecholamine metabolism. A chromatogram describing these results is presented as Figure 9.

Effects of drugs on ^{3}H-NA uptake

The preliminary experiments described allow certain conclusions to be drawn relating to NA uptake by red cells:

1) The mechanism by which NA enters the red cell is not apparently saturable at concentrations well in excess of those encountered physiologically.
2) The red cell may, therefore act as a large capacity sink for catecholamine metabolism and possibly storage (binding) and transport around the body.

The possibility therefore arises, as previously mentioned, that the discrepant results presented in the literature, independently citing either passive diffusion or carrier mediated activity as the likely routes of amine accumulation, may result from the complicating effects of cell metabolism and binding. For instance, if free NA_i is rapidly inactivated within the cell then downhill inward diffusion of NA_i will constantly be favoured. The gradient driving diffusion will increase as NA_o is raised, certainly until COMT is saturated, and as has been stated, this enzyme is known to have a high capacity for substrate. It is thus considered possible that such a component of uptake might superimpose itself upon carrier mediated uptake thus acting to mask saturation kinetics in intact red cells. It is further possible that, if there is some relationship between catabolic activity and rate of uptake, then as increasing amounts of NA are presented to the cell and accumulated, so increasing numbers of carriers may be recruited, or switched on, to mediate uptake. Hence, Vmax would be elevated and again saturation kinetics might not become evident.

Two options therefore present themselves:

1) To study NA uptake in a metabolism free system,

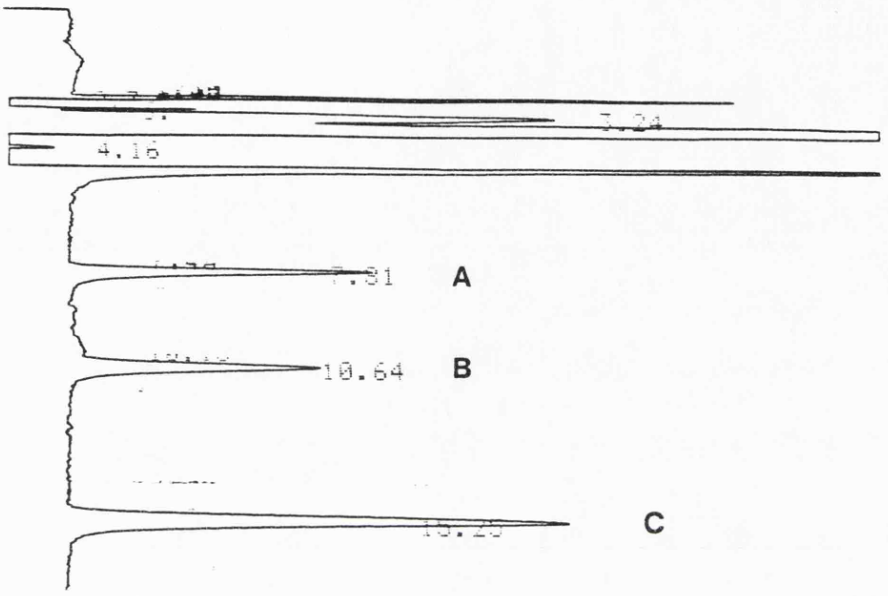
and 2) To attempt to demonstrate the presence or absence

Figure 9

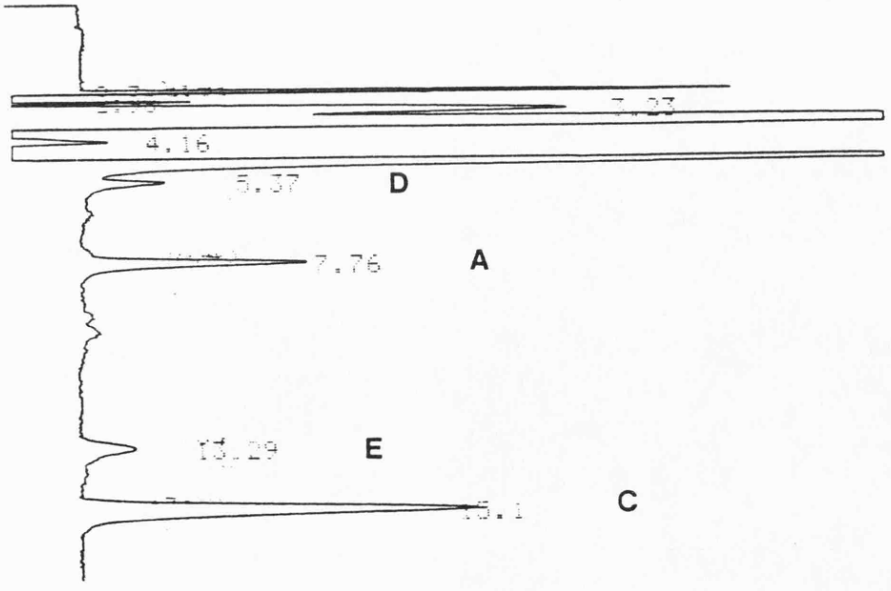
Two typical chromatograms

The upper portion of the figure shows a standard comprising 15 pmol 100 μl^{-1} NA (A), adrenaline (B), and DHB (C), in order of retention times. The lower portion shows the chromatogram obtained from a PCA-treated red cell haemolysate. NA (retention time: 7.76) and DHB (15.1) are again present. The other peaks (D and E) are presumed to be products of red cell catecholamine catabolism.
All retention times are in minutes.

START 00.00.00.00.



START 00.00.00.00.



cells by alternative strategies. The first approach to this problem was to consider the effects of drugs known to inhibit carrier mediated NA uptake in other tissues.

The effect of cocaine

Cocaine is a potent inhibitor of the well characterised neuronal transporter 'Uptake I', which is found in a variety of tissues. The concentration of cocaine reported to maximally inhibit this mechanism is in the region of 1 μM (e.g. Dengler, Spiegel & Titus, 1961). Five experiments were performed in order to ascertain the effects of 5 μM cocaine on NA uptake in fresh red cells, and the results are shown in Table 11. No effect upon accumulation of amine was noted, regardless of $[NA]_o$ in agreement with the results of Born, Day and Stockbridge (1967). Another two experiments were performed using 50 μM cocaine during prolonged incubation periods, and it was further demonstrated that such elevated concentrations of 'inhibitor' were also without effect upon NA transport. It may therefore be concluded that NA uptake into red cells proceeds via a mechanism which is not identical to Uptake I.

The effects of cortisol and reserpine

Cortisol and reserpine are known to inhibit the well characterised amine transporter found in a variety of extraneuronal tissues, e.g. heart (Iversen and Salt, 1970) and umbilical artery (Gulati and Swaramakrishna, 1975). Two experiments were performed using each of these Uptake II inhibitors over a variety of concentrations. Neither compound had any effect upon NA transport, as shown in Table 12. These results are in agreement with the observations of both Danon and Sapira (1972) and Blakeley and Nicol (1978). Hence, it may also be concluded that NA uptake into red cells is not via a mechanism identical to Uptake II.

TABLE 11

THE EFFECT OF COCAINE ON NA UPTAKE

EXPERIMENT	[COCAINE]	$[NA]_o$ = 49 nM		$[NA]_o$ = 289 nM		$[NA]_o$ = 5.39 μM	
		Control	Cocaine	Control	Cocaine	Control	Cocaine
1	5 μM	11.79	10.95	59.83	71.33	1.16	1.28
2		8.87	10.71	52.72	55.66	1.01	0.97
3		11.35	10.88	59.60	66.47	1.08	1.05
4		10.66	10.64	62.25	62.37	1.02	1.03
5		11.71	12.57			1.11	1.34
Mean		10.88	11.15	58.60	63.96	1.08	1.13
(SEM)		(0.54)	(0.36)	(2.05)	(3.32)	(0.03)	(0.07)

Units of flux: $[NA]_o$ = 49 nM, 289 nM: pmol ml cells^{-1} 15 min^{-1}.
$[NA]_o$ = 5.39 μM: nmol ml cells^{-1} 15 min^{-1}.

TABLE 12

A. THE EFFECT OF RESERPINE ON NA UPTAKE

		[RESERPINE]						
Experiment	$[NA]_o$	Control	1 μM	2 μM	5 μM	10 μM	15 μM	20 μM
1	289 nM	80.61	77.63	86.80	85.60	77.92	84.88	86.06
2	278 nM	66.38	61.68	72.13		62.28		63.69

B. THE EFFECT OF CORTISOL ON NA UPTAKE

		[CORTISOL]					
Experiment	$[NA]_o$	Control	40 μM	80 μM	120 μM	160 μM	240 μM
1	289 nM	80.61	75.35	74.04	74.70	73.43	78.28
2	278 nM	66.38	66.84	65.95	61.62	62.82	65.59

Units of flux: pmol ml cells^{-1} 15 min^{-1}.

Effects of other drugs

The effect upon NA uptake of a number of other drugs was also investigated. Drugs were chosen on the basis of their ability to act as general metabolic poisons, or their specific ability to inhibit catecholamine catabolism. The effects of ATP depletion were also considered in a subsequent series of experiments. The primary aim of this work was to discover whether NA uptake was a directly energy dependent process. The drugs utilised were: Na_2-EDTA (final concentration 1 mM), 2,4-dinitrophenol (1 mM), sodium nitroprusside (1 mM), iodoacetic acid (5 mM) and tropolone (1 mM). The effect of each agent was considered in at least two experiments.

Of the compounds chosen, only iodoacetic acid (IAA) and tropolone affected NA accumulation in any way. In the presence of IAA ^{3}H-NA uptake fell by 20-30% (Table 13), an effect which was independent of the original energetic status of the cell. The effect was both qualitatively and quantitatively similar in fresh cells and in cells obtained from cold stored blood which had been incubated overnight in a glucose free medium to further reduce any residual traces of intracellular ATP. This result is consistent with the work of Danon and Sapira (1972) in which IAA (1 mM) was noted to retard the catabolism of NA by about 30%.

The possibility also exists that IAA may additionally affect NA transport by virtue of its action as an alkylating sulphydryl reagent. Binding of IAA to sulphydryl groups situated in the membrane might cause structural changes which could lead to the occlusion of a putative NA binding site, or a reduction in the mobility of a carrier mechanism.

Results in the presence of tropolone (n=2), a specific inhibitor of COMT (Belleau & Burba, 1963), suggested that NA accumulation, as measured by HPLC-ED, was reduced. NA_i fell from 329.6 to 60.0 nmol l cells^{-1} in the presence of tropolone (NA_o = 50 μM), and from 881.4 to 527.2 nmol l cells^{-1} (NA_o = 100 μM).

This result was on one hand surprising as it might be considered that, in the absence of catabolic breakdown of

TABLE 13

THE EFFECT OF IAA ON NA UPTAKE

Experiment	$[NA]_o$	Control	+ IAA (5 mM)	% inhibition
1	50 nM	8.04	5.83	27.5
2	5.4 uM	0.67	0.50	25.4
3	5.4 uM	0.70	0.58	17.1
Mean				23.3
(SEM)				(3.2)

Units of flux: Experiment 1 : pmol ml cells^{-1} 15 min^{-1}.
Experiments 2 & 3 : nmol ml cells^{-1} 15 min^{-1}.

catecholamines, free NA_i should rise, assuming that tropolone is exerting the effect described from within the cell, and that residual traces of the drug do not interfere with HPLC extraction procedures. To explain the result, it is postulated that there may indeed exist some direct link between uptake and metabolism, of the type described by Cuatrecasas, Tell, Sica, Parikh and Chang (1974). Therefore, when metabolism is inhibited, uptake is substantially retarded by the end of prolonged (3 hour) periods of incubation.

In experiments in which ATP_i was reduced, by prolonged incubation in glucose free media, no effect upon uptake could be discerned when compared to "ATP rich" cells from the same original sample. Verification that different intracellular ATP concentrations were achieved by this procedure was provided by a spectrophotometric assay based on the method described by Bucher (1947). ATP rich cells contained 0.67 mM ATP, while ATP depleted cells contained less than 0.3 mM.

It is therefore concluded that NA uptake in red cells is not truly active, in so far as accumulation of amine is not apparently dependent upon glycolysis.

Effect of β-blockade

As previously stated, the red cell is known to contain populations of β-receptors (Rasmussen, Lake & Allen, 1975). In other tissues NA exerts some of its effects by virtue of action upon such receptors, and hence it was felt important to ascertain whether uptake might involve some interaction with β-receptors. In order to test this possibility, the effects of propranolol (10 μM, n=2) and timolol (5 μM, n=3) upon NA uptake were considered. Both compounds are β-blocking agents. $[NA]_o$ ranged between 39 nM and 5.38 μM in these experiments. No effect upon NA accumulation was noted after 15 minutes incubation in the presence of timolol. In the presence of propranolol a longer incubation period (25 minutes) also failed to reveal any effect on uptake (Table 14). Such an observation is consistent with the existence

of a NA binding site which is probably not a typical adrenergic locus (Cuatrecasas, Tell, Sica, Parikh & Chang, 1974).

Effect of temperature

Six experiments were performed to investigate the temperature dependence of red cell NA accumulation. In agreement with Born, Day and Stockbridge (1967), reduction of temperature to near 0°C inhibited influx by 93.5 ± 0.6% (n=3), as compared to data obtained at 37°C (see Table 15). It was further shown, however, that uptake was not linearly related to temperature and that a considerable portion of accumulation was inhibited by cooling to 27°C. In total, 81.5 ± 2.0% of uptake was abolished by such a 10°C drop. The resultant mean Q_{10} (flux 37°C/flux 27°C) calculated from this data was 5.64 ± 0.68. Clearly, NA uptake is highly dependent upon some temperature sensitive membrane transition which occurs between 27°C and 37°C. It is generally accepted that the accumulation of substrates by free diffusion has a relatively low dependence upon temperature, although a marked temperature dependence is not conclusive proof of active or facilitated uptake. A high Q_{10} may be derived from any process with a high energy of activation which might, for example include the opening of spaces between tightly packed phospholipid tails in the membrane bilayer. It has been shown, for instance, that the simple diffusion of water into various media may have a higher energy of activation than the facilitated diffusion of glucose into red blood cells, or the active transport of galactoside into E. coli (West, 1983). Therefore, while the results demonstrate that NA uptake is markedly temperature dependent, the data may not be interpreted as conclusive proof of the operation of a carrier.

TABLE 14

THE EFFECTS OF PROPRANOLOL AND TIMOLOL ON NA UPTAKE

1. Timolol (5 μM)

Experiment	$[NA]_o$ = 5.38 μM		$[NA]_o$ = 279 nM		$[NA]_o$ = 39 nM	
	Control	Timolol	Control	Timolol	Control	Timolol
1	1.13	1.00	47.92	51.00	7.41	7.49
2	0.83	0.76	41.46	37.93	5.03	4.82
3	0.83	0.81	47.73	45.27	5.68	6.96
Mean	0.93	0.86	45.70	44.73	6.04	6.42
(SEM)	(0.10)	(0.07)	(2.12)	(3.78)	(0.71)	(0.82)

All flux incubations were for 15 min, pH 7.4 and 37°C.

2. Propranolol (10 μM)

Fluxes measured at $[NA]_o$ = 5.38 μM.

Experiment	t_5		t_{15}		t_{25}	
	Control	Propran.	Control	Propran.	Control	Propran.
1	1.14	1.02	1.85	1.80	2.22	1.95
2			1.86	1.80		

Units of flux: $[NA]_o$ = 5.38 μM: nmol ml cells^{-1} 15 min^{-1}.
$[NA]_o$ = 279 nM, 39 nM: pmol ml cells^{-1} 15 min^{-1}.

TABLE 15

THE EFFECT OF TEMPERATURE ON NA UPTAKE

Experiment	$[NA]_o$	$Flux_{37}$	$Flux_{27}$	$Flux_4$	% decrease	Q_{10}
1	313 nM	89.55	17.60		80.3	5.09
2	313 nM	72.23	15.92		78.0	4.54
3	313 nM	132.28	18.16		86.3	7.28
4	5.38 µM	1.47		0.08	94.8	
5	279 nM	71.61		4.89	93.2	
6	39 nM	10.18		0.76	92.5	

Units of flux:

$[NA]_o$ = 5.38 µM: nmol ml cells^{-1} 15 min^{-1}.

$[NA]_o$ = 313 nM, 279 nM, 39 nM: pmol ml cells^{-1} 15 min^{-1}.

Guanethidine uptake by human red cells

The concentration dependence of guanethidine accumulation by red cells was measured at substrate concentrations of up to 150 mM under conditions essentially the same as those described for NA uptake. Four experiments were performed, one of which is illustrated in Figures 10 and 11. The kinetic constants calculated (Wilkinson, 1961) from this and the other three experiments are presented in the upper portion of Table 16.

In direct contrast to experiments studying NA uptake (see Figure 6) guanethidine influx appeared to obey simple Michaelis-Menten kinetics. Uptake was saturable, with a Vmax in the region of 10 μmol ml cells^{-1} 15 min^{-1} and a Km which ranged between 13.74 and 24.21 mM (mean 19.41 ± 2.17 mM) (Table 16). Thus, although a carrier for guanethidine on the red cell membrane may be postulated, it would appear that the maximum rate of transport of this substance is not as rapid as the speed by which NA may enter the cell. At similar concentrations (e.g. 150 mM) it is estimated that NA transport is approximately twice as fast. This may be a consequence of the fact that NA is rapidly metabolised within red cells, whilst guanethidine is not. Hence, for any given concentration of NA the chemical gradient for transport into the cell will always be greater than that for guanethidine. Alternatively, the possibility exists that the membrane has a greater affinity for NA transport, or that the two compounds enter the cell via different mechanisms. The latter consideration will be examined in further detail in Chapter 2. As has been stated, however, it seems possible that NA and guanethidine share similar routes of transport into red cells in light of the evidence presented by other workers (e.g. Blakeley & Nicol, 1978) who noted competitive interactions between the transport of other compounds structurally and functionally related to NA, such as 5-HT and histamine. It is noted that the Km for guanethidine uptake measured here is similar in magnitude to reported values for NA transport in red cells, apparently indicating a similar affinity of the cells for NA and guanethidine.

The effect of cell volume upon guanethidine transport was

not measured directly, as had been the case for NA transport. Instead, two further experiments were performed in which the concentration dependence of guanethidine uptake was measured from solutions containing varying amounts of substrate (0 - 150 mM) made isotonic to one another by the addition of appropriate quantities of mannitol.

In these experiments data also yielded good fits to the Michaelis-Menten equation. Analysis revealed that Vmax appeared unaffected (mean value 9.11 μmol ml cells^{-1} 15 min^{-1}) whilst Km was slightly increased to 30.48 mM (Table 16). It therefore seems to be the case that changes in red cell volume do not dramatically affect guanethidine accumulation, and that in this respect NA and guanethidine transport are qualitatively similar. As stated however, a rigorous examination of the effects of changes in cell volume upon guanethidine uptake was not undertaken.

Figure 10

The concentration dependence of guanethidine uptake by human red blood cells

^{14}C-guanethidine uptake was measured at 37°C, pH 7.4 from standard incubation media to which 0 - 150 mM guanethidine was added. All the points shown are means of triplicate measurements. The line joining each data point has been drawn by eye. Kinetic analysis (Wilkinson, 1961) yielded a Km of 25.45 mM and a Vmax of 8.52 μmol ml cells^{-1} 15 min^{-1}.

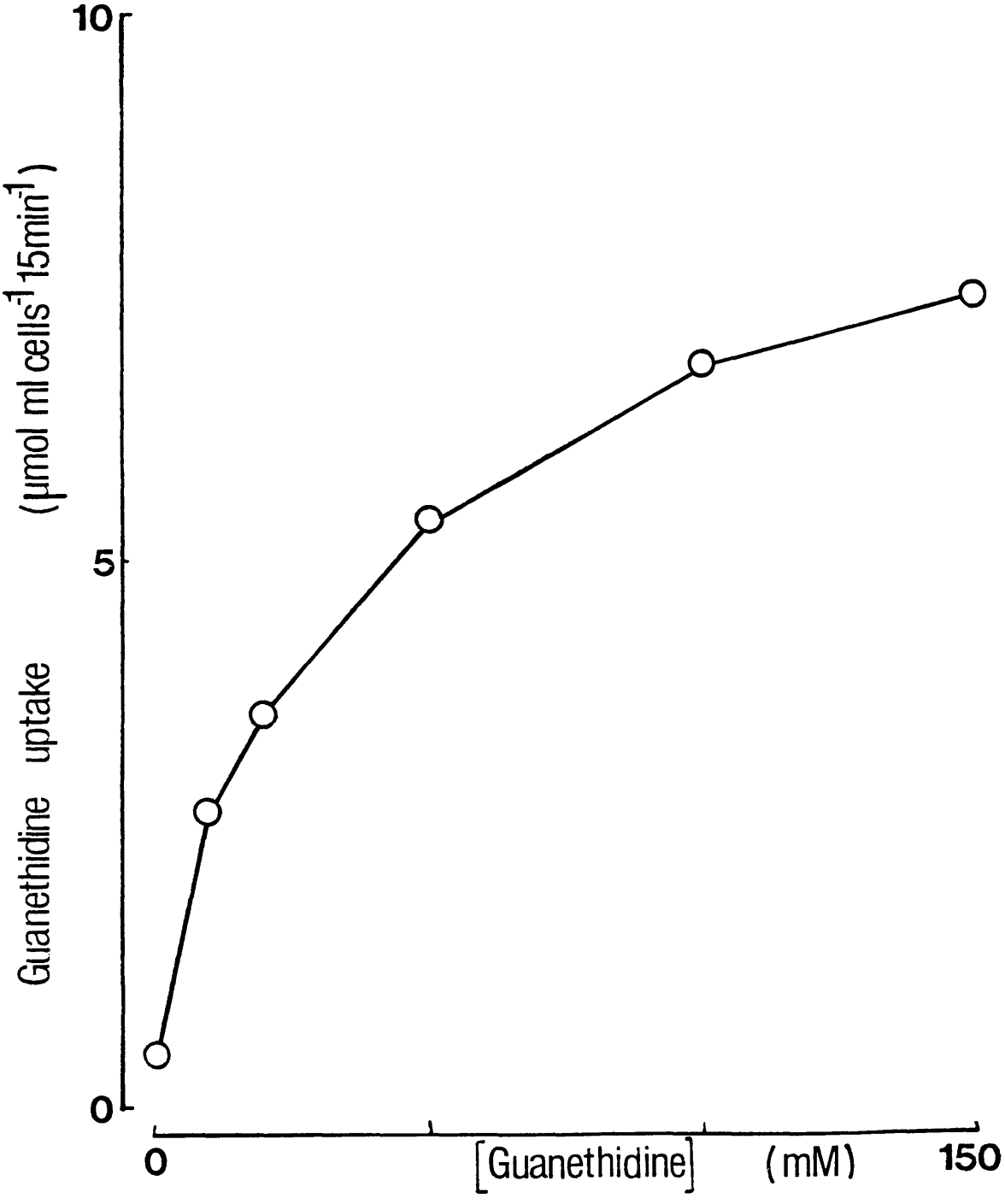

10
5
0
Guanethidine uptake (µmol ml cells^{-1} 15min^{-1})
0
[Guanethidine] (mM)
150

Figure 11

Kinetics of ^{14}C-guanethidine uptake by human red blood cells

Hanes plot of data presented in Figure 10. The line was drawn from the values of Km and Vmax obtained using the method of analysis of Wilkinson (1961). Linear regression analysis of the fit of experimental points yields a correlation coefficient of 0.997.

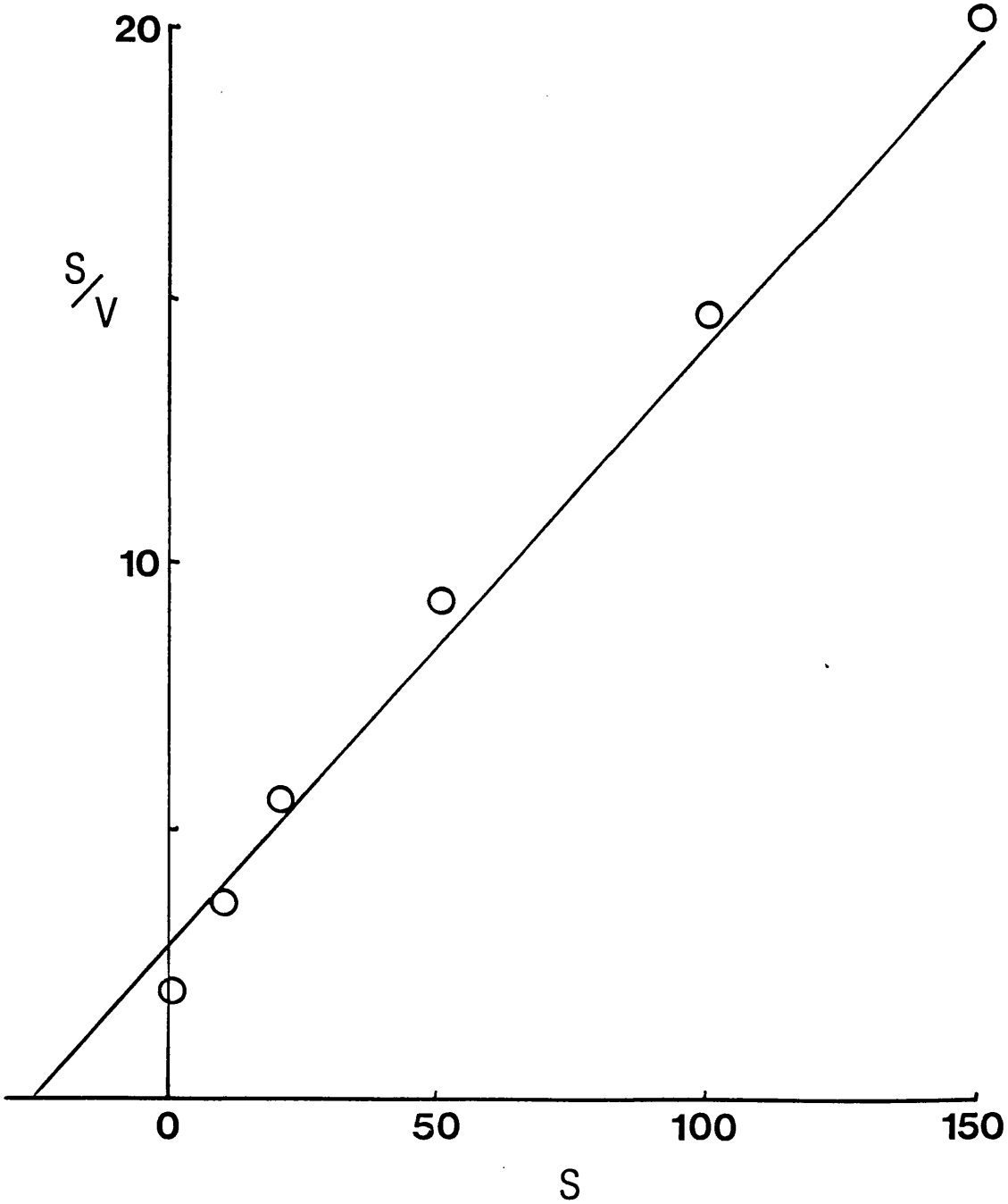

20
10
S/V
0
50
100
150
S

TABLE 16

KINETIC ANALYSIS OF GUANETHIDINE UPTAKE IN HUMAN RED CELLS

1. No osmotic balance during flux incubation

Experiment	Km (mM)	Vmax (μmol ml cells^{-1} 15 min^{-1})
1	20.60	4.96
2	24.21	13.89
3	19.08	8.69
4	13.74	10.69
Mean	19.41	9.56
(SEM)	(2.17)	(1.87)

2. Osmotic balance maintained during flux incubation

Experiment	Km (mM)	Vmax (μmol ml cells^{-1} 15 min^{-1})
1	35.50	9.70
2	25.45	8.52
Mean	30.48	9.11

Kinetic constants were obtained by the method of Wilkinson (1961).

EXPERIMENTS UTILISING RED CELL GHOSTS

Prior to studying the concentration dependence of NA uptake by ghosts in detail, a series of preliminary experiments were performed in order to assess the permeability of a typical preparation to the substrate. Figure 12 describes the transport of NA by ghosts and cells of the same origin, while Figure 13 shows a range of typical red cell NA fluxes compared to those in ghosts.

It is clear that NA uptake by ghosts is of a comparable magnitude to that measured in intact red cells, although there is a tendency towards reduced influx in ghosts. This may represent a reduction in intracellular binding capacity or metabolism. These two factors could act either separately or in unison to allow free NA_i in ghosts to rise above the levels attained in cells under identical circumstances, thus reducing the transmembrane NA gradient and consequently causing reduced uptake. It is still apparent however, from Figure 12 that the NA transporting mechanism is not saturated at substrate concentrations of up to 140 mM.

The important observation made clear from Figure 12 is that ghosting does not appear to cause red cells to become leaky to NA, and the preparation would thus seem to be a satisfactory one with which to pursue further studies pertaining to the mechanism of NA transport. NA influx at lower substrate concentrations was also measured and found to be similarly within the range of values expected from intact red cells. The uptake of 20 mM guanethidine by ghosts was also comparable to that determined in fresh red cells (see previous section). Prior to the commencement of experiments investigating the kinetics of NA transport, one further test was performed to qualitatively compare ghosts and cells.

Temperature dependence of NA uptake

Experiments previously described revealed NA uptake to be a highly temperature dependent process, to the extent that 90 - 95% of influx was inhibited by a reduction in temperature from 37°C to 4°C. By comparison, in ghosts a marginally greater percentage of

Figure 12

Comparison of NA uptake by red cells and ghosts

^{3}H-NA uptake was measured in washed red cells (closed symbols).
Units of uptake were μmol 10^{10} cells^{-1} 15 min^{-1}. Ghosts were subsequently prepared from a sample of the same cell suspension according to the method described by Richards and Eisner (1982). Uptake into these ghosts (open symbols) was then measured under identical conditions, and plotted in units of μmol 10^{10} ghosts^{-1} 15 min^{-1}. Each measurement was performed in triplicate.

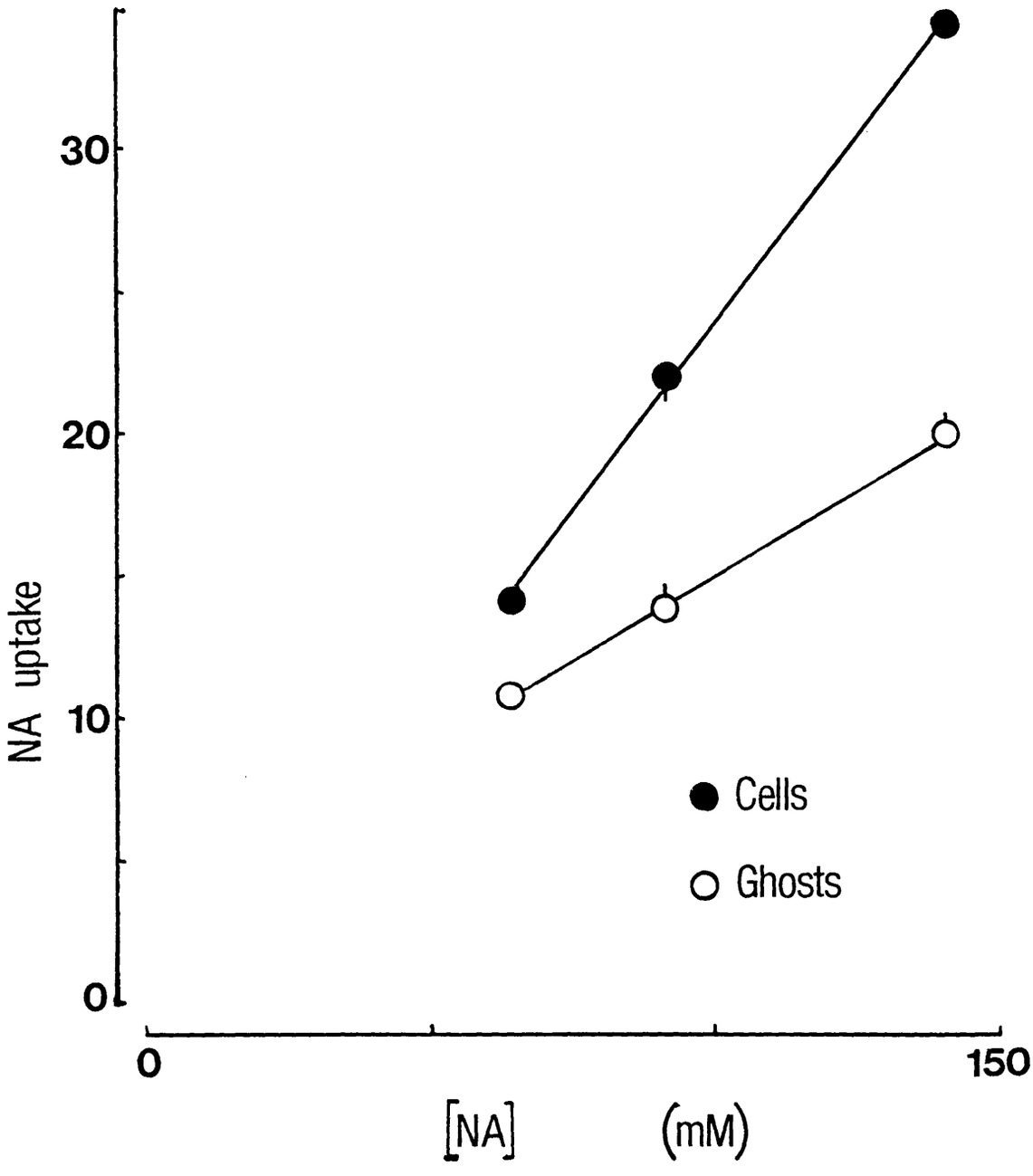
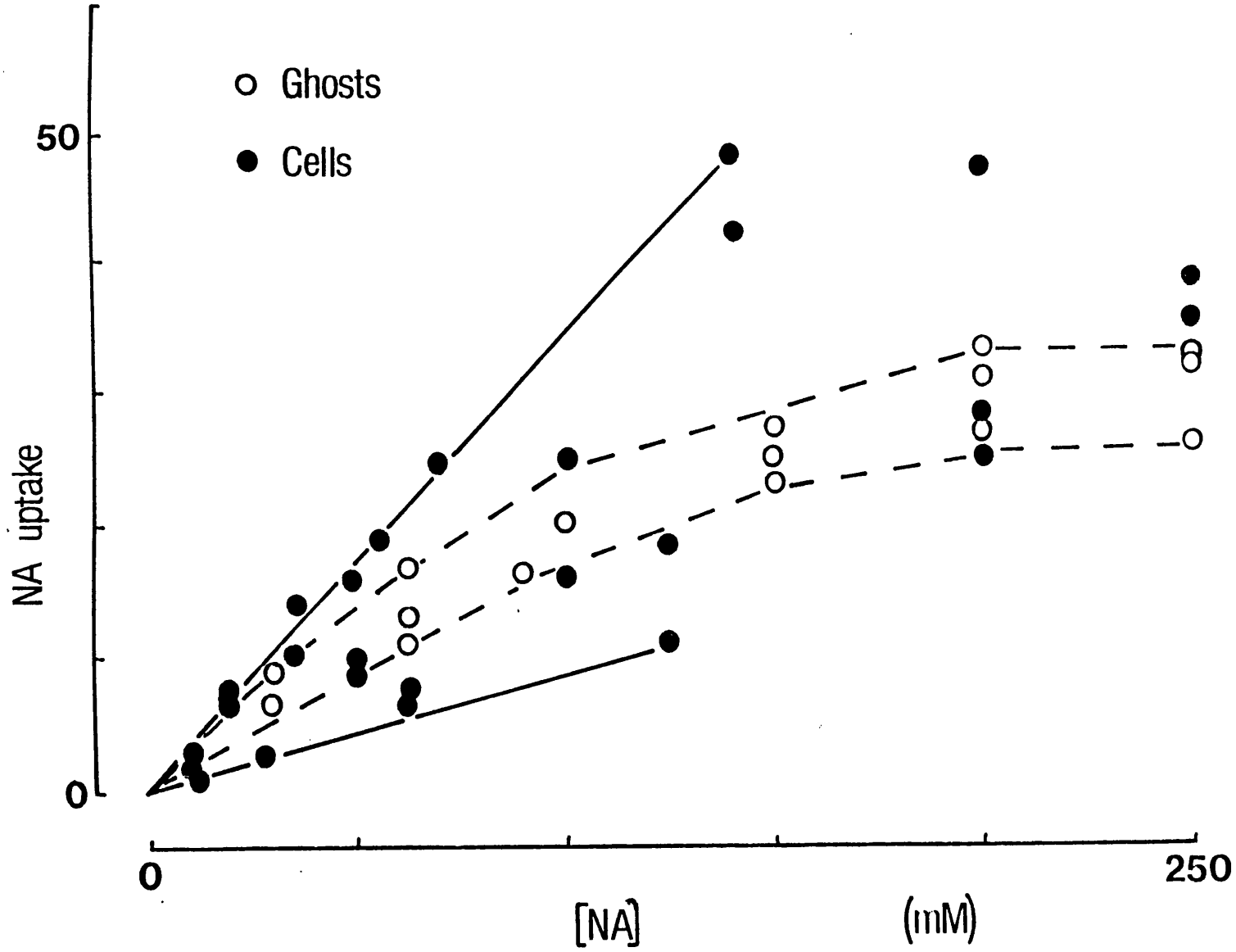

30
20
10
0
NA uptake
0
150
[NA] (mM)
Cells
Ghosts

Figure 13

Comparison of NA uptake by red cells and ghosts

Data have been plotted from a number of different experiments. Closed symbols represent cells (units of flux: μmol ml cells^{-1} 15 min^{-1}) and open symbols represent ghosts (μmol 10^{10} ghosts^{-1} 15 min^{-1}). Each point is the mean of duplicate or triplicate measurements. The lines drawn indicate the approximate ranges over which NA uptake was observed to vary.


Ghosts
Cells
50
0
NA uptake
0
250
[NA]
(mM)

uptake persisted at 4°C: cooling generally inhibited 85 - 90% of NA accumulation (n=4). Some typical data are illustrated as Figure 14.

One possible explanation for this observation could be the existence of a small number of comparatively leaky ghosts which might rapidly equilibrate with NA, even at low temperatures. This would be consistent with the observation that Type II ghosts comprised only an estimated 80% of the population, and it is possible that the remaining 20% may represent Type III ghosts which are leaky to cations and, to a lesser extent, to NA. It is clear however that, despite the small differences observed, NA uptake in ghosts at different temperatures is closely comparable to that in red cells.

Concentration dependence of NA uptake

Ghosts were exposed to concentrations of up to 250 mM NA and uptake of ^{3}H-NA measured over 15 minute periods at 37°C. These conditions were essentially the same as those employed for studies elsewhere. As for the measurement of ^{14}C-guanethidine uptake, two types of experiment were performed:

1)Incubations in which no steps were taken to osmotically balance the extracellular medium, such that tonicity increased with NA_o, and

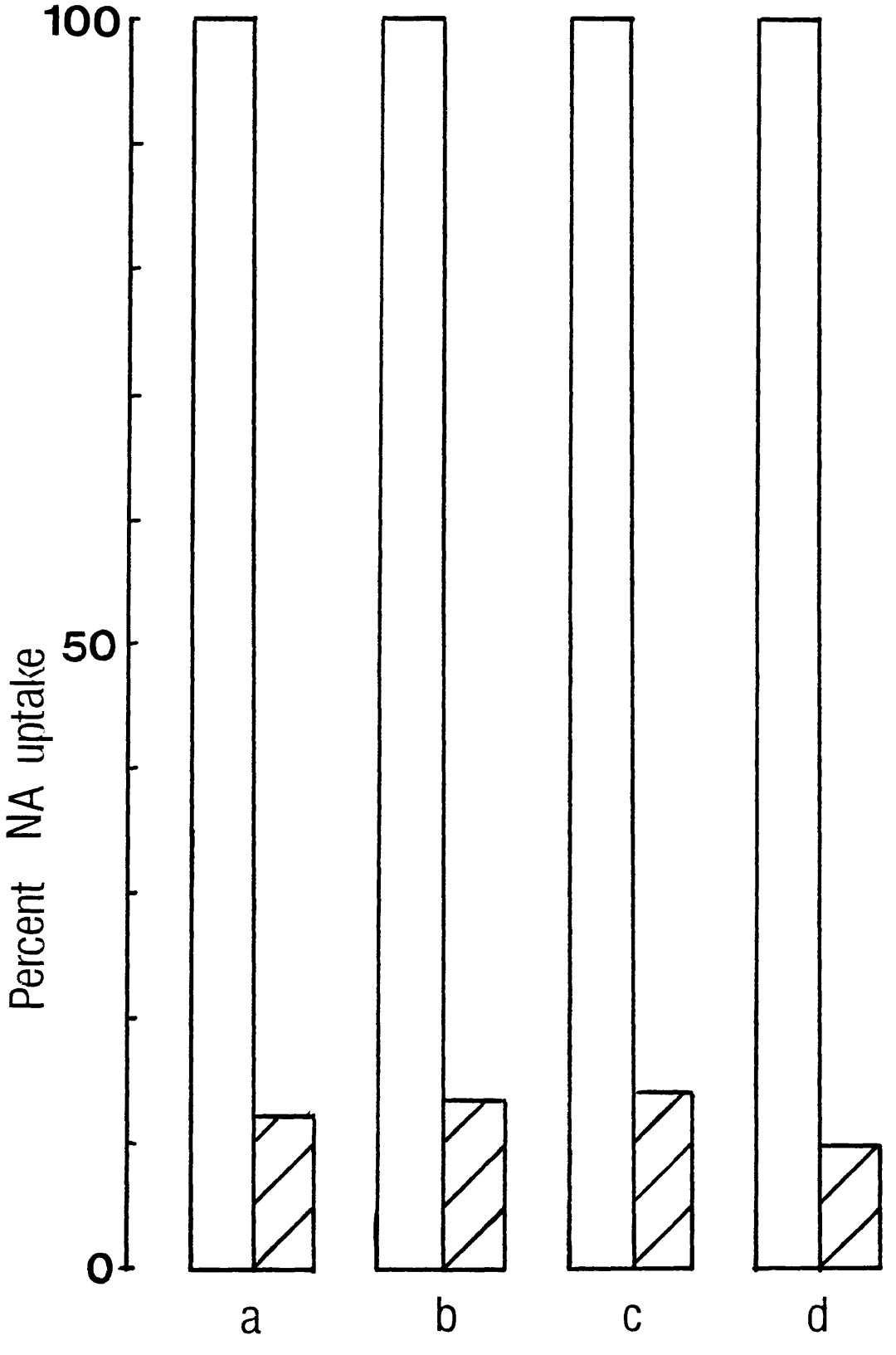
2)Incubations in which osmotic balance was maintained using sucrose or mannitol to supplement the extracellular medium. In these circumstances ghosts were suspended in solutions of varying NA_o but equal tonicity.

In both sets of experiments, results differed from work previously described involving red cells. In the experiments using ghosts, as NA_o was raised the velocity of uptake increased until a plateau (Vmax) was attained. Uptake was not linearly related to substrate concentration, but rather saturation at high NA_o (>150 mM) was observed. This result indicates the existence of some saturable system for NA uptake which would not, therefore, be consistent with a process of simple diffusion, as suggested

Figure 14

Temperature dependence of NA uptake in ghosts

^{3}H-NA uptake was measured at 37°C (open bars) and 4°C (hatched bars). The results of four separate experiments (a-d) are illustrated. Each measurement was made in duplicate and two different concentrations of extracellular NA were used. Ghosts used in a and b were suspended in media containing 100 mM NA, while those used in c and d were exposed to 62.5 mM NA.


100
50
0
Percent NA uptake
a
b
c
d

by certain previous evidence. A typical experiment is illustrated in Figure 15.

The first direct evidence for carrier mediated NA uptake by the red cell in the course of these investigations is therefore apparent. It is also noted that these results are qualitatively similar to the mechanism of guanethidine accumulation by red cells. An interesting observation is that the results differ quantitatively between experiments performed in the presence (n=6) and absence (n=10) of osmotic balance (Table 17). When extracellular osmotic balance was maintained, fluxes were generally higher such that Vmax became approximately threefold elevated. Km was enhanced to a similar degree. The kinetic parameters Km and Vmax were calculated using a direct fit method (Wilkinson, 1961). Reasonable fits were also obtained using transformations of the Lineweaver-Burk equation, for example the Hanes plot. A typical Hanes plot, derived from the data presented in Figure 15 is presented as Figure 16.

The latter result implies that ghost volume may be an important determinant of uptake, although no similar relationship could be demonstrated in intact cells.

Thus, in ghosts it appears that:

1) NA uptake is saturable and hence not mediated by simple diffusion, and

2) Ghost volume is a determinant of NA uptake.

It may therefore follow that since neither characteristic is evident in intact red cells, cellular metabolism and binding properties may profoundly influence membrane transport of NA. It is possible that either or both processes may remove NA sufficiently rapidly from the intracellular pool to allow the cell to become an infinite "sink" for NA. This option does not present itself in the case of red cell ghosts: free NA_i is able to rise and may accordingly act to modulate uptake. Hence, in ghosts, as internal volume is reduced NA_i may become elevated more rapidly thus reducing the downhill substrate gradient leading to a reduction in uptake.

TABLE 17

KINETIC ANALYSIS OF NA UPTAKE IN GHOSTS

1. No osmotic balance during flux incubation

Experiment	Km	(SEM)	Vmax	(SEM)
1	117.5	10.8	49.4	2.2
2	118.9	19.1	43.1	3.3
3	122.6	19.9	48.0	3.9
4	220.8	28.8	64.7	5.5
5	140.9	29.4	41.7	4.0
6	147.2	21.4	43.0	3.1
7	256.5	40.6	69.8	6.6
8	104.0	28.0	62.0	7.0
9	176.8	32.8	66.4	6.9
10	135.0	32.0	53.9	6.1
MEAN	154.0		54.2	
SEM	15.7		3.4	

2. Osmotic balance maintained during flux incubation

Experiment	Km	(SEM)	Vmax	(SEM)
1	749.8	147.4	145.8	26.4
2	416.8	113.5	77.4	17.2
3	344.5	113.5	63.1	14.4
4	900.8	261.2	147.6	42.7
5	761.0	193.0	155.5	42.3
MEAN	634.6		117.9	
SEM	107.6		19.6	

All values of Km are expressed as mM. All values of Vmax are expressed as μmol 10^{10} ghosts^{-1} 15 min^{-1}.
All values of Km and Vmax, and their standard errors, were obtained by the method of Wilkinson (1961).

Figure 15

The concentration dependence of NA uptake by human red cell ghosts

^{3}H-NA uptake was measured at 37°C, pH 7.4 in the presence of up to 250 mM extracellular substrate. The data was analysed according to the method of Wilkinson (1961) and observed to obey Michaelis-Menten kinetics with a Km of 141 mM and Vmax of 42 μmol 10^{10} ghosts^{-1} 15 min^{-1}. The line shown was drawn by eye and each point represents the mean of triplicate measurements. Where error bars are not shown, the error falls within the limits of the symbol.

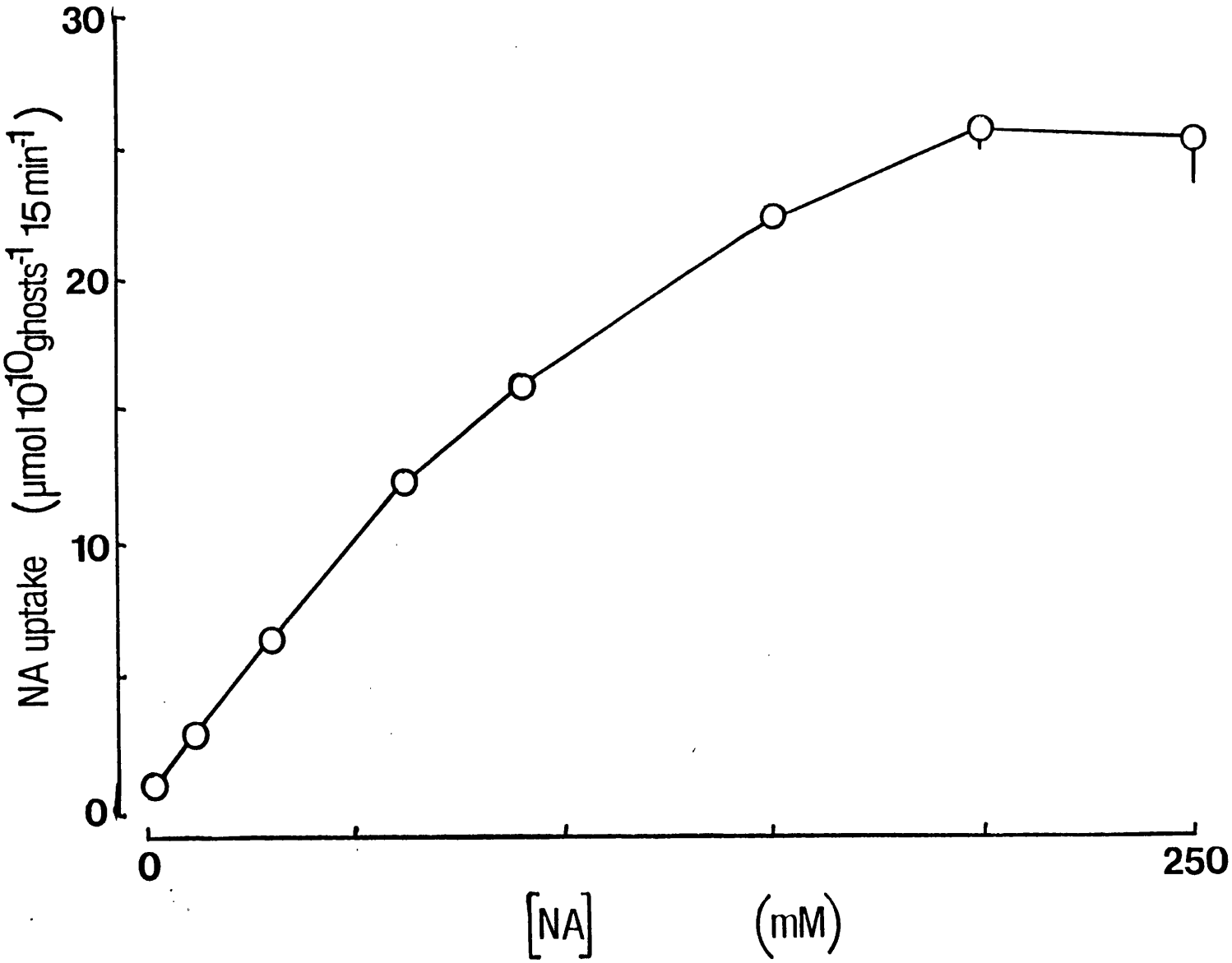
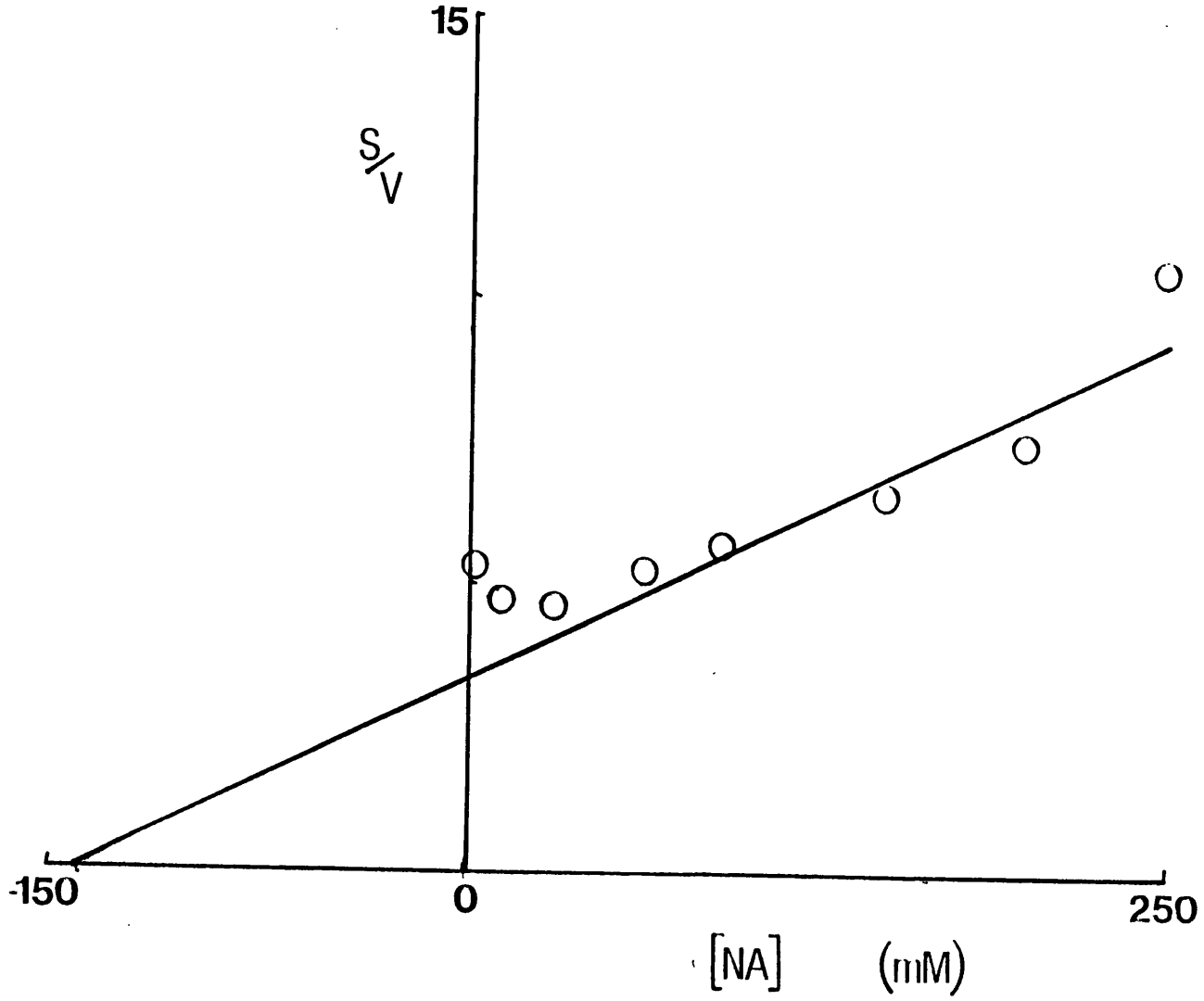

30
20
10
0
NA uptake (μmol 10^{10} ghosts^{-1} 15 min^{-1})
0
250
[NA] (mM)

Figure 16

Kinetics of ^{3}H-NA uptake by human red cell ghosts

This figure is a Hanes plot of the data presented in Figure 15. The line was drawn from the values of Km and Vmax obtained using the method of analysis of Wilkinson (1961). Linear regression analysis of the fit of experimental points yields a correlation coefficient of 0.92.


15
S/V
-150
0
250
[NA] (mM)

DISCUSSION

The results presented thus far indicate that:

1. NA rapidly enters red cells.
2. Uptake is linearly related to concentration and is shortly followed by the appearance of NA metabolites.
3. NA uptake is not a directly energy dependent process, but during prolonged (3 hour) incubations appears to be affected by disturbances in COMT activity.
4. Known inhibitors of Uptake I and Uptake II have no effect upon red cell NA handling.
5. Other adrenergic blocking agents similarly fail to modulate transmembrane NA movements.
6. Guanethidine, a very slowly metabolised analogue of NA, enters red cells by a mechanism which is saturable and obeys Michaelis-Menten kinetics.
7. When intracellular metabolism is suppressed and binding minimised (in ghosts), NA movement across the red cell membrane is qualitatively similar to guanethidine uptake.

Several conclusions may be drawn from these observations. First, it is confirmed that the mechanism of NA uptake into red cells is distinct from that present in the tissues of the sympathetic nervous system. It is also apparent that while red cells may possess adrenoceptors, that these receptors are not involved in any process mediating accumulation of amine and if there is a binding site at the membrane surface, then it would appear to be of the type proposed by Cuatrecasas, Tell, Sica, Parikh and Chang (1974).

The most interesting observation made in the course of these experiments however, concerns the apparent link between intracellular metabolism of and uptake of amine, and the masking effect of this link on the kinetics of uptake. This conclusion may be reached as the result of two separate pieces of evidence in which transport has been shown to exhibit saturation kinetics in

both of the conditions in which intracellular metabolism may not proceed, namely:

1. When metabolism is eliminated at source (in red cell ghosts) by the washout of endogenous COMT and incorporation of a specific inhibitor of COMT activity, tropolone; and,
2. When the substance entering the cell does not act as an enzyme substrate (guanethidine uptake in red cells).

It has also been demonstrated, by HPLC-ED, that treatment of intact red cells with tropolone, to inhibit COMT, retards NA accumulation at submaximal NA concentrations.

It is therefore suggested that NA may indeed enter red cells by the process of facilitated diffusion, in agreement with the findings of McQuitty and Nicol (1979), although under normal physiological conditions the carrier could not become saturated. The red cell mass may therefore act as a high capacity sink for plasma NA. It is interesting to hypothesise that in conditions such as essential hypertension, in which plasma NA may become elevated, that such rises may not simply represent increased sympathetic nervous system overflow, but also a reduced uptake by red cells due to some defect of NA transport or metabolism. This is a possibility which has been raised before. Atuk, Turner, Carey and Ayers, (1986) have also documented links between red cell NA uptake and disturbances in general catecholamine metabolism.

The high values of Km measured for both guanethidine and NA uptake indicate that the postulated carrier has a relatively low specificity for each substrate. While carrier mediated uptake is usually considered a relatively specific phenomenon, other workers have also described low affinity, saturable uptake mechanisms in the human red cell. For example, Mayrand and Levitt (1983) documented the facilitated transport of urea and ethylene glycol into the red cell: compounds which had affinity constants of, respectively, 218 mM and 175 mM. Uptake of each compound was competitively inhibited by a number of structurally related agents, and urea transport was shown to be asymmetric. A component

of urea uptake mediated by simple diffusion was also identified. It should be stressed, therefore, that the low specificity of the NA membrane transport system is thus not unique.

In conclusion, the preceding experiments would seem to suggest that at least one typical characteristic of carrier mediated uptake, that of saturation kinetics, may be assigned to the NA transporter in red cells. In addition, it would seem likely that the low affinity of this process suggests, in common with other tissues, that it might be possible to show the competition with NA of related compounds for a common binding site (e.g., see Blakeley & Nicol, 1978). This would provide further good evidence for carrier mediated uptake of NA and is a possibility which is investigated in detail in Chapter 2.

CHAPTER 2

THE EFFECTS OF STRUCTURALLY RELATED COMPOUNDS ON NA UPTAKE

INTRODUCTION

The results presented in Chapter 1 indicate that there is some evidence for carrier mediated accumulation of NA by human red cells, and ghosts, although the affinity of the carrier for substrate is extremely low. It has also been shown that a slowly metabolised analogue of NA, guanethidine, enters the red cell by a saturable mechanism. In addition, it is known that a number of other compounds related to NA (e.g. 5-HT, histamine, adrenaline, tyramine and dopamine) are all capable of crossing the red cell membrane at various rates (Born, Day & Stockbridge, 1967; Roston, 1966; McQuitty & Nicol, 1979), while the mechanism by which these compounds traverse the membrane is not fully understood. The object of the work presented in this chapter is to attempt to discern whether these compounds may be accumulated by red cells through a pathway shared with NA. This has been achieved by studying the uptake of NA by red cells and ghosts in the presence of varying extracellular concentrations of other amines. Using red cell ghosts, the effects upon NA uptake of different levels of other intracellular amines have also been investigated.

The effect of one amine on the transport of another, and the manner in which such an effect is exerted may cast considerable light upon the mechanism of movement of NA across the red cell membrane. It is possible, using suitable kinetic tools, to evaluate whether, for instance, two amines compete for a shared binding site involved in transport, or alternatively whether interactions are un- or non-competitive. Regardless of the manner of interaction, the inhibition of uptake of one compound by a related substance is not easily explained on the grounds of simple diffusion. The conclusions that may be drawn from kinetic analysis of inhibition studies are summarised in Table 18, which is taken from Engel (1981).

TABLE 18

CHARACTERISTICS OF DIFFERENT TYPES OF CARRIER INHIBITION

Type of inhibition	Inhibitor combines with	Effect on Vmax	Effect on Km	Effect on 1/V vs. 1/S plot
COMPETITIVE	E	-	↑	Convergence on ordinate
UNCOMPETITIVE	ES	↓	↓	Parallel lines
NON-COMPETITIVE	E + ES			Convergence:
i)Simple ($Ki_E=Ki_{ES}$)		↓	-	on abscissa
ii)Mixed ($Ki_{ES}>Ki_E$)		↓	↑	above abscissa
iii)Mixed ($Ki_{ES}<Ki_E$)		↓	↓	below abscissa

Despite the simple practical nature of such experiments however, investigations of red cell NA transport have often failed to probe these relationships, choosing instead to measure and compare the rates of uptake of various related compounds in isolation. The interactions of various amines have been studied in greater detail, however, in other tissues. For example, in noradrenergic neurons it has been demonstrated that dopamine is an effective substrate for the NA transport system. In mouse cerebral cortex dopamine was taken up as efficiently as NA with a Km value which was in fact slightly lower than that measured for NA (Ross & Renyi, 1966). Dopamine movements are also mediated by the so called NA transporter in isolated perfused rat heart, where the affinity for dopamine proved to be some 3 times lower than that for (-)NA, but 2 times greater than that for (+)NA (Iversen, 1963). Adrenaline is also a good substrate for the cocaine-sensitive Uptake I pathway, although its affinity is somewhat less than that of NA. As is the case with NA, the rat heart has a stereoselective preference for (-)adrenaline. Similar relationships between NA and adrenaline have also been shown in the cardiac tissues of mice, guinea-pigs and pigeons (Jarrott, 1970), but a reversed order of affinity was shown for toad heart. The latter finding may be explained on the basis that the amphibian heart contains adrenergic neurons. Reversed affinities for NA and adrenaline are also noted for the extraneuronal reserpine-sensitive Uptake II transporter, which in addition binds isoprenaline with greater avidity than NA. Alpha-methyl NA also has a greater affinity for neuronal NA binding sites than NA itself.

In the catecholamine storing vesicles of the adrenal medulla it has been shown that, as well as NA, dopamine, adrenaline and 5-HT are all taken up. The lack of specificity of this mechanism for NA is again well demonstrated by virtue of the fact that 5-HT was taken up at higher rates than any of the other compounds.

In the human platelet the uptake of ^{14}C-NA has been shown to be impaired by 5-HT, tryptamine, tyramine, guanethidine and various other compounds (e.g., Abrams & Solomon, 1969). Inhibition

was observed to be a concentration dependent phenomenon. It is interesting to note, however, that to achieve near maximal inhibition of NA uptake ($[NA]_o$ = 2.06 μM), concentrations of inhibitor in the region of 1mM were required. This is consistent with a transport mechanism of remarkably low affinity.

While compounds such as 5-HT inhibited NA uptake by up to 70%, the kinetics of inhibition were not fully investigated. It is therefore not clear whether inhibition represented competitive, non-competitive or uncompetitive interactions. Accordingly, it is impossible to assign the effects observed to either a decreased affinity for substrate binding (i.e. a change in Km), a reduced rate of carrier turnover (i.e. a change in Vmax), or a combination of both factors.

In a subsequent study, Gordon and Olverman (1978) investigated the transport of 5-HT and dopamine by human and rat platelets. These authors unveiled a relatively high affinity transporter for 5-HT (Km: in rat = 0.7 μM, in man = 1.0 μM), while uptake of dopamine was comparatively slow and involved a lower affinity process (Km: in rat = 70 μM, in man = 100 μM). It was demonstrated, however, that the presence of one compound could inhibit the uptake of the other, and that the nature of this process was competitive. The inhibition constants (Ki), defined as the concentrations of inhibitor required to half-maximally inhibit uptake of substrate, for both amines were similar to their respective Km values.

In the human red cell the nature of the possible interactions between related amines has not been elucidated. Some light has been shed upon the situation in rabbit red cells, however, as a result of the work of Blakeley and Nicol (1978), who investigated the relationship between NA and 5-HT transport in this tissue. When only (-)NA was present in the extracellular medium, uptake experiments yielded a mean apparent Km of 6.6 ± 0.4 mM and a Vmax of 0.35 ± 0.04 μmol ml cells^{-1} min^{-1}. When this series of experiments was repeated in the presence of 2.84 mM unlabelled 5-HT, NA entry was inhibited by virtue of a 60% reduction in affinity for substrate: Km rose to 10.5 ± 1.1 mM,

while Vmax remained unaltered. These observations indicated to Blakeley and Nicol that inhibition was of a competitive nature (see Table 18), and that 5-HT and NA share a common membrane binding site. Unlike the situation in the platelet, however, it was demonstrated that red cell NA transport was not affected by the presence of tyramine.

An important facet of carrier mediated transport processes is the phenomenon known as counter-transport, which occurs between competitive inhibitors of the same mechanism. Counter-transport, described for example, by Eddy (1987), is the forced movement of a substrate against its concentration gradient under specifically defined experimental conditions. The ability of a cell to counter-transport two compounds may be investigated in the following manner. First, a substrate (X) is allowed to equilibrate with a cell suspension until its intra- and extracellular concentrations are equal. If a second substrate (Y) is then chosen, either for which the carrier has a greater affinity than for X, or is added to the extracellular medium at a higher concentration than X, it should compete with X for the carriers available for inward transport. Because of this competitive inhibition of influx of X while efflux is, in the first instance, unaffected, extrusion of X may be followed against its concentration gradient as the concentration of X outside the cells rises to a higher level than that inside.

Blakeley and Nicol chose to load red cells with 5-HT because of its comparative freedom from enzymatic destruction. Addition of (-)NA to the medium bathing red cells caused ^{14}C-5-HT to leave these cells against its concentration gradient, and hence counter-transport was demonstrated.

The aims of this chapter were therefore as follows:

1. To discern whether amines which have structural similarities to NA are able to affect NA transport across the human red cell membrane.
2. If such effects can be demonstrated, are they mediated by competition for binding to some shared site, or by an alternative mechanism?

3. On the basis of this information can any structural requirements of the NA transporter be deduced?
4. If amine interactions occur, are they isolated solely at the outer facing membrane, solely at the inner facing surface, or at both?
5. What comparisons may be drawn between NA uptake in human as opposed to other red blood cells?

RESULTS

Effects of extracellular amines on NA uptake by red blood cells

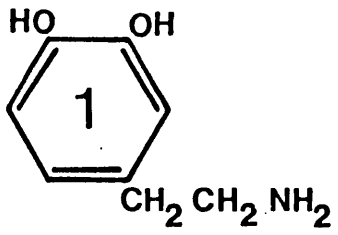
The effects of six compounds upon NA uptake into intact red cells were investigated. The compounds were chosen on the basis of their close structural similarities to NA (see Figure 17) and because most have previously been shown to accumulate within red cells. The compounds chosen were dopamine (n=1 experiment), metaraminol (n=1), guanethidine (n=1), adrenaline (n=2), 5-HT (n=2) and histamine (n=9). Of these compounds, all except dopamine effectively inhibited NA accumulation in a concentration dependent fashion and, when present at maximally effective concentrations, reduced influx to approximately 10-15% of control values. This percentage inhibition is comparable to that achieved in red cells after cooling from 37°C to 4°C (see pp. 86,89). The residual portion may therefore represent a diffusive component of uptake if it is assumed that the maximal inhibition caused by compounds such as histamine is equivalent to complete abolition of carrier mediated flux. The results are presented as Table 19 and a typical experiment is illustrated in Figure 18.

Despite its lack of effect on NA uptake at concentrations up to 10 mM in the single experiment performed, it has been postulated that dopamine enters red cells via a saturable transport mechanism. If dopamine and NA uptake are independent phenomena there would therefore seem to be some evidence for the existence of at least two facilitated transport mechanisms on the red cell membrane mediating the transfer of these compounds. This situation might be analogous to the variety of amino-acid carriers known to occur in the red cell membrane, reviewed recently by Ellory (1987). There would appear to be no obvious basis for the difference between the actions of dopamine and the other amines studied, either in terms of structure or molecular weight. Furthermore, a consideration of the pKa values of the ionisable groups on these molecules fails to suggest why dopamine should fail to inhibit NA transport.

Figure 17

Structures of NA and related compounds

1. Dopamine
2. Adrenaline
3. Metaraminol
4. Guanethidine
5. Histamine
6. 5-HT
7. NA
8. Tyramine
9. Tryptamine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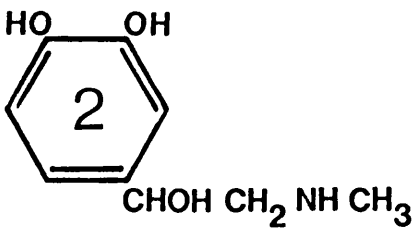
CH2 CH2 NH2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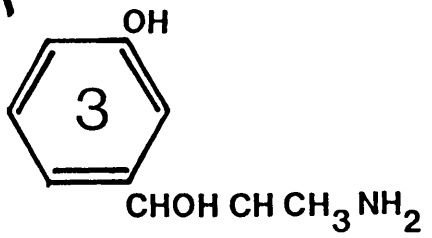
2
CHOH CH2 NH CH3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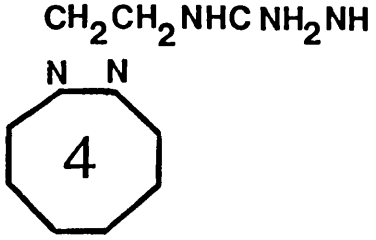
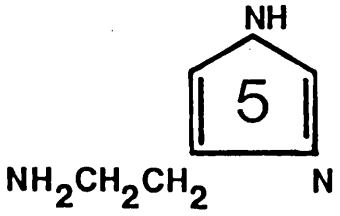
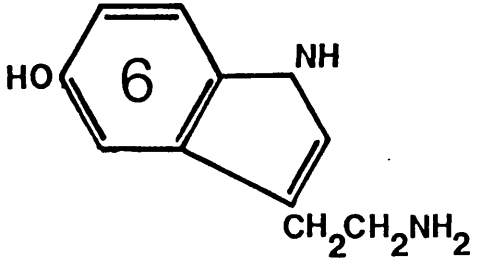
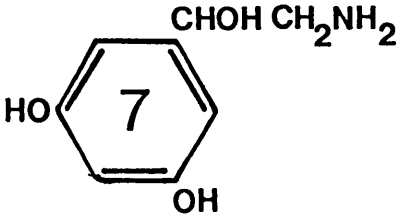
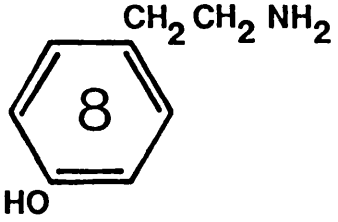
3
CHOH CH CH3 NH2


CH2CH2NHC NH2NH
N N
4


NH
5
NH2CH2CH2
N


HO
NH
6
CH2CH2NH2


CHOH CH2NH2
HO
7
OH


CH2 CH2 NH2
8
HO

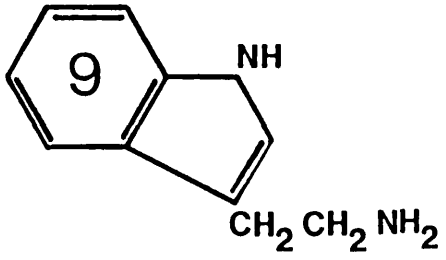

NH
9
CH2 CH2 NH2

TABLE 19

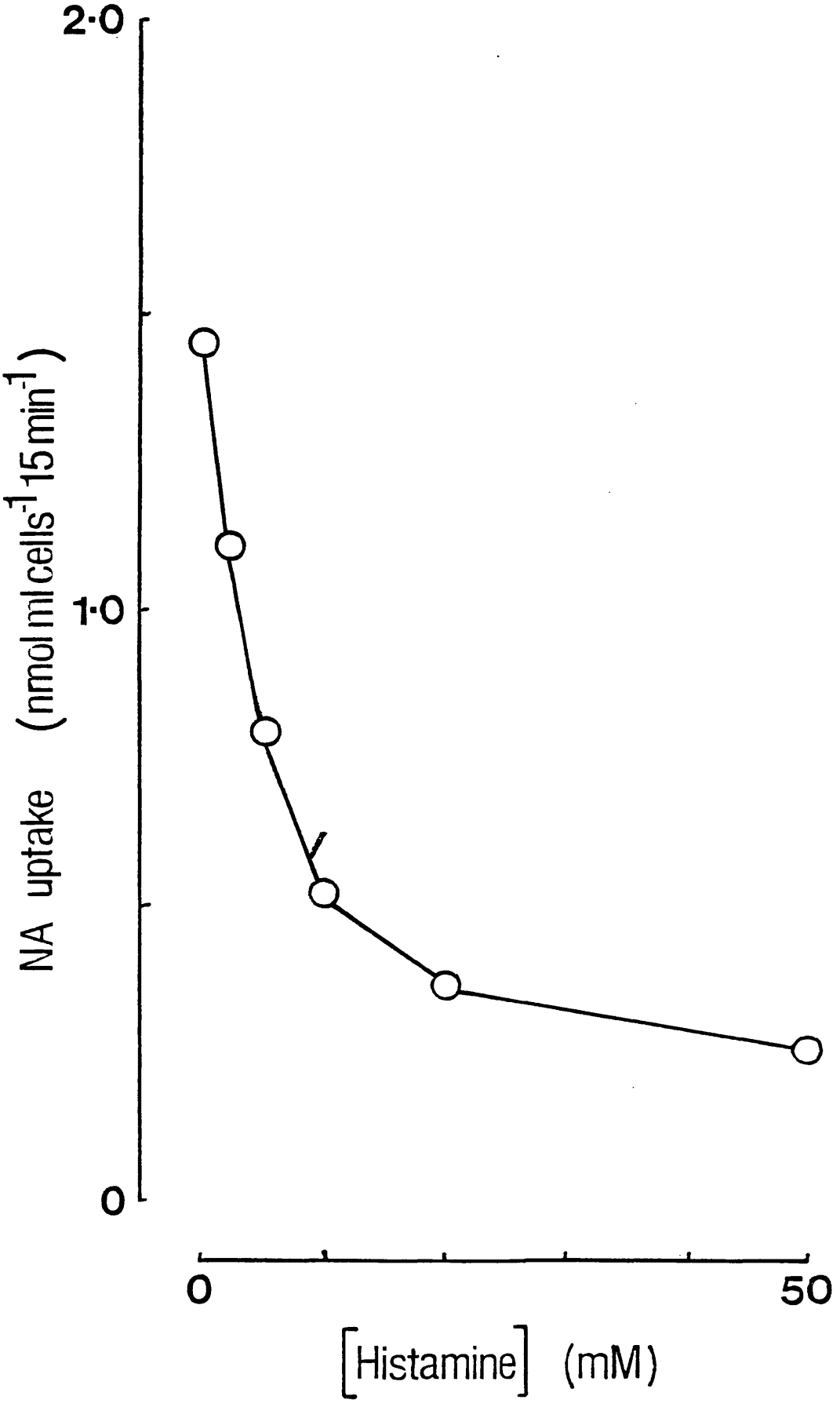
EFFECTS OF STRUCTURALLY RELATED AMINES ON NA UPTAKE

AMINE	CONCENTRATION RANGE TESTED	$[NA]_o$	MAXIMAL % REDUCTION OF UPTAKE
METARAMINOL	2 mM - 10 mM	5.39 µM	64.5
ADRENALINE	1 µM - 10 µM	100 µM	38.5
	50 µM	5.39 µM	5.3
		288 nM	7.9
		48 nM	8.3
5-HT	10 µM - 10 mM	5.39 µM	30.9
	10 mM	5.38 µM	35.0
GUANETHIDINE	10 mM - 50 mM	375 nM	20.9
		107 nM	23.4
		54 nM	19.2
DOPAMINE	10 µM - 10 mM	5.39 µM	0.0
HISTAMINE	10 µM - 1 mM	5.39 µM	15.2
	1 mM - 10 mM	5.38 µM	68.1
	1 mM - 10 mM	5.38 µM	72.3
	10 mM	5.38 µM	67.0
		279 nM	68.5
		39 nM	64.4
	10 mM	279 nM	68.9
		154 nM	63.9
		34 nM	49.8
	2 mM - 50 mM	5.38 µM	86.9
	2 mM - 50 mM	5.38 µM	82.8
	10 mM - 100 mM	5.38 µM	85.3
	10 mM - 100 mM	5.39 µM	89.8

Figure 18

Inhibition of NA uptake by related compounds

This figure illustrates the response which was typically found when μM concentrations of NA were exposed to mM concentrations of other amines. In this instance ^{3}H-NA accumulation was measured from media containing 5.38 μM NA and between 0 and 50 mM histamine. The data represent one experiment which is typical of nine performed and each point shown is the mean of duplicate determinations.


2·0
1·0
0
NA uptake (nmol ml cells^{-1} 15 min^{-1})
0
50
[Histamine] (mM)

The existence of more than one route of amine accumulation into red cells is also consistent with the work of Blakeley and Nicol (1978) and McQuitty and Nicol (1979), who worked with rabbit and human red cells respectively. In both tissues the authors concluded that, while NA uptake was carrier mediated, the influx of tyramine proceeded via a different route which was not demonstrated to saturate and presumably represented simple diffusion. In the case of the five 'active' amines considered, reduction of NA uptake only became evident when the inhibitory substance was present at concentrations in the mM range. This effect was relatively independent of NA_o which varied over a wide range within certain experiments. This suggested for the first time that the mechanism of inhibition might not be competitive in nature. This seems likely as, if the degree of inhibition caused by for example histamine is independent of $[NA]_o$, it is presumably also independent of the level of substrate occupancy of carrier sites at which competitive interactions would occur. The data also demonstrate that, irrespective of their mode of action, the inhibitors chosen have a relatively low affinity for the postulated NA transporter.

Kinetic analysis of data obtained from eight further experiments was performed using the method of Dixon (1953). This enabled the inhibition constants for histamine (n=4) and 5-HT (n=4) to be evaluated. As previously stated, the inhibition constant, Ki, is defined as the concentration of inhibitor required to half maximally inhibit NA uptake. The mean values obtained were respectively, 4.50 ± 0.79 mM and 15.07 ± 1.83 mM (see Table 20). Histamine is thus apparently a more potent inhibitor of NA transport than 5-HT, despite being taken up less quickly into red cells (e.g. Born, Day & Stockbridge, 1967). The points of intersection of the Dixon plots from which Ki values were calculated (see Figure 19, for example) fell both marginally above and below the abscissa, with a mean y-axis coordinate value of -0.021 ± 0.017. This value is not significantly different to zero, which strongly suggests that inhibition is non-competitive in nature.

TABLE 20

KINETICS OF INHIBITION OF NA UPTAKE BY RELATED AMINES

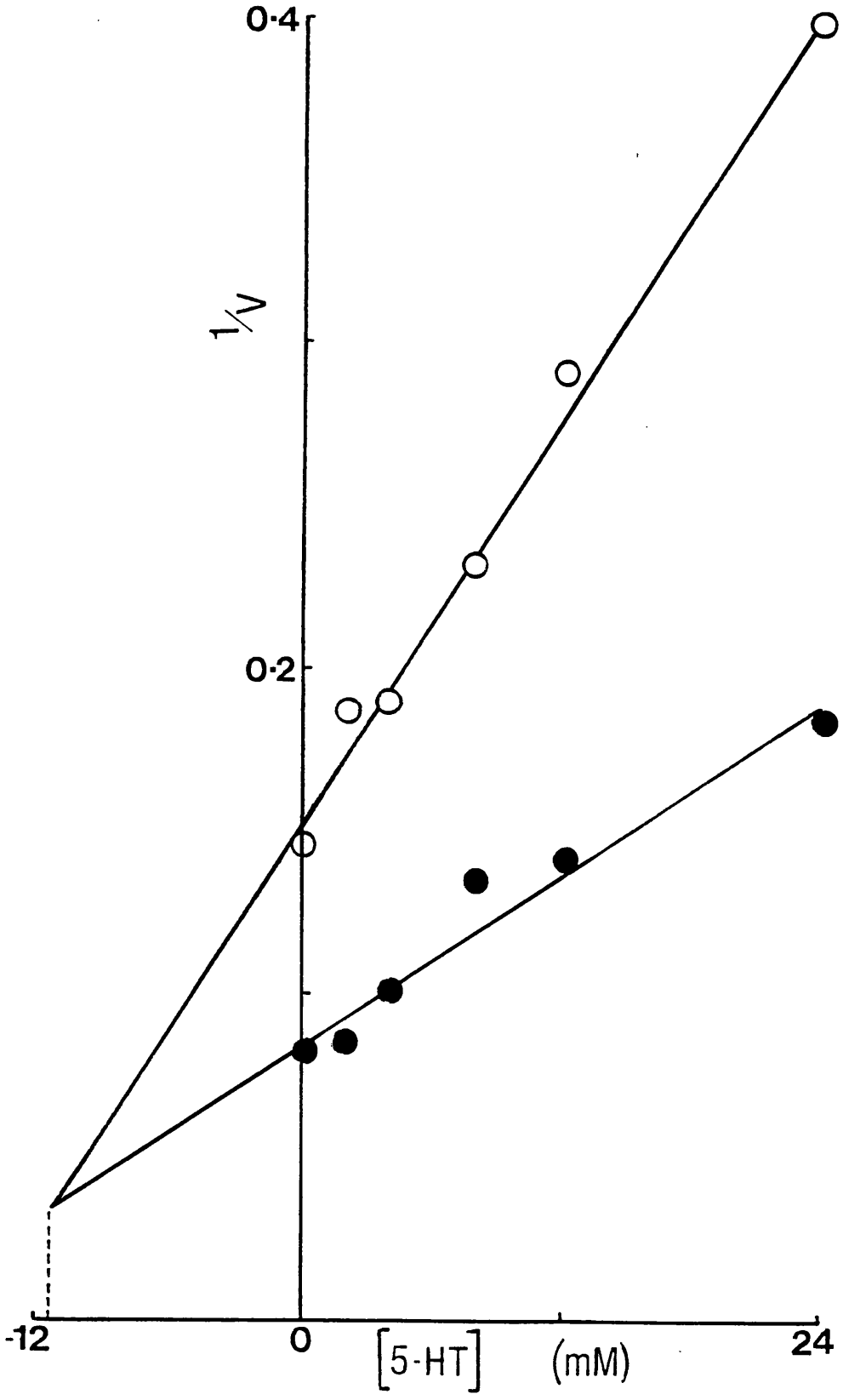
INHIBITORY AMINE	EXPERIMENT	Ki (mM)	y-AXIS INTERSECT
5-HT	1	11.67	0.057
	2	18.91	-0.009
	3	12.22	-0.035
	4	17.48	-0.107
	Mean	15.07	-0.024
	(SEM)	1.83	0.034
HISTAMINE	1	5.33	-0.059
	2	5.22	-0.022
	3	2.14	0.012
	4	5.30	-0.007
	Mean	4.50	-0.019
	(SEM)	0.79	0.015

Kinetic analysis was performed by the method of Dixon (1953). Ki is defined as the concentration of inhibitor (5-HT or histamine) required to inhibit uptake of substrate (NA) by half.
These experiments were performed at similar substrate concentrations to those described in Table 19.
The value of the y-axis intersect (see Figure 19) indicates the nature of the inhibition observed. A y-axis intersect value not significantly different from zero, as above, indicates a non-competitive pattern of inhibition.

Figure 19

Kinetics of inhibition of red cell NA uptake by 5-HT

^{3}H-NA uptake was measured from media containing 39 nM NA (open symbols) and 79 nM (closed symbols) in the presence of various concentrations of 5-HT (0 - 24 mM). The data from one experiment, which is typical of four, has been plotted according to the method of Dixon (1953), and each data point represents the mean of duplicate determinations. The lines drawn were calculated by linear regression analysis and in this instance are observed to intersect marginally above the abscissa. Downward extrapolation on the point of intersection, as indicated by the dotted line, allowed the inhibition constant (Ki) for this experiment to be estimated as 11.67 mM 5-HT.


0·4
0·2
1/V
-12
0
[5-HT]
(mM)
24

Effects of extracellular amines on NA uptake by red cell ghosts

As can be seen from Table 18, various types of non-competitive inhibition occur, and it is once again possible to distinguish between these options on the grounds of kinetic evidence. It was therefore decided to investigate the effects of a known inhibitor of influx, histamine, on the kinetics of NA transport in ghosts. To this end a preliminary experiment was performed to verify that ghosts retained the capacity to respond to inhibitory amines in the same manner as cells. Resealed ghosts were incubated in media containing 2 - 10 mM metaraminol, and NA influx was measured over the standard 15 minute incubation period. The result of this experiment is shown in Figure 20, and it can be clearly seen that the reduction in NA uptake attributable to metaraminol in ghosts is both qualitatively and quantitatively similar to that observed in intact red cells. In both ghosts and cells 10 mM metaraminol inhibited NA accumulation by approximately 65%.

Ghosting therefore, does not apparently affect the ability of the red cell to respond to this type of NA uptake inhibitor, and this result furthermore supports the notion that amine interactions are localised to the level of the membrane.

In studying the activation of uptake by NA with and without the presence of an inhibitor (histamine), it was necessary , as also had been the case with experiments detailed in Chapter 1, to test substrate concentrations of up to 250 mM. To achieve meaningful results it was also necessary to utilise considerable amounts of inhibitory amine. The final concentration of histamine used in these experiments was 50 mM.

Two experiments were performed, one of which is illustrated in Figure 21. In both experiments the effect of histamine was to reduce both Km (which fell from a mean of 381 mM to 258 mM) and Vmax (which fell from a mean of 70.1 to 30.5 μmol 10^{10}ghosts^{-1} 15 min^{-1}). Referring to Table 18, these results are once more consistent with a non-competitive pattern of inhibition. It would also seem to be the case that the effect of histamine upon NA uptake is of the mixed non-competitive form,

Figure 20

The effect of metaraminol on NA uptake by red cell ghosts

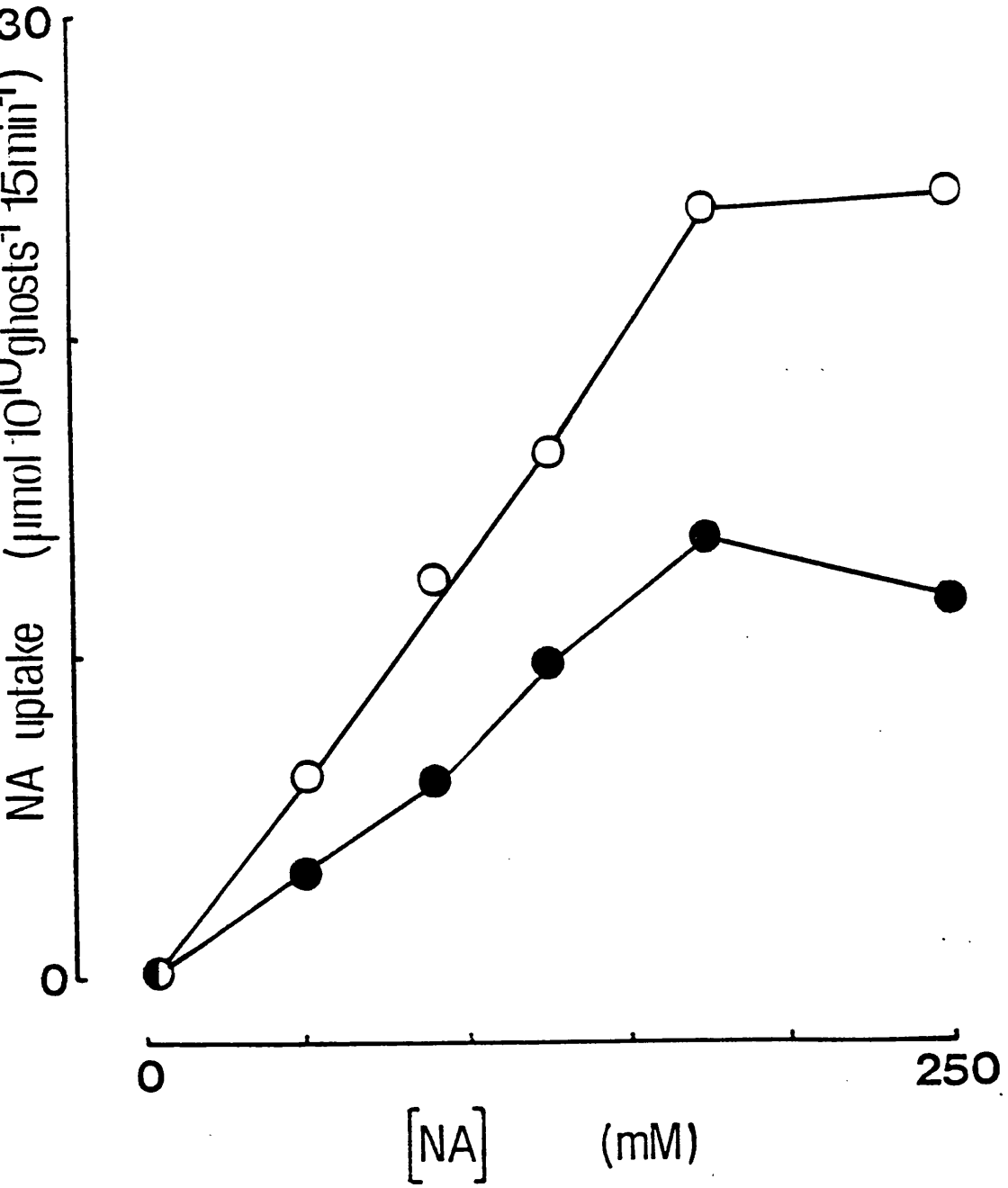
^{3}H-NA uptake by red cell ghosts was measured 37°C, pH 7.4 from media containing 5.36 μM NA and 0 - 10 mM metaraminol. Metaraminol had previously been shown to inhibit NA uptake in intact red cells in a manner qualitatively similar to that illustrated in Figure 18. Each point drawn represents the mean of triplicate measurements within one experiment and where standard error bars are not shown the error falls within the limits of the symbol.

2·0
1·0
0
NA uptake (nmol ml ghosts^{-1} 15min^{-1})
0
10
[Metaraminol] (mM)

Figure 21

The effect of histamine upon the kinetics of NA transport in red cell ghosts

The activation of ^{3}H-NA uptake in ghosts by external substrate was determined in the presence (closed symbols) and absence (open symbols) of 50 mM histamine. Two experiments were performed, one of which is illustrated in the figure. Kinetic parameters were calculated using Wilkinson (1961).
In the presence of histamine Vmax fell from 63.1 to 24.3 μmol 10^{10}ghosts^{-1} 15 min^{-1}, while Km fell from 345 to 199 mM. Each point represents represents the mean of triplicate measurements.


NA uptake (μmol·10^10 ghosts^-1 15min^-1)
30
0
0
250
[NA] (mM)

such that Ki_{ES} is less than Ki_{E}. In other words, the evidence suggests that the affinity for inhibitor is greater for the carrier-substrate complex than for the carrier alone. This would imply that the binding of substrate (NA) causes some conformational change in the membrane which enhances the ability of the inhibitor (histamine) to bind.

Implications of extracellular inhibition studies

A non-competitive inhibitor (I) is characterised by being able to bind both to a carrier (C) or a carrier-substrate complex (CS). Inhibition therefore gives rise to either CI or CSI (Figure 22), and it is assumed that the latter is unable to break down and release S. In this type of inhibition, I clearly does not bind to the same site as S and it is therefore possible, although not always the case, that the affinity of C for S may remain unaltered in the presence of I (see Table 18). Vmax is, however, always decreased. Examples of non-competitive inhibition are rather less common than examples of competitive inhibition in the case of one substrate reactions, although they naturally become increasingly more common in multi-substrate reactions, where greater numbers of binding sites may be involved. A non-competitive pattern of inhibition in one substrate reactions is however, more common than the third type of inhibition (uncompetitive) where the inhibitor binds only to the carrier-substrate complex. An example of non-competitive inhibition in a biological system, where inhibitor is bound to the membrane near the substrate site, is the inhibition by corticosteroids of sugar transport in human red blood cells (Kotyk & Janacek, 1975). In this situation the inhibitor prevents access of the substrate to carrier but is itself unaffected in its own binding to carrier by the presence of substrate.

If the implications of the results are briefly considered, it first becomes apparent that NA uptake in human and rabbit red cells is qualitatively different. In rabbit red cells 5-HT and NA compete for a common binding site on a shared carrier (Blakeley & Nicol, 1978). In the human red cell no such competition can be

Figure 22

Schematic representation of non-competitive inhibition

This figure is redrawn from Engel (1981).

Key: C = carrier

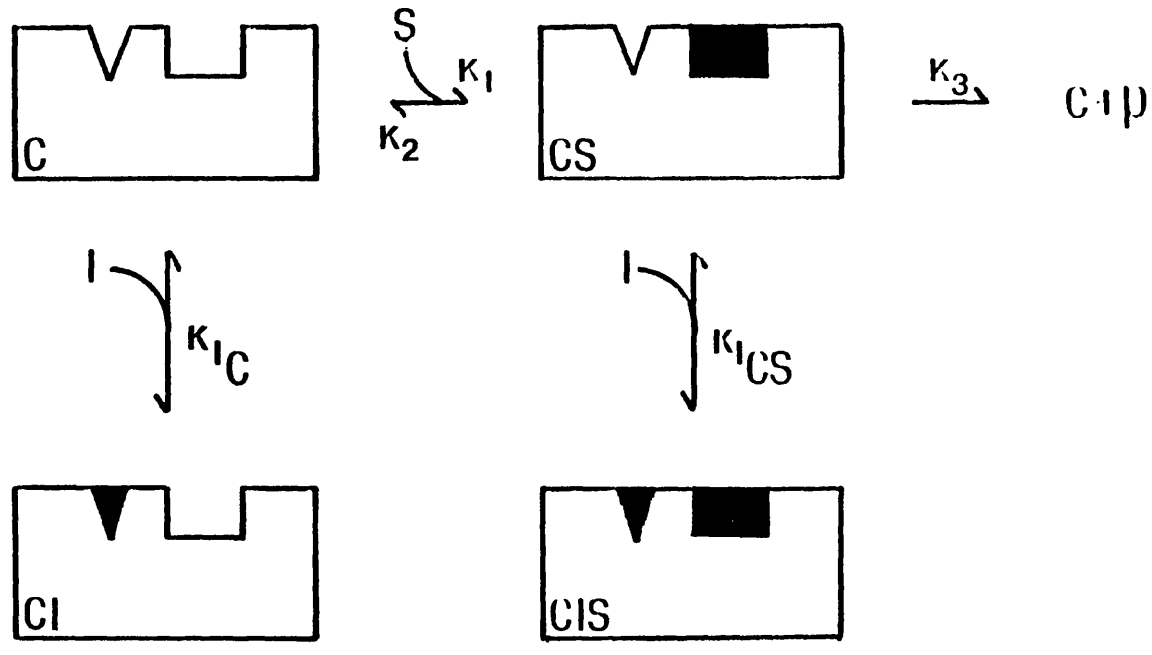
S = substrate

P = product

I = inhibitor

$K_{x,y}$ = equilibrium constants

Binding of a non- competitive inhibitor may occur both to the unoccupied carrier molecule (C) and the carrier-substrate complex (CS). In the former instance, inhibitor binding precludes substrate binding, while in the latter instance substrate release is inhibited. I and S bind to separate sites.


S
K_1
K_2
C
CS
K_3
I
K_{IC}
I
K_{ICS}
CI
CIS

observed, and it is difficult to believe that this is the result of having chosen a poorly matched group of potential inhibitors. It seems unlikely that there exists a family of compounds more closely structurally related to NA than certain of those depicted in Figure 17.

Another consequence of failing to identify competitive inhibition of NA uptake is the fact that counter-transport must remain impossible to demonstrate in this system, assuming of course that the mechanism is able to operate bi-directionally. To date no strong evidence exists to the effect that NA efflux from human red cells may be mediated by facilitated diffusion, as influx. Only Born, Day and Stockbridge (1967), Banaschak and Bluth (1978) and Zimon, Sheps, Hazelrig, Schirger and Owen (1966) have provided any evidence for the release of NA from red cells. In all three cases, NA efflux was measured by leaching NA-loaded cells in amine-free media.

The data presented by Zimon, Sheps, Hazelrig, Schirger and Owen (1966) is controversial, as these authors only measured the appearance of extracellular ^{14}C, and no rigorous attempts were made to elucidate whether or not this radioactivity truly represented NA alone. In fact, it would seem likely from the observations made in Chapter 1, that considerable portions of collected ^{14}C may have been NA metabolites. Born, Day and Stockbridge (1967), and Banaschak and Bluth (1978) did measure NA outside the cell however, although their data suggested that efflux was a significantly slower process than uptake. This might arise due to intracellular binding and metabolism of substrate, but could also represent an absence, or scarcity, of outward facing transporters. Alternatively, the carrier for NA could be bi-directional but exhibit a considerably lower affinity for substrate at the inner surface of the membrane. Clearly, the symmetry or otherwise of NA transport might therefore be the focus of considerable further attention, and to this end it was decided to perform another series of experiments designed to investigate the effects of intracellular amines upon NA uptake.

Effects of intracellular amines on NA uptake by red cell ghosts

The effects of intracellular amines upon NA uptake were studied in red cell ghosts so as to minimise problems arising from binding and metabolism of inhibitors. Ghosts were resealed to contain 1 mM NA and between 0 and 25 mM histamine or 5-HT. Isotonicity between different batches of ghosts was maintained by incorporating appropriate amounts of intracellular mannitol, and NA uptake was measured after 5 and 15 minute incubation periods from media containing 1 mM NA.

It has been shown that inhibition of NA uptake in human red cells is non-competitive, such that the binding of inhibitor to either the carrier or carrier-substrate complex precludes the binding or release of substrate, and hence, effectively, transport. If this assertion is correct then it is clearly possible that the inhibitory compounds could also bind to the intracellular facing carrier sites, thus retarding the return of the carrier to the extracellular surface, and therefore similarly acting to inhibit inward substrate (NA) movements. Therefore, a similar pattern of concentration dependent inhibition of inward NA transport could be observed, whichever side of the membrane the inhibitor acts from. Counter-transport, the driven uphill movement of a substance by an oppositely facing concentration gradient of a competitor should not occur, as previously described, in the presence of a non-competitive inhibitor.

The effects of intra-ghost histamine upon NA accumulation

Experiments were performed in order to consider the effects of both intracellular histamine and 5-HT. The majority of experiments were performed using histamine, and this was for two reasons:

1. Dixon plot analysis (pp. 116 - 118) suggested that histamine is a more potent inhibitor of NA transport than 5-HT, and
2. Histamine permeates red cells more slowly than 5-HT (eg. see Born, Day & Stockbridge, 1967) and therefore it should be possible to maintain intra-

ghost histamine at higher levels than 5-HT for longer periods of time. (The assumption was made that ghost and red cell permeabilities were equivalent, as has been shown to be the case for NA).

Five experiments were performed using ghosts resealed to contain 0, 5, 10 or 25 mM histamine. It should be noted that the concentrations of inhibitors chosen were such that, in the first instance, Ki was adequately exceeded. Each experiment indicated that, when present at the inner surface of the membrane, histamine inhibited NA uptake.

At 5 mM histamine, NA uptake fell by 4.8 ± 7.3% (after 5 minutes) and by 0.2 ± 7.2% (after 15 minutes). At 10 mM histamine, uptake fell by 18.2 ± 6.2% (5 minutes) and 10.1 ± 2.8% (15 minutes). At 25 mM histamine, uptake was reduced by 24.1 ± 6.5% (5 minutes) and 15.8 ± 5.0% (15 minutes). Therefore, inhibition was both time and concentration dependent, such that:

1. Inhibition was more pronounced after 5 minutes than 15 minutes at each concentration tested (5 mM histamine, $P<0.01$; 10 mM histamine, $P<0.0625$; 25 mM histamine, $P<0.05$; paired t-test), and
2. Inhibition was more pronounced as intra-ghost histamine was increased.

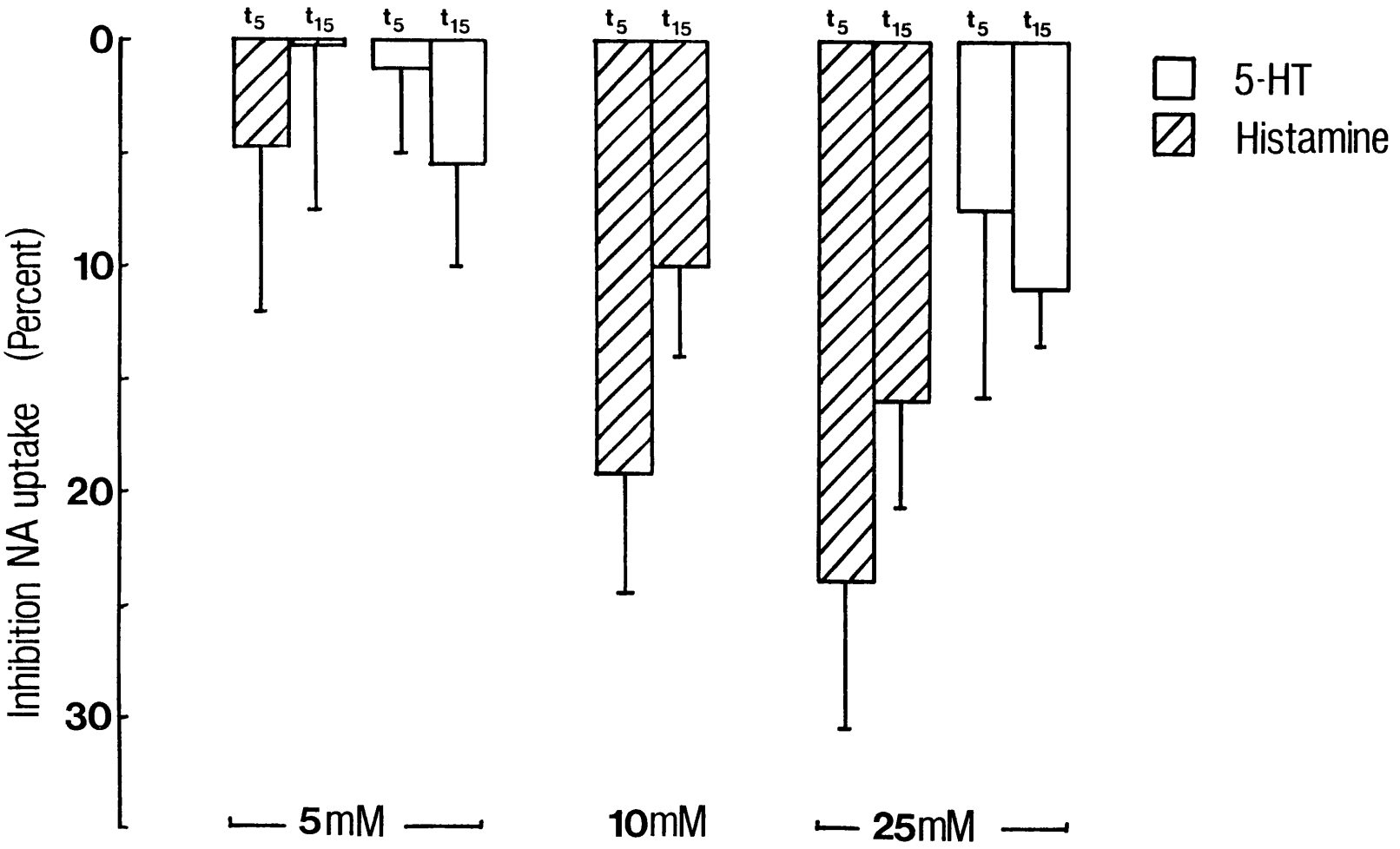
The data are illustrated in Figure 23.

The time dependence of the effect may be explained on the basis of leakage of histamine into the external medium during the course of the experiment. Consequently, after 15 minutes of incubation, the concentration of intra-ghost histamine may be less than that remaining after 5 minutes, and accordingly the degree of inhibition is reduced. The phenomenon is thus transient, which in turn suggests that the effects of histamine upon NA uptake are at least partially reversible. It is assumed that none of the effects observed were caused by extracellular amine: it seems unlikely that this would play any part in these responses because, due to the low haematocrit and the consequent dilution effects,

Figure 23

The effect of intra-ghost amines on NA uptake

^{3}H-NA uptake by ghosts was measured at 37°C, pH 7.4 after 5 and 15 minute incubations. Ghosts were prepared to contain 5, 10 or 25 mM histamine (shaded bars) or 5-HT (open bars). Internal and external NA was 1 mM in every case, and intra-ghost osmolarity was made constant in all experiments by using appropriate amounts of mannitol. Inhibition of NA uptake by intra-ghost amines has been expressed as a percentage.
The data shown are means of five (histamine) and four (5-HT) experiments.


Inhibition NA uptake (Percent)
0
10
20
30
t_5 t_{15} t_5 t_{15}
t_5 t_{15}
t_5 t_{15} t_5 t_{15}
5mM
10mM
25mM
5-HT
Histamine

extracellular concentration will remain small. The results should therefore represent interactions at some intracellular site.

The results of these experiments also tend to suggest that higher concentrations of histamine are required to block NA transport from the inner as opposed to the outer membrane surface. Histamine (25mM) at the intracellular surface caused a mean inhibition of uptake of 24.1 ± 6.5%, while at the external surface it has been shown that a greater degree (50%) of inhibition may be caused by lesser amounts of amine (Ki = 4.50 ± 0.79 mM). These two sets of experiments were not performed under identical conditions, as in cells it was not possible to expose both surfaces of the membrane to specific (in this case 1 mM) concentrations of NA. Previous evidence suggests, however, that the inhibitory effects of histamine and related amines are not profoundly influenced by [NA] (see page 115 & Table 19), consistent with the hypothesis that two separate binding sites are involved in the response. The data suggest that the NA transporter may therefore be asymmetrical in at least one aspect of its behaviour, namely the binding of histamine.

It may be argued in the above instance that ghosts loaded with 25 mM histamine might rapidly lose amine, such that the affinities for intra- and extracellular inhibition of NA uptake are, in fact, more similar than it would appear at first sight. This is not the case. If one were to assume equal velocities for influx and efflux of amine, then after 5 minutes ghosts loaded with 25 mM histamine should still contain in the region of 20 mM inhibitor. Indeed, since all the evidence available suggests that the tendency is for amines to leave cells more slowly than they enter, this estimate of 20 mM may be an underestimate.

The effects of intra-ghost 5-HT upon NA uptake

Four experiments were performed to investigate the effects of intra-ghost 5-HT upon NA uptake. The concentrations of 5-HT studied were 5 mM and 25 mM. At 5 mM 5-HT NA uptake was inhibited by 1.2 ± 3.8% (5 minutes) and 5.5 ± 4.6% (15 minutes): P=0.2039. At 25 mM 5-HT NA uptake was inhibited by 7.3 ± 8.4% (5 minutes)

and 10.9 ± 2.7% (15 minutes): P=0.3954. Statistical analyses were again performed using Student's paired t-test.

The inhibition observed with 5-HT (see Figure 23) was less marked than that obtained with histamine, as might be predicted if 5-HT is leached from the ghost very rapidly. If this hypothesis is correct, it would appear that, even after 5 minutes of incubation, most of the intra-ghost 5-HT is dissipated. This is reflected by 2 observations:

1. The 'effects' caused by 5-HT frequently fell comfortably within 5% of control values, and therefore within the bounds of reasonable experimental error, and
2. There was no significant difference between uptakes measured at 5 minutes and 15 minutes.

In light of the data obtained using histamine to inhibit NA uptake these results are not surprising. There is no reason to doubt that if sufficient quantities of 5-HT could be maintained within the ghost, then similar results would be obtained. It appears, however, that 5-HT is lost from the ghosts too rapidly to cause any significant effects upon NA uptake from the ghost interior. This observation does however, remain consistent with the hypothesis that the effects of the inhibitory amines studied are reversible, as discussed previously.

DISCUSSION

The results presented in this Chapter indicate that:

1. A number of compounds related in structure to NA are able to inhibit NA movement across the red cell membrane. This behaviour is evident both in intact red cells and ghosts.
2. The kinetics of inhibition of NA uptake by these compounds, is of the mixed non-competitive variety such that the inhibitor may bind to either C or CS, but has a greater affinity for the latter.
3. Inhibition of NA uptake may be mediated from either side of the cell membrane.
4. Binding of inhibitors would appear to be reversible, as the degree of inhibition declines as inhibitors leach from the ghosts.
5. Counter-transport could not be demonstrated, as none of the compounds tested exhibited inhibition with competitive kinetics.
6. NA transport in human red cells would appear to be qualitatively different to that described in rabbit red cells, in which NA and 5-HT compete for a common binding site.

A question which obviously arises at this stage is that, if the existence of more than one amine transport mechanism in the red cell membrane is postulated, then how many such pathways might exist? The effects of six different amines upon NA uptake have been considered in this chapter. Is it therefore the case that each of these compounds may enter the human red cell via its own specific route or, as seems perhaps more likely, do some of these other amines exhibit competitive interactions with each other? In view of the work already presented pertaining to guanethidine uptake in Chapter 1, it was considered of interest to ascertain whether:

1. Histamine might inhibit guanethidine uptake in intact red blood cells, and
2. How the kinetics of such inhibitions might be described.

The single experiment performed (Figure 24) showed that histamine was indeed able to inhibit guanethidine transport, and that similar concentrations of inhibitor were required as for studies of NA uptake. The nature of the inhibition observed was different however, as it was clearly competitive (Figure 25). Vmax was unaffected by histamine (8.67 compared to 8.52 μmol ml cells^{-1} 15 min^{-1}), whilst Km was increased by a factor of approximately 3 in the presence of the inhibitory amine (85.81 mM compared to 25.45 mM). The interactions between histamine and guanethidine therefore conform more closely to the classical expected behaviour of related compounds which cross membranes by the same route. Unfortunately it was not possible to further pursue this line of investigation due to financial limitations and the cost of ^{14}C-guanethidine. Thanks are extended to Ciba-Geigy (Basle, Switzerland), for the generous gift of ^{14}C-guanethidine, which enabled some of the work in this thesis to be carried out.

As has been previously stated, it was considered surprising in the first instance that none of the inhibitors studied appeared to compete with NA for a shared binding site. Since these compounds are all effectively accumulated by red cells it would seem likely, therefore, that there exists more than one amine transport mechanism in the red cell membrane. This situation thus shares some similarities with the rather complex picture of amino acid transport into the mammalian red cell, currently being built up, and reviewed recently by Ellory (1987).

There also exists a certain similarity in structure between the amines studied here and the amino acids. For instance, all amino acids characteristically possess a terminal -NH_2 group, a moiety which is commonly found in the NA uptake inhibitors studied in this thesis. In addition, many amino acids are also ring structures, in common with the compounds depicted in Figure 17

Figure 24

The effect of histamine on red cell guanethidine uptake

^{14}C-guanethidine uptake was measured in the absence (open circles) and presence (closed circles) of 50 mM histamine. Each data point is the mean of triplicate determinations, and the curves have been drawn by eye. Analysis, (Wilkinson, 1961) yielded Km values of 25.5 mM (control) and 85.8 mM (histamine); Vmax values were 8.52 (control) and 8.67 µmol ml cells^{-1} 15 min^{-1} (histamine). The data was transformed and redrawn as a Hanes plot - see Figure 25.

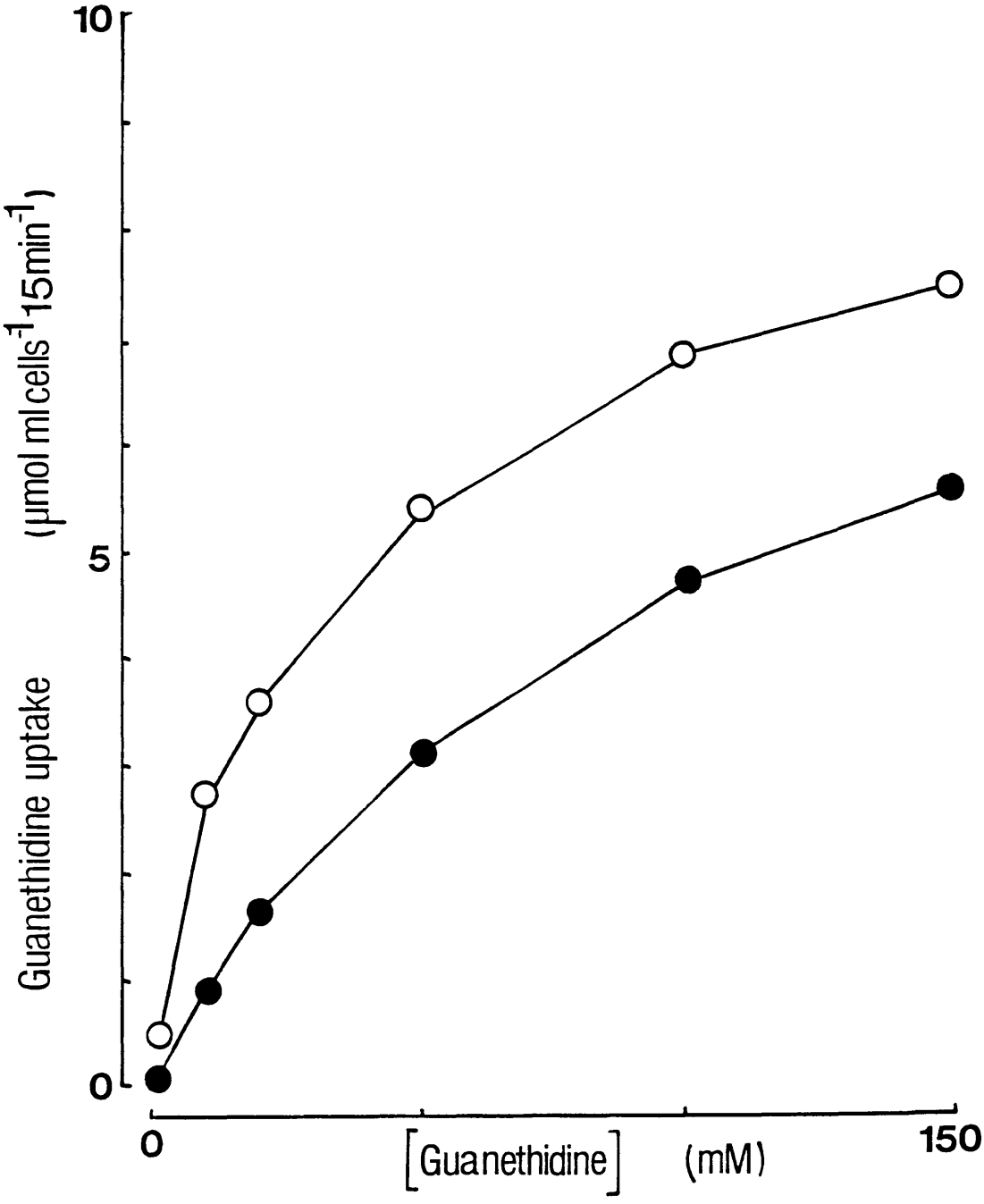
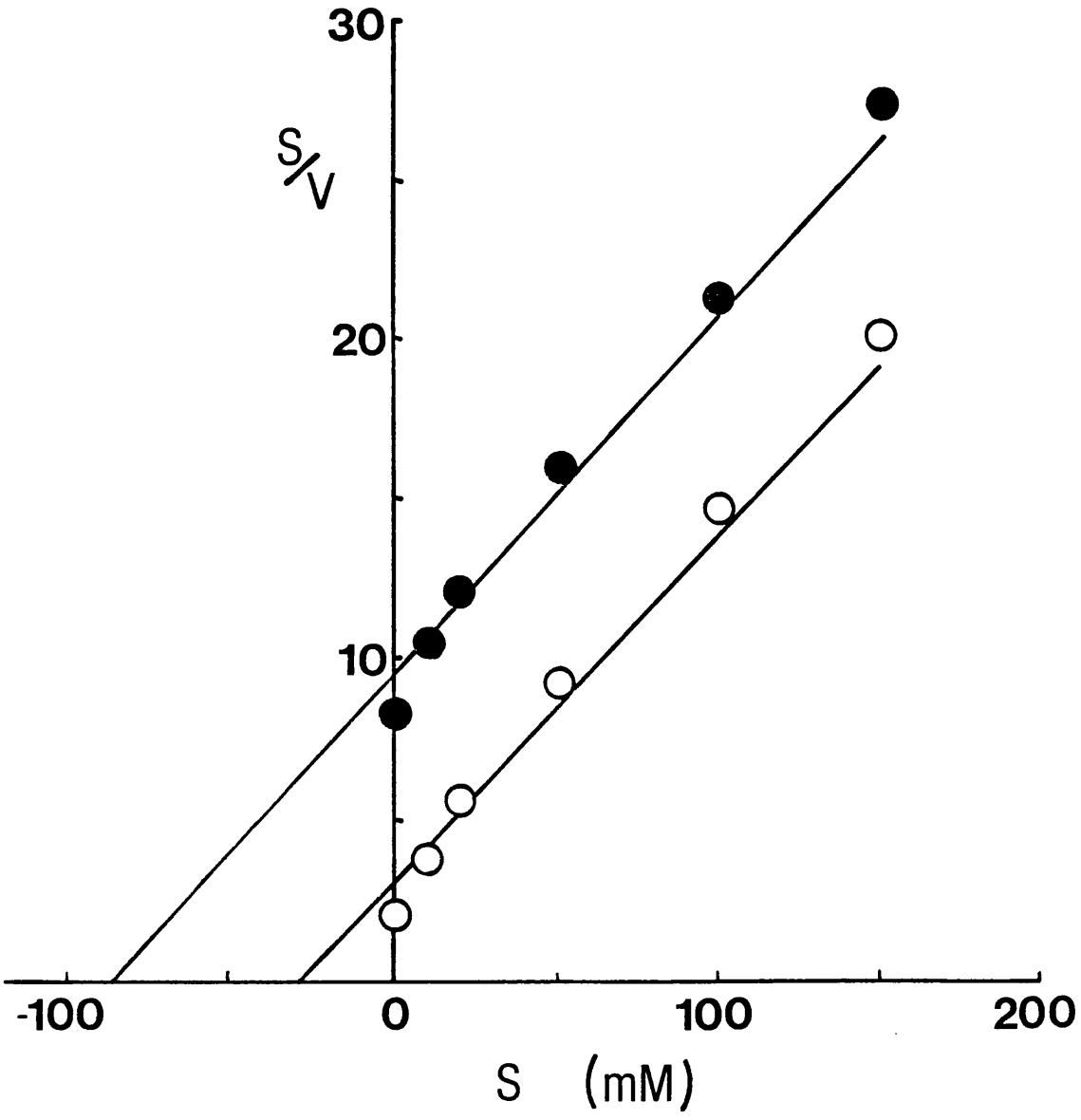

10
5
0
Guanethidine uptake (μmol ml cells^{-1} 15min^{-1})
0
150
[Guanethidine] (mM)

Figure 25

Kinetics of inhibition of guanethidine uptake by histamine

Data from Figure 24 are presented as a Hanes plot. Kinetic parameters were calculated from raw data using the method of Wilkinson (1961). The lines shown have been drawn to fit the calculated points of intersection on the ordinate and abscissa. Open circles represent control results, whereas filled circles represent results obtained in the presence of 50 mM histamine.


30
S/V
20
10
-100
0
100
200
S (mM)

(e.g. histidine and tyrosine), and may act as precursors for the biologically active amines. For example, tryptophan is a precursor of 5-HT.

The effects of amino acids upon NA uptake were not investigated, although a review of the recent literature suggests that NA and amino acid transport pathways are not shared. In general, amino acid carriers exhibit saturation kinetics with Km values in the μM range. Hence, the NA uptake process characterised in this thesis is a mechanism with a much lower affinity for substrate. In addition, the majority of amino acid carriers recognised to date transport neutral amino acids, while most NA at physiological pH exists in the cationic form.

The transport of amino acids, and many other substances, is often markedly dependent upon sodium. The cation dependence of NA uptake by human red cells is unknown and hence it was felt important to investigate this relationship, in an attempt to further elucidate the mechanism of and factors affecting NA transport in this system. These experiments are described in Chapter 3.

In conclusion, whilst the results presented in Chapter 2 pose many questions as to the precise nature of NA transport, and indicate many avenues for future study, the data remain consistent with a model of facilitated diffusion. The observed interactions of other amines are difficult to explain in terms of simple diffusion.

CHAPTER 3

CATION DEPENDENCY OF NA TRANSPORT

INTRODUCTION

Numerous studies have demonstrated that the carrier mediated transport of solutes across biological membranes may depend upon the presence of certain ionic species. It is also often the case that the movements of such solutes may be tightly coupled to those of ions, whose own downhill electrochemical gradients may provide energy capable of driving the uphill transport of the second species. For instance, the dissipation of a downhill Na gradient may drive concentrative glucose uptake in the same direction, but against its own electrochemical gradient - so called secondarily active transport (e.g. in the small intestine, Schultz, 1977). Coupled ion-substrate movements in the same direction are termed co-transport, and in the systems elucidated to date it would appear that Na is the most commonly co-transported species. Alternatively, downhill cation transport movement may drive uphill movement of a given substrate on a shared carrier in the opposite direction (counter-transport)(e.g. Eddy, 1987). In this chapter the relationships between NA uptake, transmembrane Na gradients, and the effects of Na transport inhibitors are investigated in an attempt to discern whether NA uptake in the human red cell is a cation dependent process. Attention is also paid to the effects upon NA transport of some other cations: K^+, Mg^{2+} and Ca^{2+}.

Na-dependence of NA uptake in other tissues

It has been asserted that neuronal NA accumulation is Na-dependent and that this requirement is absolute; Na cannot be effectively replaced by Li^+, K^+, Rb^+, Cs^+ or choline (Bogdanski & Brodie, 1966; Paton, 1971). It should be stressed, however, that the precise mechanism of Na-dependent NA accumulation may vary from tissue to tissue. For instance, it has been shown that removal of Na from the external bathing medium may on the one hand

decrease the maximal velocity (Vmax) of uptake without altering the apparent affinity of carrier for substrate (e.g. White & Paton, 1972), or alternatively, increase the apparent affinity (reduce the apparent Km) without affecting Vmax (e.g. Bogdanski, Tissari & Brodie, 1970).

The consequences of substrate movements in response to varying transmembrane ionic gradients include the fact that both exit and entry of NA from nerve granules may be modulated by similar protocols. For example, a reduction in $[Na]_o$ was shown to accelerate the efflux of NA from the cytoplasm of adrenergic neurons (Paton, 1973), whilst a similar reduction in $[Na]_o$ impaired the 60 second accumulation of NA (White & Paton, 1972; Logan & O'Donovan, 1974). These results are both in response to perturbation of the transmembrane Na gradient, and both yield the same net effect - a reduction of intracellular NA. Investigators should, therefore, be wary of attributing effects to unidirectional movements when, in fact, impaired accumulation may represent the sum of reduced influx coupled to accelerated efflux.

More recently, the cation dependence of NA uptake has been investigated in rat vasa deferentia noradrenergic neurons and the PC12 clonal cell line, which both possess the characteristic cocaine sensitive NA 'pump' (Bonisch, Fuchs & Graefe, 1986; Friedrich & Bonisch, 1986). In both studies the Km for NA uptake was inversely proportional to $[Na]_o$. Therefore, as the outer face of the membrane was exposed to increasing amounts of Na, its affinity for NA increased. Furthermore, in the former of the two studies (Bonisch, Fuchs & Graefe, 1986), the Km for NA release was similarly inversely proportional to $[Na]_i$.

Similar studies have also been performed in extraneuronal tissues, for example, arterial smooth muscle and collagen. (Gillespie & Towart, 1973). These authors concluded that the ionic requirements for NA uptake (Uptake II) in such tissues were of relatively low specificity, to the extent that some uptake persisted when muscle was perfused with isotonic sucrose alone. Omission of Na from the perfusate (using choline or sucrose as replacements) partially inhibited uptake, although it was

demonstrated that Li was able to completely satisfy the 'Na requirement'. Uptake II is therefore a less cation dependent process than Uptake I.

The cation dependence of NA uptake by human red blood cells has not been systematically investigated, an omission which may in part represent the previous lack of hard evidence for carrier mediated transport. Some workers have focussed their attention upon the effects of cation transport inhibitors, specifically ouabain, on NA uptake however, and these results will be discussed later in this introduction.

The K-dependence of NA uptake

The neuronal accumulation of NA (Uptake I) is dependent upon external K and is optimal at concentrations of about 1.5 - 5.0 mM (Paton, 1971). Rb and Cs may substitute for K, but not Li. The documented effects of K on NA transport suggest that variations in concentration primarily affect the maximal velocity of uptake rather than affinity. A reduction in $[K]_o$ reduced Vmax for NA accumulation in synaptosomes (White & Paton, 1972), while a large increase in $[K]_o$ decreased the Vmax for metaraminol uptake in rabbit heart slices (Sugrue and Shore, 1969). Metaraminol is a compound known to be transported by Uptake I. In both instances the apparent Km was unaffected.

Omission of K from the experimental medium also impaired intraneuronal storage of NA (Bogdanski & Brodie, 1969) and accelerated the efflux of amine from adrenergic neurons (Paton, 1973). Effects of K replacement upon net NA movements may therefore, as for the Na^+ ion, be a consequence of both impaired uptake and enhanced efflux. In addition, it should be noted that exposure of certain tissues to K-free media may significantly inhibit ouabain-sensitive Na pumping, and alterations in NA transport may arise secondarily to disturbance of the Na gradient.

In contrast, the extraneuronal uptake of NA was unaffected by omission of K_o (Gillespie & Towart, 1973), although the authors discuss the possibility that rapid K leakage from smooth muscle may make it effectively impossible to maintain a genuinely K-free

medium. High (100 mM) K, however, partially inhibited accumulation, possibly as a consequence of partial membrane depolarisation.

Ca- and Mg-dependence of NA uptake

There is reputedly little evidence to suggest that either Ca or Mg modulate neuronal NA transport (Paton, 1972). However, it has been demonstrated in adrenergic nerve granules suspended in an isotonic buffered medium containing sucrose and K phosphate (5 - 30 mM) that 6 mM Mg further enhances stimulated NA uptake attributable to K phosphate. Ca, on the other hand, tends to increase release and inhibit uptake (von Euler, 1980).

The effect of K phosphate is biphasic - NA uptake is greater in 30 mM phosphate than isotonic phosphate - and it has been shown that Mg is also only effective in the lower range of phosphate concentrations. It has been suggested that since Mg is necessary for ATP-dependent uptake, that the effect of Mg in such a medium is in some way associated with ATP in the granules. This is consistent with the observations of Colburn and Maas (1965), who predicted that ATP-metal-NA complexes might be of considerable biological significance at physiological pH, as long as [Mg] exceeded [NA].

The effect of Ca is more obscure and complicated by the fact that, when added to phosphate-containing media, turbidity or precipitation may occur in conjunction with increased rates of release.

Effects of Na transport inhibitors

If NA uptake was Na dependent and the transmembrane Na gradient was therefore of regulatory significance to this process, then it might be expected that a disturbance of this gradient by cation transport inhibitors would affect NA movements. This theory was originally tested by Dengler, Michaelson, Spiegel and Titus (1962), who were able to demonstrate inhibition by ouabain of NA accumulation in adrenergic tissues. This finding has been confirmed by numerous subsequent studies. The action of ouabain is

non-competitive (Maxwell, Ferris & Burcsu, 1976) and is effected by an elevation of Na_i, while Na_o remains constant or falls. Hence the inward downhill Na gradient is reduced.

A necessary consequence of this hypothesis is that the action of ouabain is time dependent, which was verified by Tissari, Schonhoffer, Bogdanski and Brodie (1969). Furthermore, the accumulation of metaraminol by rabbit heart slices was more sensitive to inhibition by ouabain than that occurring in rat heart slices (Giachetti & Shore, 1966), a finding that parallels the ouabain sensitivity of the Na,K-ATPases in the two preparations.

Because of the ensuing increase in Na_i, ouabain treatment should also theoretically increase efflux of NA, an observation made at both granular (Bogdanski & Brodie, 1969) and extra-granular (Paton, 1973) intraneuronal sites. It should be noted however, that an induced inward Na gradient did not produce a net inward flux of metaraminol in ouabain treated slices (Paton, 1971) and hence, while the evidence concerning the action of ouabain is copious, care should be exercised in interpreting experimental results. It appears that not all results may be simply attributable to alterations in the Na gradient.

Various authors have studied the action of ouabain on NA uptake by red cells. Danon and Sapira (1972) and Bluth and Banaschak (1976), both found no effect of ouabain on the interactions of NA with the human red cell, while Blakeley and Nicol (1978) described a small (percentage not quoted) inhibitory effect of ouabain on NA accumulation by rabbit red cells. A possible reason for the negative results quoted in these studies could be that the length of time for which the cells were exposed to ouabain was insufficient to induce significant changes in the Na gradient. Hence, in the following experiments, the action of ouabain was studied both in the short and long term, as were the effects of other inhibitors of Na transport.

RESULTS

EXTRACELLULAR CATION MANIPULATIONS

Na replacement experiments

The experiments to be described were performed using N-methyl-D-glucamine (NMDG) to isosmotically replace extracellular Na. Despite certain limitations in common with other supposedly inert organic cations (e.g. trimethylphenylammonium, arginine and choline) NMDG is currently the Na substitute of choice (Blackstock and Stewart, 1986).

It was clearly seen that the effect of Na substitution upon NA uptake was dependent upon the extracellular catecholamine concentration. When NA_o was high (in the mM range), where saturation kinetics for uptake in ghosts have previously been demonstrated, replacement of Na inhibited uptake by red cells as shown in below:

	NA uptake (μmol ml cells^{-1} 15 min^{-1})		
$[NA]_o$	145 mM NaCl	145 mM NMDGCl	% change
250 mM	41.93	36.18	↓ 13.7
200 mM	31.19	26.44	↓ 15.2
125 mM	23.66	21.45	↓ 9.3

(All data are means of triplicate estimations).

These observations are consistent with the existence of a Na dependent mechanism for NA transport, but it should be noted that the percentage of total influx dependent upon Na_o was only of the order of 10 - 20%, and that to see this response Na_o had to be almost entirely replaced. There was no graded effect upon NA accumulation as Na was varied between 0 - 145 mM.

In contrast, at intermediate NA concentrations (from approximately 250 nM to 10 μM) the effect of Na substitution upon NA uptake was different. In these experiments uptake appeared to be either unaffected or stimulated in the presence of NMDG and was

therefore seemingly inversely concentration dependent with regard to Na_o. In two experiments out of five conducted a stimulation of between 10 and 30% of NA uptake was observed.

The extent of this Na dependent effect became much enhanced as NA_o was further reduced, in a third series of experiments, to the lowest concentration possible allowing for the specific activity of the isotope. The final concentration achieved was 49 nM. In total four experiments were performed in which Na replacement was observed to bring about a large stimulation of uptake as shown below:

		NA uptake (pmol ml cells^{-1} 15 min^{-1})		
	$[NA]_o$	145 mM NaCl	145 mM NMDGCl	% change
	49 nM	7.68	12.46	↑ 62.2
	49 nM	7.35	11.66	↑ 58.6
	49 nM	7.36	17.97	↑ 144.2
	49 nM	7.94	18.91	↑ 138.2
MEAN		7.58	15.25	↑ 100.8
(SEM)		(0.84)	(1.86)	(23.4)
				P<0.02

There seems to exist, therefore, a dramatic effect upon NA uptake when Na_o is replaced by NMDG at lowest NA_o concentrations investigated. The magnitude of this effect is reduced as NA_o is increased, and at the highest concentrations considered the qualitative nature of the response is also altered. The stimulation in NA uptake noted at low concentrations in Na-free media becomes an inhibition. The possible implications of these results are discussed elsewhere.

K replacement experiments

The majority of incubations performed in this thesis were in media containing 5 mM KCl. In experiments designed to determine the effects of different concentrations of K upon NA uptake in intact red cells, K was either absent or present at various levels

by varying extracellular NaCl, normally 145 mM, which accordingly ranged between an upper limit of 150 mM (K-free incubations) and a lower limit of 100 mM. Previous results suggested that complicating effects of Na substitution did not become evident until Na_o was reduced considerably below this point. It was observed (Table 21) that neither removal nor supplementation of K_o affected uptake, and it therefore appears that NA transport in red cells is not a K specific process.

Effects of Ca and Mg

Flux incubations were routinely performed in the nominal absence of extracellular divalent cations. In three experiments, at various $[NA]_o$ (5.36 μM - 1 mM) incubation media were supplemented by the addition of Mg (1 mM) and/or Ca (2.5 mM). Both were added as chlorides. No effect upon NA uptake was observed (Table 22). It therefore appears that NA uptake in human red cells is not dependent upon Ca or Mg, and is not affected by the presence of physiological levels of either ion.

INTRACELLULAR CATION MANIPULATIONS

In light of the results obtained in the previous section in which reversal of the Na gradient appeared to affect red cell NA uptake, a second series of experiments was performed. The object of these studies was to attempt to further probe the effect by loading cells with Na to levels far in excess of normal physiological amounts. This was achieved by exposing cells to the ionophore nystatin as described in Methods.

NA uptake could then be measured in the presence of both large inwardly and outwardly directed Na (and K) gradients, which was not possible in fresh red cells. Two populations of cells were therefore produced: those rich in Na (at the expense of K), and those rich in K (at the expense of Na). K was used as both the intra- and extracellular Na substitute in these experiments due to the uncertain nature of the interactions between NMDG and nystatin. The characteristics of these cells are also described in Methods.

TABLE 21

EFFECT OF VARYING $[K]_o$ ON ^{3}H-NA UPTAKE

Experiment	$[NA]_o$	$[K]_o$ (mM)	NA uptake
1	5.35 μM	5.0	0.98
		50.0	1.01
2	5.35 μM	0.0	1.56
		5.0	1.57
	250 nM	0.0	77.82
		5.0	74.04
	10 nM	0.0	2.90
		5.0	2.84

Units of flux:
$[NA]_o$ = 5.35 μM: nmol ml cells^{-1} 15 min^{-1}.
$[NA]_o$ = 10 nM, 250 nM: pmol ml cells^{-1} 15 min^{-1}.

All measurements are means of duplicate determinations. In Experiment 1, additional measurements were made at intermediate K_o concentrations. These were indistinguishable from values obtained at 5 mM and 50 mM K_o.

TABLE 22

THE EFFECT OF CALCIUM AND MAGNESIUM ON ^{3}H-NA UPTAKE

Time of incubation	Control	+1 mM $MgCl_2$	+2.5 mM $CaCl_2$
15 min	1.87	1.97	1.97
45 min	3.07	3.05	3.05

Flux measurements were made from solutions containing 5.36 µM NA.
Units of flux: nmol ml cells $^{-1}$ 15 min^{-1}.
All points are means of duplicate determinations.

NA uptake by nystatin treated cells

NA uptake was measured in both 'high Na' and 'high K' cells. For both populations three conditions were investigated:

1) NA uptake from a physiological medium (145 mM NaCl, 5 mM KCl).
2) NA uptake from a Na-free medium (150 mM KCl).
3) NA uptake from a K-free medium (150 mM NaCl).

Hence a wide variety of different cation gradients were imposed on the cells. In addition, uptake under each condition was measured at three different NA concentrations. A total of four experiments were performed.

The results showing NA uptake into nystatin treated cells are illustrated in Table 23. Essentially, none of the variations in Na and/or K gradients appeared to affect NA uptake in these cells. For instance, although mean uptakes measured in high Na (K-free) media appeared greatest in both 'high K' and 'high Na' cells, this was not a consistent observation in each experiment.

Hence, there would appear to be no obvious Na dependence of NA uptake when K is used as the substitute cation. Neither is there any K dependence of NA uptake when Na is used as a replacement. This result clearly contrasts with the effects of replacing Na_o with NMDG and the implications of this finding are considered in the Discussion section of this chapter.

With regard to the possible Na dependence of NA accumulation, however, it was interesting to note that, despite the increased passive permeability of these cells to Na (see Methods), NA fluxes were within what would be considered the normal range for red cells. This finding suggests that the route of NA uptake is unlikely to be shared with the Na leak pathway unveiled by nystatin.

TABLE 23

NORADRENALINE UPTAKE BY NYSTATIN-TREATED RED CELLS

Incubation medium:	100 μM NA_o			1.04 μM NA_o			43 nM NA_o		
	A	B	C	A	B	C	A	B	C
HIGH K CELLS	15.48 (0.42)	14.34 (0.58)	14.58 (0.27)	0.182 (0.008)	0.167 (0.007)	0.156 (0.007)	7.92 (0.56)	6.84 (0.23)	6.68 (0.19)
HIGH Na CELLS	15.23 (0.48)	14.65 (0.67)	14.30 (0.39)	0.169 (0.005)	0.163 (0.004)	0.149 (0.004)	7.14 (0.13)	6.65 (0.15)	6.28 (0.19)

All values are means from four experiments. Standard errors are shown in brackets.

Units of ^{3}H-NA flux: 100 μM and 1.04 μM NA_o: nmol ml cells^{-1} 15 min^{-1}.

43 nM NA_o: pmol ml cells^{-1} 15 min^{-1}.

External cation conditions: A - 150 mM NaCl; B - 145 mM NaCl/5 mM KCl; C - 150 mM KCl.

Cation dependence of NA uptake in ghosts

In an attempt to derive kinetic data pertaining to the apparent Na dependence of NA uptake witnessed in NMDG media, uptake was measured in ghosts suspended in either:

1. Na media, or
2. Na-free (NMDG) media.

Similar experiments were also performed, using intact cells, to investigate the possible Na dependence of uptake of the related compound guanethidine, which has already been demonstrated to enter red cells by a saturable mechanism.

Two initial experiments were performed to study the effects of Na substitution on NA uptake at substrate concentrations which ranged from 3 - 62.5 mM. Using K as the substitute cation, no effect upon uptake could be observed. Conversely, ghosts suspended in NMDG media, both at similar and greater substrate concentrations, showed an inhibition of uptake which was similar to that observed in intact cells.

A total of 4 experiments were performed to elucidate the effects of Na substitution on the kinetics of NA uptake. The data is summarised in Table 24, and a typical experiment is illustrated in Figure 26.

The effect of replacing Na_o was primarily upon the apparent affinity for NA uptake, which was reduced in NMDG media. This was represented by an approximately 50% increase in the value of Km from 180 ± 35 mM (Na media) to 296 ± 63 mM (NMDG media) ($P<0.02$). The Vmax for uptake was not significantly altered by substitution of NMDG for Na. The Na dependent fraction of uptake (calculated as the difference between fluxes measured in the presence and absence of Na) did not exhibit any conventional concentration dependence with regard to NA and in most experiments was biphasic. Na dependent uptake rose to approximately 150 mM NA_o, above which point a marked decline in flux was observed.

The results suggest that, as in intact red cells, replacement of Na_o by NMDG reveals a Na dependent uptake mechanism which is not apparent when K is used as the extracellular substitute.

TABLE 24

SODIUM DEPENDENCE OF ^{3}H-NA UPTAKE BY GHOSTS

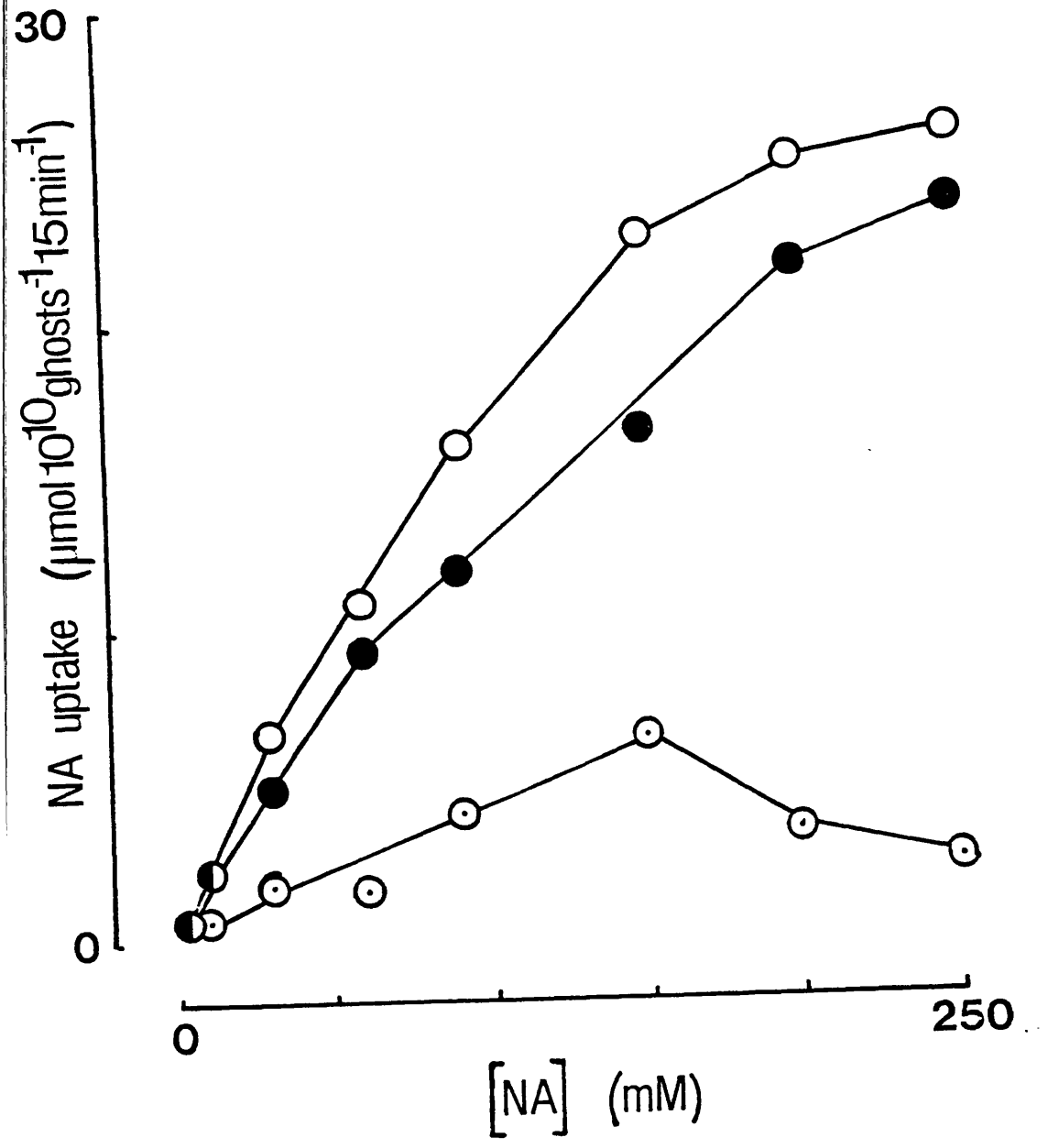
Experiment	Km (mM)		Vmax (μmol 10^{10}ghosts^{-1} 15 min^{-1})	
	Na	NMDG	Na	NMDG
1	176 ± 20	307 ± 31	46 ± 3	53 ± 4
2	281 ± 33	460 ± 70	66 ± 5	75 ± 9
3	141 ± 29	258 ± 54	42 ± 4	50 ± 6
4	122 ± 18	159 ± 28	38 ± 3	37 ± 3
MEAN	180	296	48	54
(SEM)	(35)	(63)	(6)	(8)

$P<0.02$

Figure 26

The Na dependence of ^{3}H-NA uptake by red cell ghosts

The concentration dependence of ^{3}H-NA uptake by red cell ghosts was measured from media containing 145 mM NaCl (open symbols), or from media in which Na had been isosmotically replaced by NMDG (filled symbols). The difference, which represents Na dependent transport, is shown by the dotted circles. The data illustrated are from one experiment, typical of four that were performed at pH 7.4 and 37^{o}C. All points shown are means of triplicate determinations.


30
0
NA uptake (μmol 10^10 ghosts^-1 15min^-1)
0
250
[NA] (mM)

Guanethidine uptake

Four experiments were performed to investigate the effects of Na substitution upon guanethidine uptake by red cells, and the results are illustrated in Table 25. When Na_o was replaced, Vmax fell slightly from a mean value of 10.20 ± 1.26 to 9.24 ± 0.70 μmol ml cells^{-1} 15 min^{-1} (approximately 10%). The effect upon Km was inconsistent, although clearly dissimilar to that noted for NA uptake by ghosts. In the presence of Na Km was 26.06 ± 3.44 mM and fell to 23.70 ± 2.78 mM in NMDG media. The overlap of standard errors for both Km and Vmax suggests that there is probably little Na dependence of guanethidine uptake, and hence implies that despite certain functional similarities the two compounds do not enter human red cells by identical routes. This is consistent with data already presented in Chapter 2. A typical experiment is illustrated in Figure 27.

Effects of cation transport inhibitors

The effects of 4 cation transport inhibitors upon NA uptake were investigated. Each inhibitor was added to the reaction mixture at the concentration known to maximally inhibit the relevant transport pathway, and where a vehicle was required to aid solubility (e.g. DMSO, or ethanol) suitable controls were performed. The compounds studied were ouabain (Na-K pump inhibitor), bumetanide (Na-K co-transport inhibitor), phloretin (Na-Na exchange inhibitor) and amiloride (Na-H exchange inhibitor). For the sake of convenience, the results obtained with amiloride will be discussed in Chapter 4, where other features of the pH dependence of NA uptake are also considered.

Ouabain

In two initial experiments red cells were exposed to ouabain (0.1 mM) and NA uptake measured over a 15 minute period. $[NA]_o$ ranged between 49 nM and 5.39 μM. No effect was observed, and it therefore appeared that NA transport was not dependent upon continued active Na-K pump function. The experiment was repeated twice, but prior to measurements of NA uptake, cells were

TABLE 25

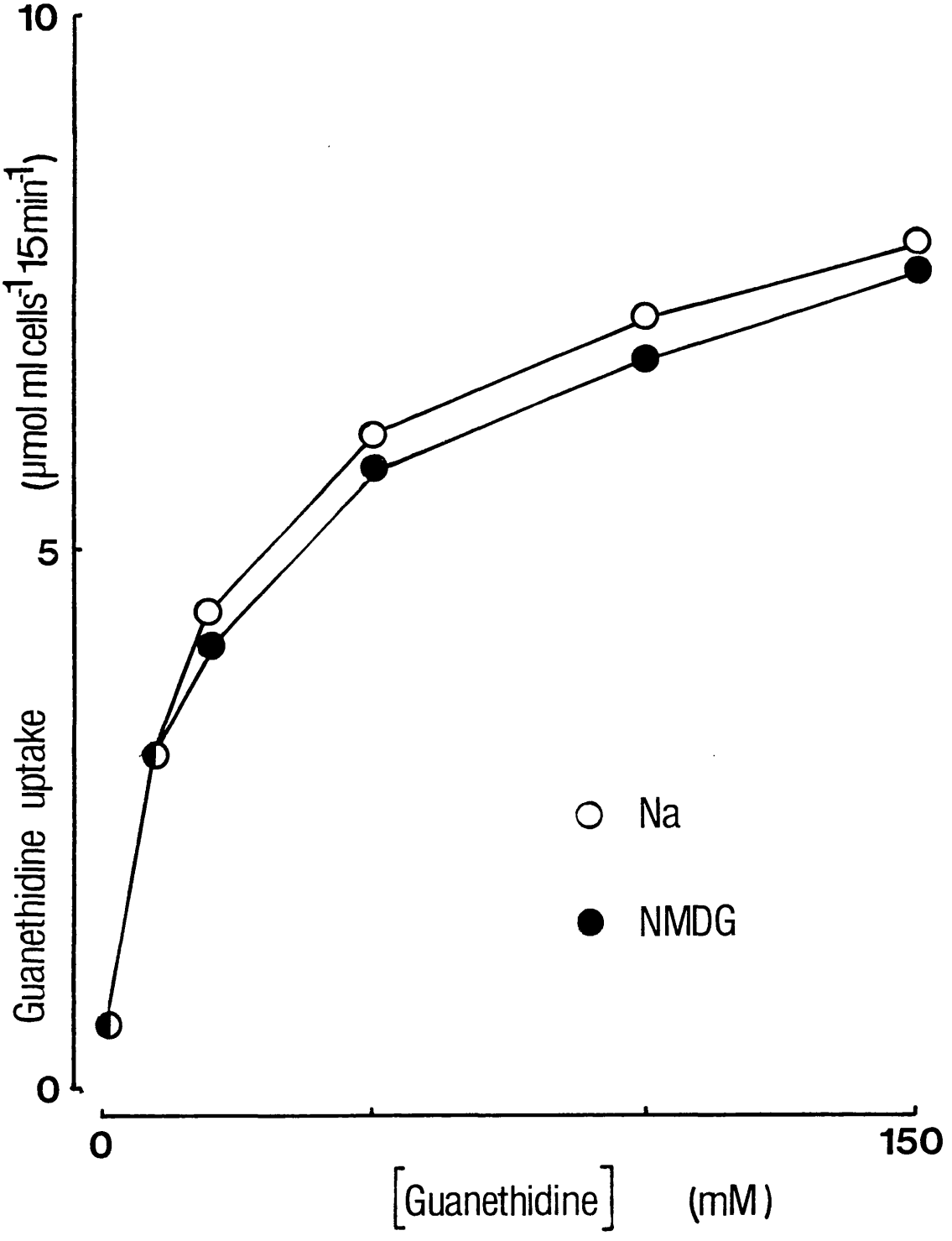
SODIUM DEPENDENCE OF GUANETHIDINE UPTAKE BY RED CELLS

Experiment	Km (mM)		Vmax (μmol ml cells^{-1} 15 min^{-1})	
	Na	NMDG	Na	NMDG
1	24.21	18.40	13.89	11.31
2	19.08	19.44	8.69	8.31
3	35.50	27.80	9.70	8.50
4	25.45	29.17	8.52	8.84
MEAN	26.06	23.70	10.20	9.24
(SEM)	(3.44)	(2.78)	(1.26)	(0.70)

Figure 27

The Na dependence of guanethidine uptake by human red cells

The concentration dependence of ^{14}C-guanethidine uptake by red cells was measured from media containing 145 mM NaCl (open symbols), or from media in which Na had been isosmotically replaced by NMDG (filled symbols). The data illustrated are from one experiment, typical of four performed. All points shown are means of triplicate determinations.


10
5
0
Guanethidine uptake (µmol ml cells^{-1} 15min^{-1})
Na
NMDG
0
150
[Guanethidine] (mM)

incubated for 2½ - 3½ hours in the presence of ouabain, over which period $[Na]_i$ should significantly rise. Uptake was also measured after 15, 30 and 45 minutes - some cells were therefore exposed to ouabain for periods in excess of 4 hours. Again, no significant changes in NA transport were observed. A typical experiment is illustrated in Figure 28.

Bumetanide

Two experiments were performed to investigate the acute effect of bumetanide (0.1 mM) upon NA transport. Bumetanide is a potent and specific inhibitor of human red cell Na-K-Cl co-transport, with an apparent Ki value of 0.16 μM (Chipperfield, 1986). In both cases bumetanide caused a modest reduction in uptake, although the mean percentage inhibition remained less than 5% - within the limits of reasonable experimental error. The effect of bumetanide was also studied in the presence of ouabain to discern whether the two compounds might have some additive action upon NA uptake. The result was unchanged (see Figure 28). It was therefore apparent that inhibition of Na-K-Cl co-transport did not affect NA accumulation.

Phloretin

Phloretin was dissolved in ethanol and incubated with red cells at a final concentration of 0.2 mM. At this level phloretin maximally inhibits the ouabain insensitive electroneutral Na-Na exchange pathway in human red cells (e.g. Duhm and Becker, 1977). Uptake was measured at 15, 30 and 45 minutes during each of the 3 experiments performed. A typical experiment is shown in Figure 29. As with bumetanide, a small ($5.31 \pm 1.9\%$, $n = 9$ observations) inhibition of uptake was observed in the presence of phloretin. It therefore seems unlikely that NA uptake is significantly coupled to or affected by ouabain insensitive Na-Na transport activity in the red cell membrane.

Figure 28

The effect of Na transport inhibitors on red cell ^{3}H-NA uptake

The time course of ^{3}H-NA uptake by red cells was measured under control conditions (circles), in the presence of 0.1 mM ouabain (diamonds) and in the additional presence of 0.1 mM bumetanide (squares). The extracellular medium contained 5.32 μM NA, and each data point represents the mean of duplicate determinations. One experiment, typical of a number performed, is illustrated.

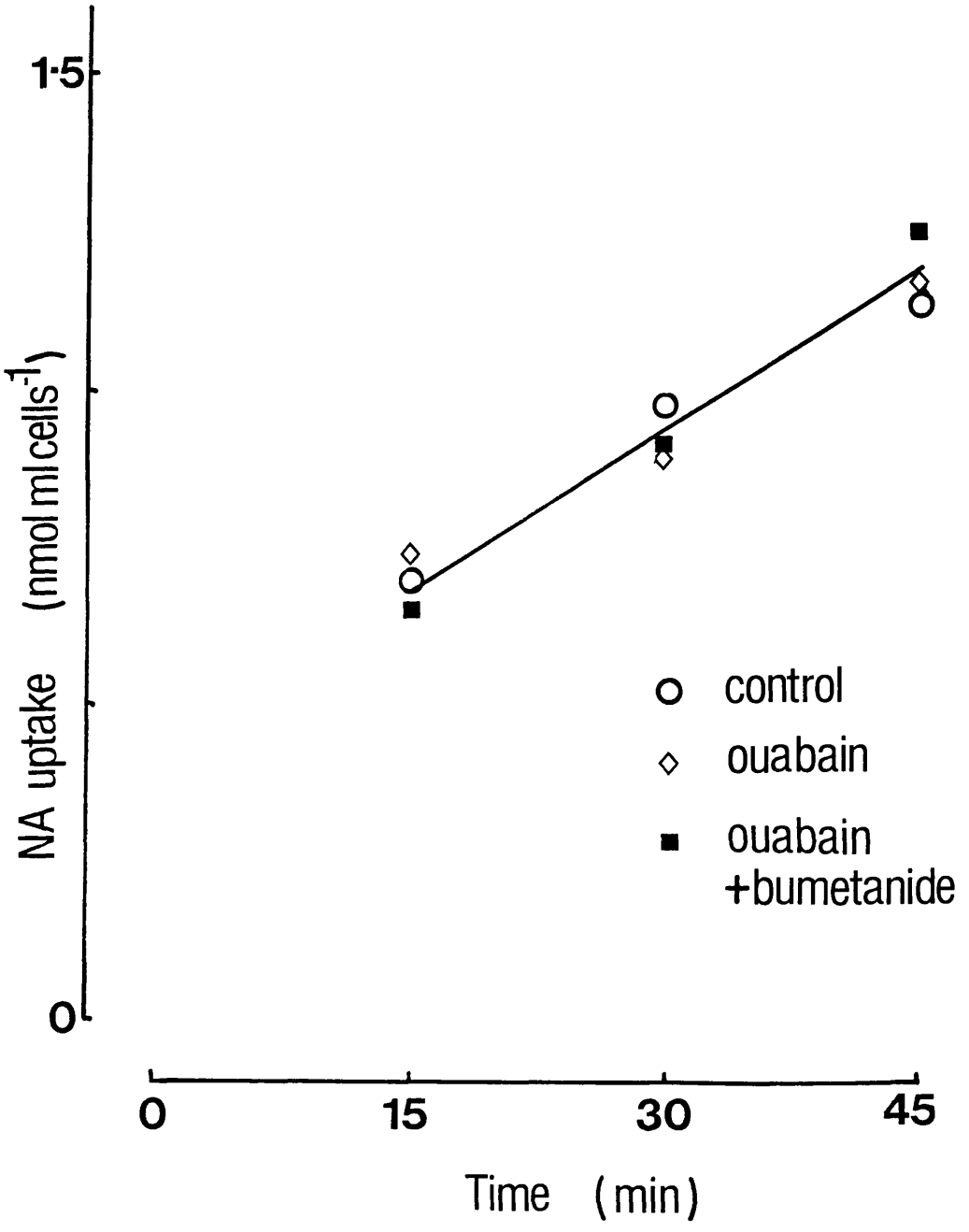
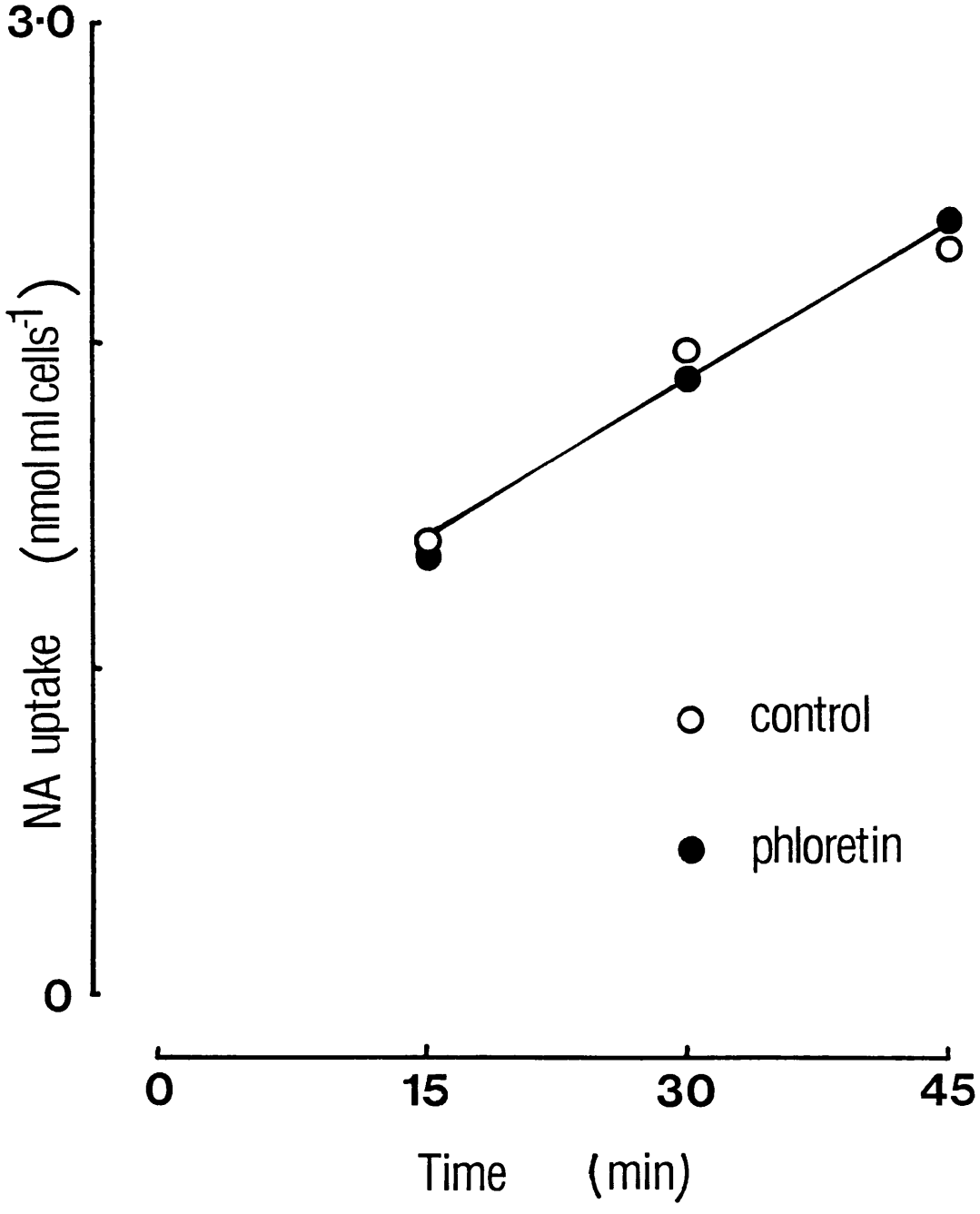

1·5
0
NA uptake (nmol ml cells^{-1})
0
15
30
45
Time (min)
control
ouabain
ouabain
+bumetanide

Figure 29

The effect of phloretin on red cell ^{3}H-NA uptake

The time course of ^{3}H-NA uptake was measured in the presence (filled symbols) and absence (open symbols) of 0.2 mM phloretin from media containing 5.32 µM NA. Each point represents the mean of duplicate determinations. One experiment, typical of three performed, is illustrated.


3·0
0
NA uptake (nmol ml cells^{-1})
control
phloretin
0
15
30
45
Time (min)

DISCUSSION

It has been demonstrated in both intact red cells and reconstituted ghosts that replacement of Na by NMDG in the incubation medium at high (1 - 250 mM) NA concentrations results in a modest inhibition of catecholamine uptake. The functionally related compound guanethidine does not exhibit similar Na dependence.

Kinetic analysis (Wilkinson, 1961) suggests that this effect represents a reduction in affinity of the transport mechanism for NA, while the maximal velocity of uptake remains unaltered. It was noted that saturation kinetics for NA uptake persisted even in the absence of Na and it may therefore be the case that the Na dependent pathway comprises only a small proportion of the total uptake *in vivo*. Naturally, caution should be exercised in attributing results obtained under such unphysiological conditions, but one certain conclusion may be drawn, namely that NA transport differs substantially from the classical Na dependent carrier mediated transport of compounds such as glucose. The results also contrast markedly with those, for instance, of Friedrich and Bonisch (1986) who noted that Na_o was an absolute requirement for cocaine sensitive NA transport. Further evidence has therefore been presented to differentiate between the neuronal NA "Uptake I" pathway, and the route of NA transport in human red cells. It should be noted, however, that many other carrier mediated transport systems with zero or negligible Na dependence have been characterised, for example those mediating the uptake of certain amino acids in sheep red cells (Young, Ellory & Tucker, 1976). NA transport in human red cells is, therefore, not unique in this respect.

It would be interesting to discern whether the Na dependent fraction of NA uptake represents a second mechanism operating alongside the Na independent component. As can be seen, the Na dependent fraction does not appear to obey conventional saturation kinetics in the range of concentrations studied (Figure 26) and hence it is not possible to measure kinetic parameters for this component. Furthermore, if two such carriers coexisted in the same

membrane it might be expected that the inhibition of one would lead to a reduction in the overall capacity for transport, represented by a decline in Vmax. This is not the case here.

The effect of Na substitution was apparently complicated as NA was reduced to levels which more closely approximated to the physiological norm. At these lower levels, replacement of Na was seen to have the opposite effect upon NA uptake - a reproducible and often large stimulation of uptake was observed. In case this result was in some way artefactual due to the nature of the substitute itself, a control experiment was performed using choline, instead of NMDG, to replace Na_o. In this experiment, performed at 5.38 μM NA_o, graded replacement of Na_o was without effect on NA transport (in agreement with results previously described) until Na_o approached zero. Uptake in the presence of 145 mM Na_o was measured as 1.49 nmol ml cells^{-1} 15 min^{-1}, compared to 1.82 nmol ml cells^{-1} 15 min^{-1} in a medium containing 10 mM Na plus 135 mM choline. This represented a stimulation of just over 20% which is both qualitatively and quantitatively similar to that shown in NMDG media. It was therefore concluded that the data obtained with NMDG were not a specific consequence of using that particular substitute, while the actions of other replacement cations were not considered. The need to carefully select appropriate Na substitutes, as shown by Blackstock and Stewart (1986), is thus stressed, although it is repeated that there is good agreement between results obtained for NA uptake whichever replacement is utilised. It should also be emphasised that the methodological problems, associated with organic cations and described by Blackstock and Stewart (1986) were due to an increase in passive permeability to Na and K under certain conditions. Studies here have shown that Na and K leak pathways do not apparently mediate NA transport, thus further validating the use of NMDG as a Na substitute.

A possible alternative explanation for the concentration dependent effects observed may involve a consideration of the intracellular binding capacity of the red cell for NA. At extremely high $[NA]_o$, comparatively large amounts of NA enter the

cell. It is therefore conceivable that the binding capacity of the red cell (and certainly the ghost) may approach saturation. Hence, free NA_i may become elevated under conditions in which an outward facing Na gradient has already been imposed. Assuming that the uptake mechanism is able to transport NA out of, as well as into, the cell, exchange diffusion of NA will be stimulated, presumably both by Na dependent and independent routes. The magnitude of this flux is unknown, but two assumptions can be made:

1) Efflux of ^{3}H-NA will reduce the apparent influx, and
2) This effect will become less pronounced at lower $[NA]_o$ where free NA_i will be elevated to a lesser extent.

This speculative hypothesis however, does not adequately explain the results and it would be informative to pursue further lines of investigation, such as the kinetic effects of Na substitution at the inner surface of the membrane. An important, and unanswered, question involves the determination of the symmetry of NA transport.

It was interesting to note that, despite the apparent Na dependence of NA uptake, a variety of induced Na gradients in nystatin treated cells failed to affect NA transport. This may be a consequence of two factors. Firstly, the possibility exists that the rapid dissipation of cation gradients from the leaky cells may in some way mask the effects observed elsewhere. This seems unlikely, in that Na substitution in ghosts did elicit a response and ghosts were similarly leaky to cations. The possibility also exists that nystatin in some way impairs the NA transport capacity of cells with which it interacts, and that this impairment persists when nystatin is washed away. Again, this hypothesis seems unlikely, as the fluxes measured in these cells were within what would be considered the normal range. Again, if it is assumed that NMDG is truly acting as an inert cation (or equally inert as choline) it may be concluded that NA uptake appears unable to distinguish between Na and K. The possibility therefore arises that a small proportion of NA accumulation by human red cells has

a relatively non-specific requirement for simple monovalent cations. It would seem that organic monovalent cations cannot adequately fill this role, and neither are divalent cations (e.g. Mg, Ca) apparently essential for uptake.

One further revealing piece of evidence may be seen from these experiments, and that is the fact that NA transport in cells leaky to Na is not similarly elevated. (It is also the case that NA influx values similar to those obtained from intact red cells may be measured in ghosts prepared by the method described by Richards and Eisner (1982), despite the similarly elevated leakiness of these ghosts to Na. Furthermore, preparation of 'tighter' ghosts (Bodemann & Passow, 1972) does not affect NA uptake). It may therefore be concluded that the Na leak pathway is not important for NA uptake despite the fact that a certain proportion of NA exists in the cationic form at physiological pH. The effects of pH upon NA uptake are discussed in detail in the next chapter.

If such a leak pathway is not involved in mediating NA uptake, then NA must presumably cross the membrane via either a lipid soluble route, or a specific membrane protein. Saturation kinetics imply the functional existence of the latter, although transport in vivo is almost certainly a combination of the two components. The precise location and nature of such a protein is beyond the scope of this study, but results obtained with various inhibitors of cation transport preclude the direct involvement of the Na-K pump, Na-K co-transporter and Na-Na exchange mechanisms in NA movements. Neither does the inhibition of any of these mechanisms appear to influence NA transport in any significant manner.

It may therefore be concluded that NA uptake by human red cells is influenced by the cationic composition of the extracellular environment. It is equally clear, however, that the cation dependence of NA uptake in this tissue is less marked than that observed in Uptake 2, which is in turn less marked than that documented in Uptake 1.

CHAPTER 4

ANION DEPENDENCY AND EFFECTS OF pH UPON NA TRANSPORT

INTRODUCTION

Effects of anions upon carrier mediated transport

In contrast to the numerous studies of cation dependent membrane transport phenomena, the effects of anion substitutions have been less thoroughly investigated. In some respects this omission is surprising. For instance, the generally accepted stoichiometry of ouabain sensitive Na-K exchange (3 Na out : 2 K in) in the red cell requires the additional outward translocation of a monovalent anion if electroneutrality is to be maintained. Alternatively, electroneutrality might be maintained by the simultaneous inward movement of a positively charged species. Therefore, although the Na pump cannot actively mediate Cl movements, it may be in part responsible for the passive transmembrane distribution of anions, and it follows that other mechanisms which transport positively charged substrates may similarly affect the Cl gradient. However, the extent to which simple inorganic anions such as Cl may be involved in carrier mediated solute transfer was not recognised until comparatively recently (e.g. Ellory, Dunham, Logue & Stewart, 1982).

Much of the recent work pertaining to anion dependent cation transport has been performed in relation to the diuretic sensitive Na-K co-transport mechanism, which was originally described in the red cell by Wiley and Cooper (1974). It was subsequently observed by Chipperfield (1980), that ouabain insensitive ^{42}K influx was inhibited to similar degrees both by the application of furosemide (1 mM) and by removal of Cl_o. Similar effects of furosemide, a diuretic, and Cl_o replacement upon ^{24}Na efflux were reported, although direct evidence for simultaneous ^{36}Cl transport by the same system in red cells was not presented. This was due to the fact that the rapidity of ^{36}Cl movements in these cells made such measurements technically difficult.

Chloride dependent transport of Na and K has since been described in a variety of tissues in addition to the red cell, including many epithelia, ascites tumour cells, nerve and muscle (for review, see Ellory, Dunham, Logue & Stewart, 1982). Specifically, in mammalian red cells, two major anion dependent cation transport systems have been defined, namely K-Cl and Na-K-Cl. The recent literature cites evidence that various other substances also exhibit anion dependent transport characteristics. For example, passive Pb transport by red cells is strongly stimulated by HCO_3, and is inhibited if Cl_o is replaced by ClO_4 (Simons, 1986). The increased transport of Pb in HCO_3 media may be abolished by the application of low concentrations of 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid (DIDS), an inhibitor of carrier mediated anion transport, which points to the involvement of this mechanism in HCO_3 stimulated uptake.

That NA and anions have some physiological inter-relationship is evidenced by the fact that in rats approximately 53% of NA circulates in a conjugated form, and that the major conjugate, both at rest and under stress, is the sulphate (Alexander, Yoneda, Vlachakis & Maronde, 1984). A similar situation exists in human plasma (Jenner & Rose, 1973). Also of considerable importance to the work undertaken in this chapter are the results of Friedrich and Bonisch (1986). These authors studied the uptake of ^{3}H-NA into reserpine pretreated PC-12 cells (a clonal cell line which possesses Uptake I). It was noted that uptake was abolished when extracellular Cl was replaced by isethionate, and that this effect was concentration dependent, with increases in [Cl] progressively enhancing NA uptake. Of the substitute anions tested, only Br and SCN were able to partially mimic the transport stimulating effects of Cl (with about 40% and 20% effectiveness, respectively) while virtually no transport at all could be detected in cells suspended in NO_3, SO_4 and $MeSO_4$ media. The effects of Cl substitution were further investigated in a series of experiments designed to elucidate the kinetic consequences of Cl replacement, and the results indicated that the apparent Km for NA transport progressively decreased, and the Vmax

increased, with increases in $[Cl]_o$. Furthermore, the stimulation of the initial rate of uptake of NA by increasing $[Cl]_o$ obeyed saturation kinetics when determined at several different $[NA]_o$. The concentration of Cl_o which caused half maximal stimulation of uptake decreased with increases in the concentration of NA. The authors therefore postulated the direct participation of the Cl ion on the PC-12 cell NA transporter, which also appears to co-transport Na (as described in Chapter 3).

Although the bulk of NA at physiological pH exists in the charged (cationic) form, the anion-dependence of NA transport by red cells has not previously been investigated. In view of this gap in the understanding of the process, and in light of the results of Friedrich and Bonisch (1986), the effects of Cl replacement were investigated both at saturating and non-saturating concentrations of NA, and in both intact red cells and ghosts. In addition, the effects of the anion exchange inhibitor DIDS upon NA uptake were considered. It should be noted that the consequences of altering the transmembrane Cl gradient are to affect both the transmembrane potential difference, and pH gradient (e.g. Cotterrell & Whittam, 1971), if Cl is replaced by an impermeant species such as gluconate. Because of this close link between Cl^- and H^+ ion distribution it was therefore felt appropriate to also consider the effects of pH_o upon NA uptake in this chapter.

Effects of anions upon non-carrier mediated transport

It is important to realise that the effects of anion substitution do not necessarily reflect the removal of a substrate for a given system. For instance, Brand and Whittam (1985), extending the work of Funder and Wieth (1967), observed that replacement of Cl_o by NO_3 caused a stimulation of passive ^{22}Na influx measured in the presence of both ouabain and furosemide. This effect may be attributed to an alteration of the micellar structure of the red cell membrane, induced by NO_3, and a change in the electrostatic surface charge . Such a shift in potential will, accordingly, influence the permeability of the cell to a variety of charged

species.

In addition, effects of other anion substitutions may be secondary to cell volume perturbations. This arises, for example, if Cl is replaced by an impermeant anion such as gluconate (Everall & Whittam, 1983). Yet another possible explanation for such effects would be deleterious effects upon cell metabolism (Funder & Wieth, 1967). Abnormal anion transport may also have implications for intracellular pH homeostasis, as previously mentioned (e.g. Hladky & Rink, 1977).

In the experiments to be described, the effects of both anion substitution and anion transport inhibition were studied. Anion substitutions were performed both in red cells and ghosts in an effort to ascertain whether effects upon uptake were mediated at the membrane level, or were secondary to intracellular events.

The effects of pH upon NA transport

Most biological processes exhibit a degree of pH dependence, although some effects are complex to interpret. Variations in extracellular pH may be of significance with respect to NA transport in a number of ways. The first observation that pH influenced NA uptake was made by Schanker, Nafpliotis and Johnson (1961) who noted a marked stimulation of uptake as extracellular pH was elevated, and attributed this response both to an increase in influx via the lipid domain, and enhanced intracellular binding to haemoglobin. This interpretation of the effects of pH upon NA uptake does not, however, take into account all of the relevant factors, as discussed below.

Molecular species of NA transported

Most biogenic amines, including NA, possess two ionisable groups, an amine group (pKa of 9.98) and a phenolic group (pKa of 8.82). The pKa is defined as the pH at which 50% of that particular moiety, in this instance either $-NH_2$ or $-OH$, exists in the ionised state. It is therefore the case that at physiological pH four NA species exist, all of which are theoretically capable of being transported. The four species of amine are: 1) the cationic

species, the predominant species at neutral pH; 2) the neutral species, which is highly lipophilic, formed by deprotonation of the nitrogen; 3) the zwitterionic species, formed by deprotonation of one of the ring hydroxyl groups; and 4) the anionic species, formed by deprotonation of the nitrogen and a ring hydroxyl group. The effects of pH upon the ratio of ionised to unionised forms of NA, as calculated from the Henderson-Hasselbalch equation, are shown in Table 26. Clearly, as pH is elevated the simple diffusion of lipophilic NA into the red cell will become enhanced, assuming that the concentration gradient favours uptake. A second possibility also exists however, in that the NA carrier might also preferentially transport the neutral species which, at physiological pH, only constitutes 0.1% of the total amine. Johnson, Carty & Scarpa (1984) suggest that in chromaffin granules isolated from the adrenal medulla, it is precisely this species which is most readily accepted by the NA transporter. A plot prepared by these authors of apparent Km versus external pH indicates an increase in carrier affinity for NA as pH_o is elevated, with the steepest section of the curve lying in the range pH 6-7. As can be seen from the table, this is also the region in which the ratio of ionised to unionised catecholamine is most markedly pH dependent.

From the work described in the literature there is no evidence to the effect that the postulated red cell NA carrier favours either one form of substrate or another. The evidence is clear, however, that investigators have independently both postulated carrier mediated uptake of NA, and observed an increased rate of uptake of amine as extracellular pH is raised. Therefore, in an attempt to discern the affinity of the mechanism for different forms of substrate, the activation of uptake of NA has been studied in ghosts at different pH values. Additional circumstantial evidence for the involvement of pH sensitive carrier mediated NA uptake has also been sought.

TABLE 26

IONIC CHARACTER OF NORADRENALINE

pH	pKa = 8.82		pKa = 9.98	
	$[A^-]/[HA]$	% ionised substrate	$[A^-]/[HA]$	% ionised substrate
6.6	166	99.4	2399	100.0
7.0	66	98.5	955	99.9
7.4	26	96.3	380	99.7
7.8	10	90.9	151	99.3
8.2	4	80.0	60	98.4
pKa	1	50.0	1	50.0

% ionisation and ratios were calculated from the Henderson-Hasselbalch equation:

$$pH = pKa - \log_{10}([A^-]/[HA])$$

pKa values for NA were obtained from Dawson, Elliot, Elliott and Jones (1974).

Effects of H movements

The hypothesis presented by Johnson, Carty & Scarpa (1984), suggests that NA uptake into chromaffin granules may, furthermore, be directly responsive to H^+ gradients produced, for example by an H^+-translocating ATPase. The red cell is also able to transport H^+, via the electrically silent Na-H exchange mechanism, the physiological role of which is to restore pH_i in the event of an acid load. The mechanism is inhibited by amiloride, and a number of structurally related analogues (see Benos, 1982; Friedrich, Sablotri & Burckhardt, 1986).

It was therefore decided to investigate the relationship between NA transport and Na-H exchange by measuring uptake in the presence and absence of amiloride (1 mM). These experiments were performed at a variety of extracellular pH values which were chosen so that Na-H exchange would be activated to different extents in each condition. This protocol was designed such that any non-specific actions of amiloride might be detected (e.g. effects observed at high pH_o levels where Na-H exchange is silent). The use of amiloride as a specific inhibitor of this transport pathway is considered in greater detail in the discussion section of this chapter. The effects of another inhibitor of Na-H exchange, dicyclohexylcarbodiimide (DCCD), were also studied under similar conditions.

Effects of binding and metabolism

As previously discussed (Chapter 1), the manner by which NA enters the red cell may be influenced both by intracellular binding and metabolism. These considerations are of particular relevance to this chapter, as both have been cited as pH-dependent events. As mentioned earlier, Schanker, Nafpliotis & Johnson (1961) suggested that an elevation of pH_o might accentuate intracellular binding to haemoglobin. Banaschak and Bluth (1978) suggested that such binding plays an important role in the transport of catecholamines in the bloodstream where, it is assumed, they are protected from oxidation. Binding is, therefore, reversible. Blakeley and Nicol (1978) proposed an ion trapping mechanism by which catecholamines

might be sequestered in the intracellular space. In this model, NA is considered to enter the cells from a medium the pH of which is greater than that of the cell interior. From a consideration of pH and pKa effects, the NA within the cell becomes ionised to a greater extent, and is hence less permeant. The molecule is, effectively, trapped.

Furthermore, Raymond and Weinshilboum (1975) demonstrated that red cell catechol-O-methyl transferase activity was pH-dependent, with a maximum activity at pH 7.6-7.7 which declined to either side. (As pH was reduced, half maximal activity was reached at approximately pH 6.9).

Accordingly, the experiments designed to investigate the kinetics of NA uptake in ghosts at different pH values were also analysed to discern whether pH effects might be mediated intracellularly, or at the membrane level. Any effect of pH upon NA uptake in ghosts may be taken to indicate:

1. That the pH-dependence observed in intact red cells is not wholly a consequence of altered metabolism (because ghosts are metabolically inert) and

2. That, similarly, any pH-dependent effect in red cells cannot be explained solely on the basis of a change in intracellular binding capacity and/or affinity of enzyme for substrate.

RESULTS

THE EFFECT OF ANION SUBSTITUTION ON NA UPTAKE BY HUMAN RED CELLS

^{3}H-NA influx

In a series of preliminary experiments (n=3), six different monovalent anions were used to isosmotically replace Cl_o: NO_3, SCN, $MeSO_4$, HCO_3, formate and acetate. Cl_o was entirely replaced in all conditions except in the presence of HCO_3, where only partial replacement (25 - 94mM) could be achieved whilst still maintaining a satisfactorily constant extracellular pH. Of the anions tested, SCN, acetate and formate had no effect upon NA transport, as opposed to NO_3, $MeSO_4$ and HCO_3, all of which yielded a reproducible stimulation of uptake over 15 minutes. Stimulation of uptake was not a transient phenomenon and persisted in incubations performed for periods of up to one hour.

Although the effects of NO_3 and $MeSO_4$ were in some cases small, they were consistent. NA uptake was always enhanced in the absence of Cl_o. Furthermore, stimulation of uptake in $MeSO_4$ media was always greater than that caused by NO_3 (see Figure 30). The effect of NO_3 was, however, highly significant, causing an increase in flux from 0.99 ± 0.03 to 1.10 ± 0.07 nmol ml cells^{-1} 15 min^{-1} ($[NA]_o$ = 5.4 μM, n=9, P<0.02) and from 46.7 ± 4.0 to 53.1 ± 4.4 pmol ml cells^{-1} 15 min^{-1} ($[NA]_o$ = 0.28 μM, n=14, P<0.001). The effect of $MeSO_4$ was also significant. Anion substitutions were similarly effective in experiments which utilised either fresh cells or those obtained from bank blood.

In experiments in which varying amounts of HCO_3 were used to replace Cl_o, stimulation of uptake was far more marked. In four experiments at 25 mM HCO_3 ($[NA]_o$ = 1-20 μM), mean uptake increased by 100.3 ± 20.3%. Interestingly, however, when HCO_3 was further elevated to 94 mM, at constant extracellular pH, the stimulation of uptake observed was less. These results are summarised in Table 27.

Simultaneous measurements of ouabain- and bumetanide-insensitive ^{22}Na uptake by cells incubated in identical media again suggested that the pathways of passive Na influx and NA accumulation by red cells are not the same, in agreement with the

results presented in Chapter 3. A typical experiment is shown in Table 28. In the first instance, it is noted that the effects, in order of stimulation, are reversed with NO_3 and $MeSO_4$ when Na and NA fluxes are compared. A considerable (approximately 4-fold) stimulation of Na uptake was also observed in red cells incubated in SCN media, in agreement with Funder and Wieth (1967), whereas this substitution had no effect upon NA movements.

Net transport experiments

Three experiments were also performed in order to ascertain the resultant concentrations of free $[NA]_i$ after incubating red cells in media containing Cl, NO_3 or $MeSO_4$. Incubations were performed over three hour periods in the presence of 100 μM NA_o and after this time $[NA]_i$ was determined by HPLC-ED. Surprisingly, the results were dissimilar to those obtained using tracer influx techniques. Accumulation of NA in cells exposed to NO_3 was markedly enhanced (between 100-400%), while accumulation of NA from $MeSO_4$ media showed little difference from that observed in control conditions (see Figure 31). If initial unidirectional influx in $MeSO_4$ media is enhanced, yet resultant intracellular concentrations remain unaffected, it would seem reasonable to postulate that free NA_i is being removed at a faster rate from these cells - possibly by efflux, metabolism or binding. In contrast, the elevated $[NA]_i$ demonstrated in cells in NO_3 media might arise from an inability of such cells to remove NA by the usual routes.

Thus, it is postulated that the 10-20% stimulation of tracer influx in NO_3 media may, during prolonged incubations, result in a 300-400% elevation of NA_i levels, compared to Cl controls, possibly via a deleterious effect of NO_3 upon COMT activity. This point is of interest as NO_3-containing media are known to suppress other branches of cell metabolism, for example glycolysis (Funder and Wieth, 1967).

Figure 30

The effect of anion substitution upon red cell NA uptake

^{3}H-NA uptake by red cells was measured from media containing 0.28 - 0.30 μM NA. The results from 7 experiments are shown, in each of which influx was determined in the presence of either 150 mM Cl or equivalent osmotic quantities of NO_3 or $MeSO_4$. The points drawn are the means of duplicate measurements.

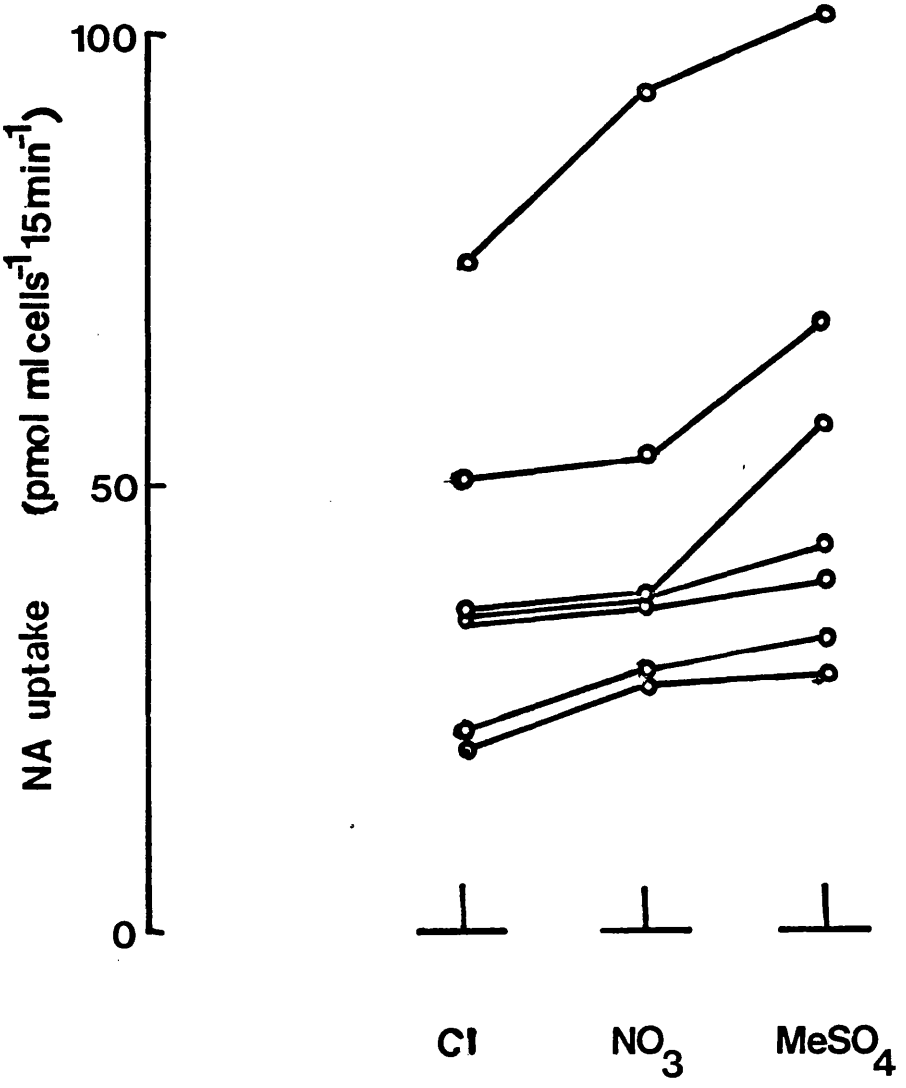

100
50
0
NA uptake (pmol ml cells^{-1} 15min^{-1})
Cl
NO_3
$MeSO_4$

TABLE 27

THE EFFECT OF HCO_3 ON NA UPTAKE BY RED CELLS

Experiment	$[NA]_o$	Control	25 mM HCO_3	94 mM HCO_3
1	1 μM	0.178	0.337	0.285
2	1 μM	0.197	0.303	0.250
3	20 μM	4.17	10.48	
4	20 μM	4.28	8.85	

Units of flux: nmol ml cells^{-1} 15 min^{-1}

All values are means of three separate data points.

The extracellular pH in these incubations was maintained by constantly gassing cell suspensions with:
i) 95% CO_2/5% O_2 when HCO_3 was 25 mM.
ii) 80% CO_2/20% O_2 when HCO_3 was 94 mM.

TABLE 28

THE EFFECTS OF ANION SUBSTITUTION ON ^{22}Na AND ^{3}H-NA UPTAKE

Anion (A^-)	^{22}Na UPTAKE	% STIMULATION	^{3}H-NA UPTAKE	% STIMULATION
Cl	1.99		9.45	
NO_3	3.15	58.3	10.03	6.1
$MeSO_4$	2.53	27.1	12.14	28.5
HCO_3	8.07	305.5	28.50	201.6

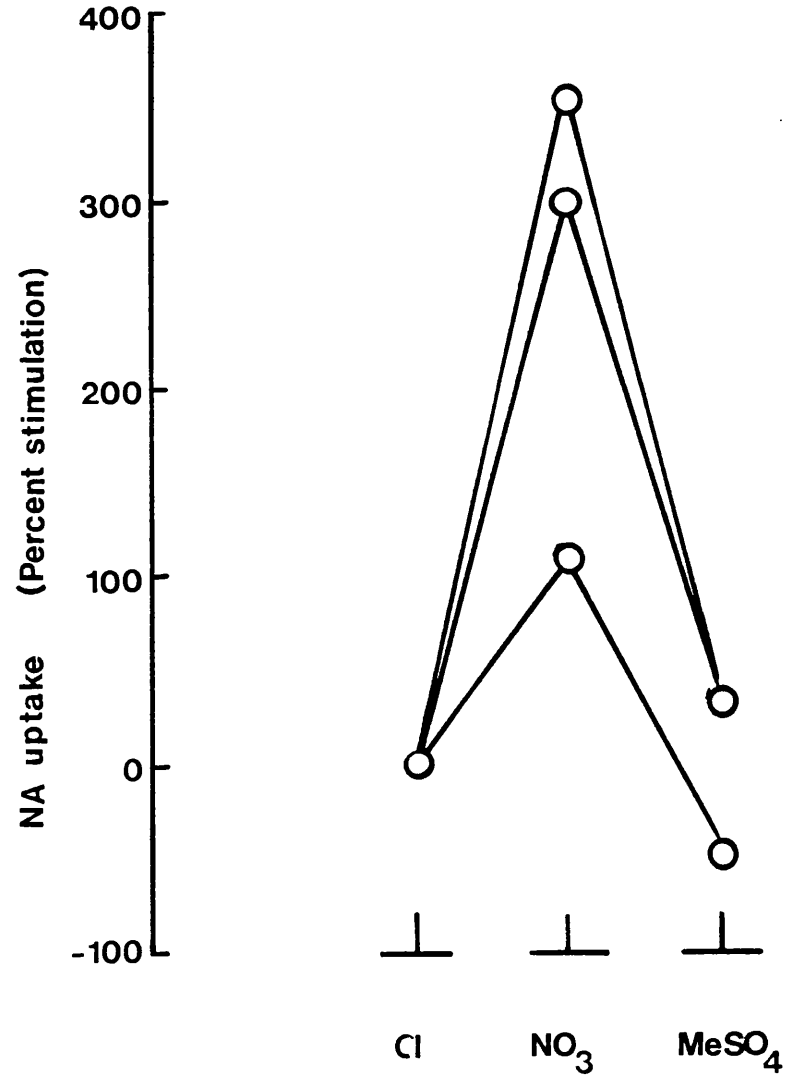
$[Na]_o$ = 145 mM; units of flux: μmol ml cells^{-1} 15 min^{-1}
$[NA]_o$ = 25 μM; units of flux: nmol ml cells^{-1} 15 min^{-1}
$[A^-]_o$ = 145 mM, except HCO_3 = 25 mM

All points represent the means of three determinations.

Figure 31

The effect of anion substitution upon red cell NA uptake

Net NA accumulation by red cells was measured by HPLC-ED after 3 hour incubations in media containing 100 μM NA. Red cells were suspended in solutions containing 150 mM Cl, or osmotically equivalent amounts of NO_3 or $MeSO_4$, as shown. Three experiments were performed and each point drawn is the mean of duplicate measurements.


400
300
200
100
0
-100
NA uptake (Percent stimulation)
Cl
NO3
MeSO4

The effect of tropolone upon NA uptake

To test the hypothesis that stimulated ^{3}H-NA uptake in NO_3 and $MeSO_4$ media might be a consequence of abnormal catecholamine metabolism, the effects of anion substitutions were therefore investigated in red cells exposed to tropolone, an inhibitor of COMT (Belleau and Burba, 1963). In two experiments, addition of tropolone to the incubation media did reduce the expected increase in flux, but some stimulation of uptake persisted (Table 29). Therefore it is possible that the effects of these anions may be partially attributable to disturbances in catecholamine metabolism especially since the action of tropolone appears to display some latency (see Chapter 1). In light of this observation it was felt appropriate to investigate the effects of anion substitution upon NA-uptake in a metabolism-free system (the ghost), and these experiments are presented below.

The effects of anion substitution on NA uptake by ghosts

Three preliminary experiments were performed in order to ascertain that ghosts remained responsive to known effects of anion substitution. This was assessed by measuring the time course of ^{22}Na uptake into ghosts suspended in either NO_3 or Cl media. After an initial rapid phase of uptake, ^{22}Na influx remained linear with time for periods of up to 70 minutes, and uptake was always greater in NO_3 media, despite the enhanced leakiness to cations of the preparation. It was therefore concluded that results for ^{22}Na uptake in ghosts were qualitatively similar to those noted in cells (e.g. Brand and Whittam, 1985). A typical experiment is illustrated in Figure 32.

NA transport studies

Two groups of experiments were performed - some at comparatively low NA concentrations (i.e. non-saturating), and others at concentrations at which saturation kinetics for NA uptake have previously been demonstrated (see Chapters 1 and 3). Results were independent of the substitute anion utilised (NO_3 or $MeSO_4$), although in the majority of experiments $MeSO_4$ was used to

TABLE 29

THE EFFECT OF TROPOLONE ON NA UPTAKE IN THE PRESENCE OF Cl, NO_3 AND $MeSO_4$

EXPERIMENT	ANION					
	Cl		NO_3		$MeSO_4$	
	-Tropolone	+Tropolone	-Tropolone	+Tropolone	-Tropolone	+Tropolone
1	16.71	17.04	18.38	17.14	22.27	20.89
2	15.07	15.44	16.83	16.09	22.85	21.24

$[NA]_o$ = 100 μM

Units of flux: nmol ml cells $^{-1}$ 15 min^{-1}

Tropolone, where present, was at a concentration of 3 mM.

All values are means of three separate data points.

Figure 32

The time course of ^{22}Na uptake by red cell ghosts

^{22}Na uptake into red cell ghosts was measured from media containing either 150 mM Cl (open symbols) or 150 mM NO_3 (closed symbols). Each point is the mean of triplicate determinations. To obviate the need to correct for simultaneous tracer efflux due to the Na pump, cells were haemolysed and ghosts resealed in ATP-free solutions. Uptake measurements were subsequently performed in the presence of 10^{-4}M ouabain. The experiment illustrated is one which is typical of three performed.

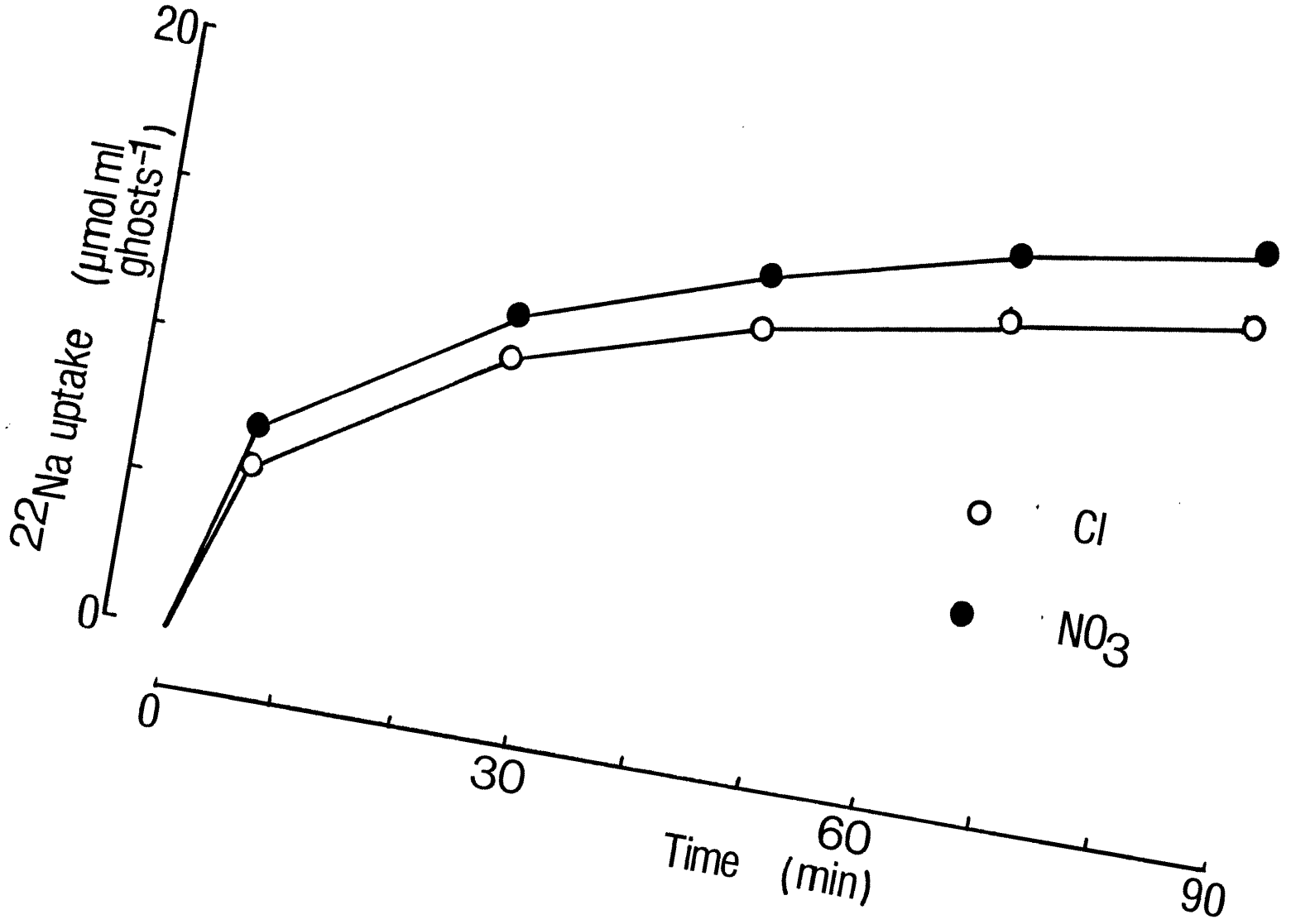
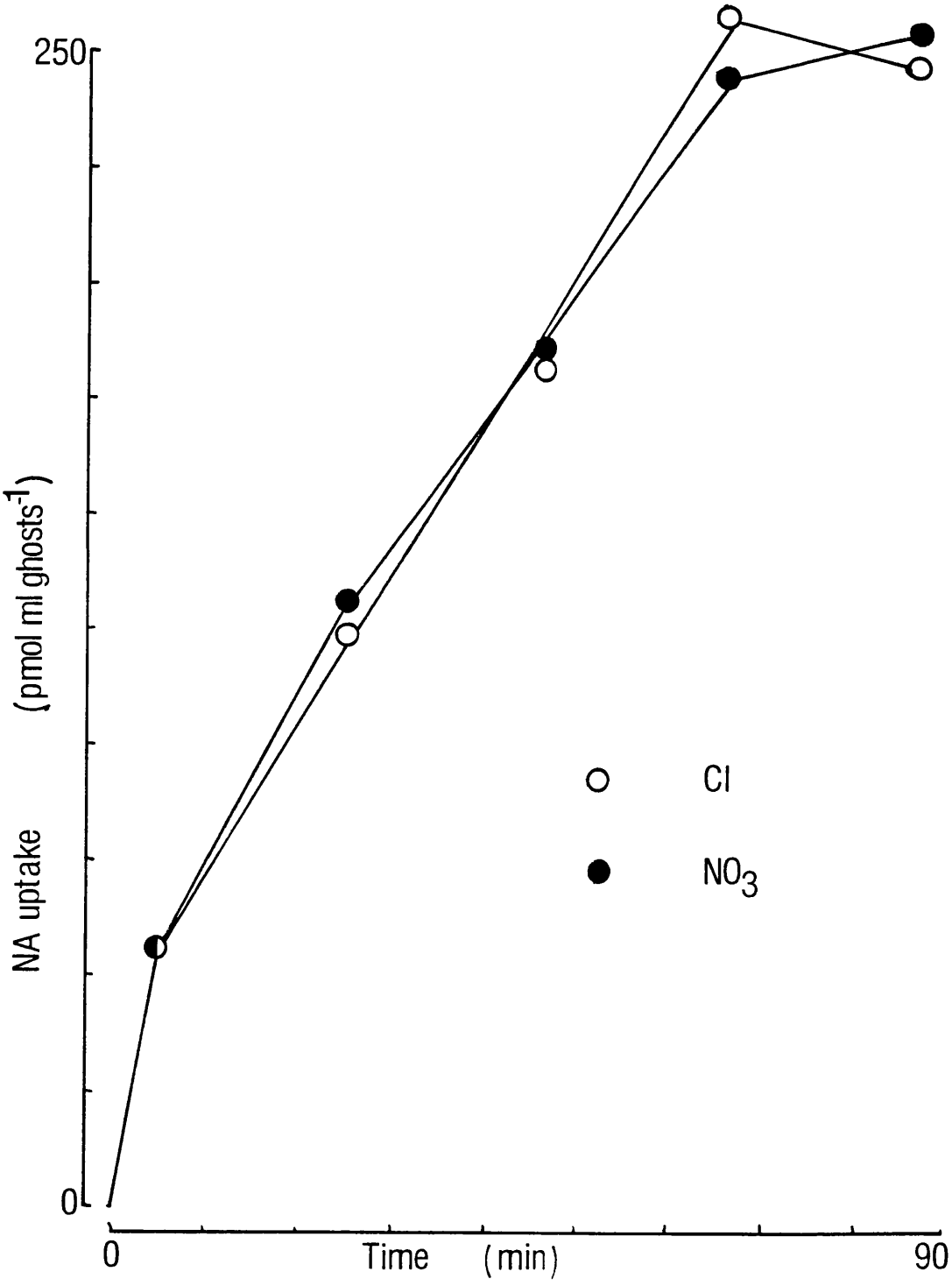

20
0
22Na uptake (μmol ml ghosts-1)
Cl
NO3
0
30
60
90
Time (min)

Figure 33

The effect of anion substitution upon the time course of NA uptake by red cell ghosts

^{3}H-NA influx was measured from media containing 280 nM NA. Open symbols represent measurements made in the presence of 150 mM Cl and closed symbols represent measurements in Cl-free conditions (Cl replaced by NO_3). Each point is the mean of duplicate determinations. One of two identical experiments is illustrated.


250
0
NA uptake (pmol ml ghosts^{-1})
0
Time (min)
90
Cl
NO_3

replace Cl_o. Two time courses for ^{3}H-NA uptake were produced and one is illustrated in Figure 33. Unlike the effects of anion substitution upon ^{22}Na uptake, NA transport was apparently not influenced.

Similarly, in experiments at high $[NA]_o$ no effect of anion substitution upon kinetic parameters for uptake could be observed. In Cl media Km was 164 ± 46 mM compared to 180 ± 30 mM in $MeSO_4$ media. Vmax was 54.1 ± 8.1 µmol 10^{10} ghosts^{-1} 15 min^{-1} in Cl media, and 56.5 ± 6.0 µmol 10^{10} ghosts^{-1} 15 min^{-1} in $MeSO_4$ media. The data (mean ± SEM of 3 experiments) is illustrated in Table 30. Hence the maximal velocity of uptake and the apparent affinity of NA for transport in the red cell ghost appears to be independent of the major extracellular anion. This observation is clearly not consistent with those of Friedrich and Bonisch (1986) using PC12 cells, and hence provides further support for the hypothesis that the route of NA entry into red cells is not via the well characterised cocaine-sensitive Uptake I pathway.

The effects of anion substitution upon guanethidine uptake

It was suggested in Chapter 3, on the basis of differing effects of cation substitutions, that NA and the functionally related compound guanethidine might enter the red cell by separate routes. In order to further compare the mechanisms of uptake of the two compounds, ^{14}C-guanethidine influx into red cells was studied in both Cl and $MeSO_4$ media. Kinetic data was derived (Table 31), and one of three experiments is illustrated in Figure 34. It is clear that the replacement of Cl has no effect upon the maximal rate of uptake of guanethidine and it also seems unlikely that there is any large effect upon the affinity of substrate for transport under similar circumstances. Although the mean value for Km is reduced in $MeSO_4$ media (indicating an increase in affinity under these conditions), the effect was not reproducible and the standard errors of both groups are large. It seems, therefore, that the kinetics of both NA and guanethidine accumulation are not influenced by anion substitutions.

TABLE 30

THE EFFECTS OF ANION SUBSTITUTION UPON THE KINETICS OF ^{3}H-NA UPTAKE IN GHOSTS

Experiment	Chloride		Methyl sulphate	
	Km Mean(SEM)	Vmax Mean(SEM)	Km Mean(SEM)	Vmax Mean(SEM)
1	257 (41)	69.8 (6.6)	239 (28)	66.9 (4.8)
2	118 (11)	49.4 (2.2)	165 (24)	56.5 (4.3)
3	119 (19)	43.1 (3.3)	137 (20)	46.1 (3.5)
MEAN(SEM)	164 (46)	54.1 (8.1)	180 (30)	56.5 (6.0)

Kinetic constants and their associated standard errors were calculated by the method of Wilkinson (1961).

Km: mM.
Vmax: μmol 10^{10} ghosts^{-1} 15 min^{-1}.

TABLE 31

THE EFFECTS OF ANION SUBSTITUTION UPON THE KINETICS OF ^{14}C-GUANETHIDINE UPTAKE IN HUMAN RED CELLS

Experiment	Chloride		Methyl sulphate	
	Km Mean(SEM)	Vmax Mean(SEM)	Km Mean(SEM)	Vmax Mean(SEM)
1	13.7 (2.0)	10.7 (0.4)	14.0 (1.5)	12.3 (0.4)
2	35.5 (4.8)	9.7 (0.5)	22.7 (2.1)	9.5 (0.3)
3	25.5 (2.6)	8.5 (0.3)	22.9 (4.0)	8.9 (0.5)
MEAN (SEM)	24.9 (6.3)	9.6 (0.6)	19.9 (2.9)	10.2 (1.0)

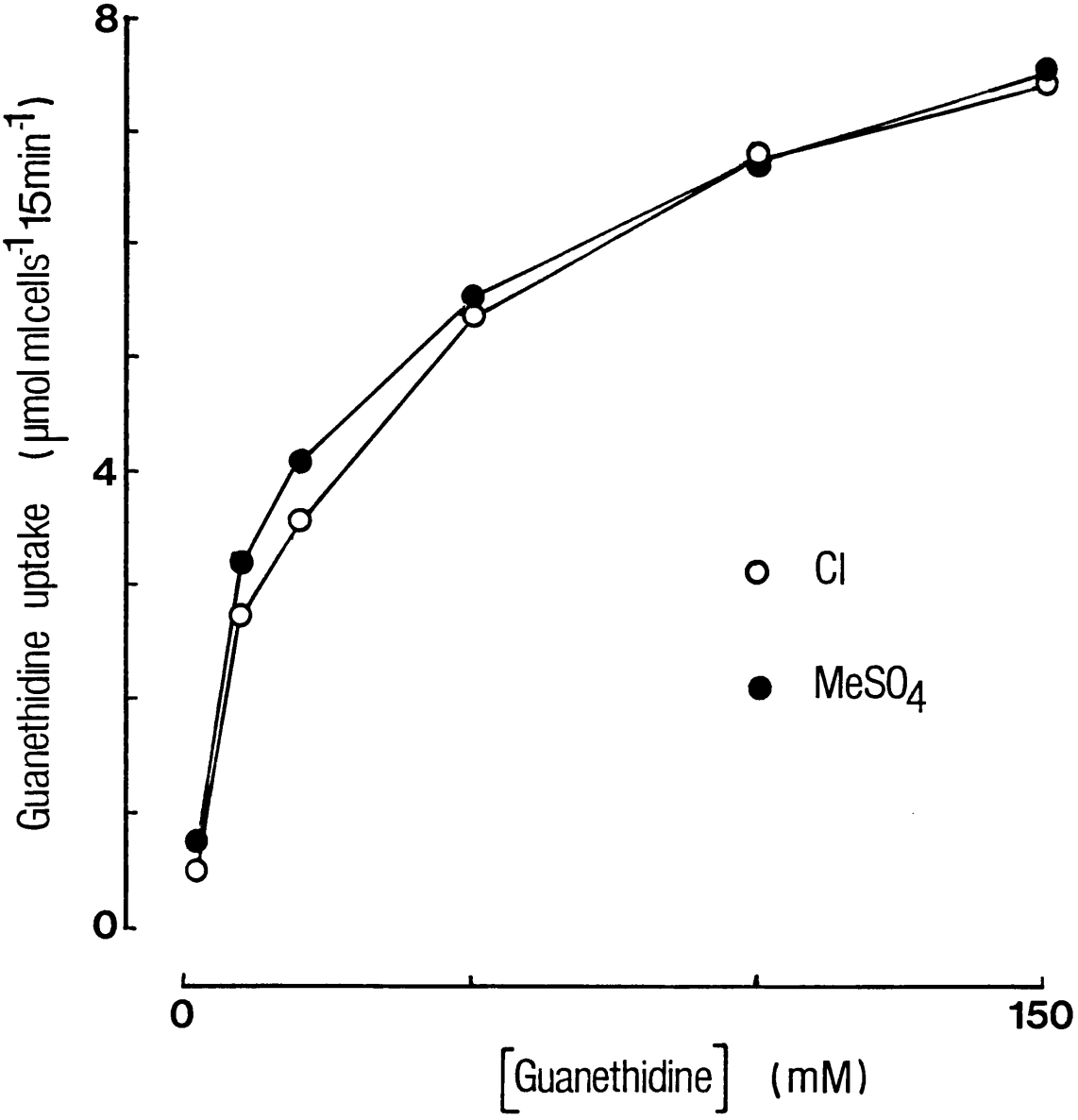
Kinetic constants and their associated standard errors were calculated by the method of Wilkinson (1961).

Km: mM.
Vmax: μmol ml cells^{-1} 15 min^{-1}.

Figure 34

The effect of anion substitution on kinetics of guanethidine uptake by red cells

^{14}C-guanethidine influx into red cells was measured from media containing predominantly Cl (open symbols) or $MeSO_4$ (closed symbols). The curves have been drawn by eye and each point is the mean of triplicate measurements. This experiment is representative of three performed.


8
4
0
Guanethidine uptake (μmol ml cells^{-1} 15min^{-1})
Cl
$MeSO_4$
0
150
[Guanethidine] (mM)

It is interesting, in conclusion, to note two points:

1. That no anionic substitution performed appeared at any time to inhibit NA uptake. (In other words, uptake in Cl media may be considered "basal".)
2. That accumulation of NA by red cells *in vivo* may be considerably faster than the values presented *in vitro* in Cl media, due to the presence of HCO_3 in the plasma.

The effect of DIDS upon NA uptake by red cells

Since it has been shown that NA uptake by red cells is influenced by different anionic substitutions, it was considered important to investigate the effect of DIDS, a potent anion exchange inhibitor, on this system under various experimental conditions. The effect of DIDS upon NA accumulation by ghosts was not investigated as it has been shown in the previous section that anion substitution failed to affect uptake in this system.

Six experiments were performed in which cells were exposed to DIDS prior to uptake measurements in either Cl, NO_3 or $MeSO_4$ media. The extracellular concentration of DIDS (10 μM) was adequate to substantially inhibit carrier-mediated anion transport (Ki = approximately 1 μM; Knauf, 1979). The results of these experiments are presented in Figure 35.

In the control (DIDS-free) incubations, replacement of Cl by either NO_3 or $MeSO_4$ significantly enhanced NA uptake, as expected from previous results ($P<0.02$ for both anions, as compared to Cl). It was further observed in the presence of DIDS that the results of anion substitution were affected such that the relative stimulation disappeared. In the first instance this appeared to suggest some small portion of NA uptake in NO_3 and $MeSO_4$ media might be mediated by the anion exchange mechanism. However, it also became apparent that the application of DIDS in fact stimulated control (Cl) NA uptake (from 47.9 ± 5.9 to 52.8 ± 7.4 pmol ml cells^{-1} 15 min^{-1}, $P<0.02$) and additionally, that further increasing [DIDS] to concentrations of up to 100 μM enhanced this effect. One experiment which is typical of four is illustrated in Figure 36. The degree of stimulation of NA uptake was not found to

Figure 35

The effect of DIDS on NA uptake by red cells

^{3}H-NA uptake into red blood cells was measured from media containing either Cl (open bars), NO_3 (slashes) or $MeSO_4$ (squares), in the presence and absence of 10 μM DIDS. DIDS-treated cells were incubated with the stilbene for 30 minutes at 37°C prior to uptake measurements. The data are the means of six experiments in which extracellular NA was 280 nM. * indicates $P<0.02$, compared to the control (Cl) condition and significance was assessed using Student's t-test for paired data.

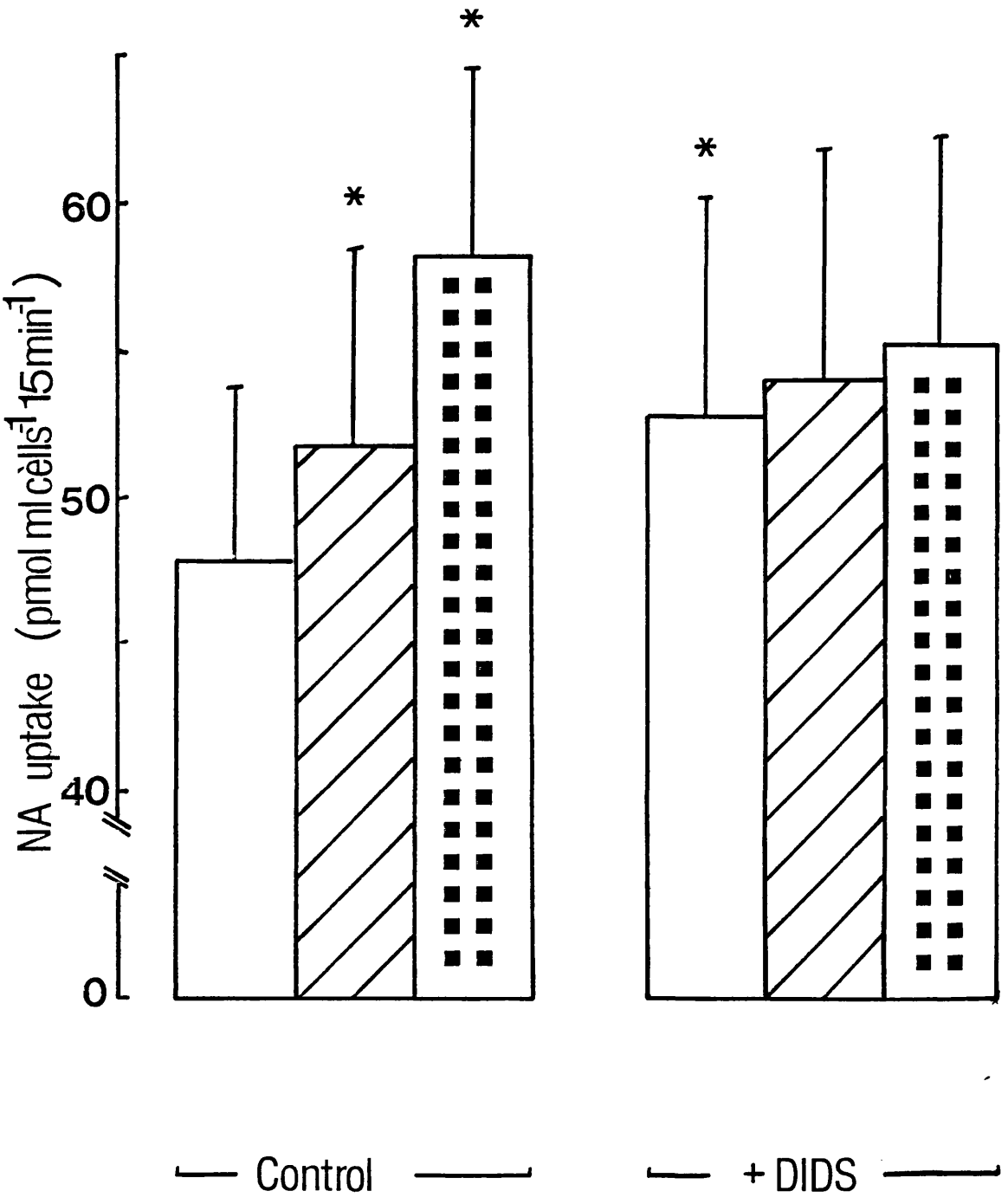
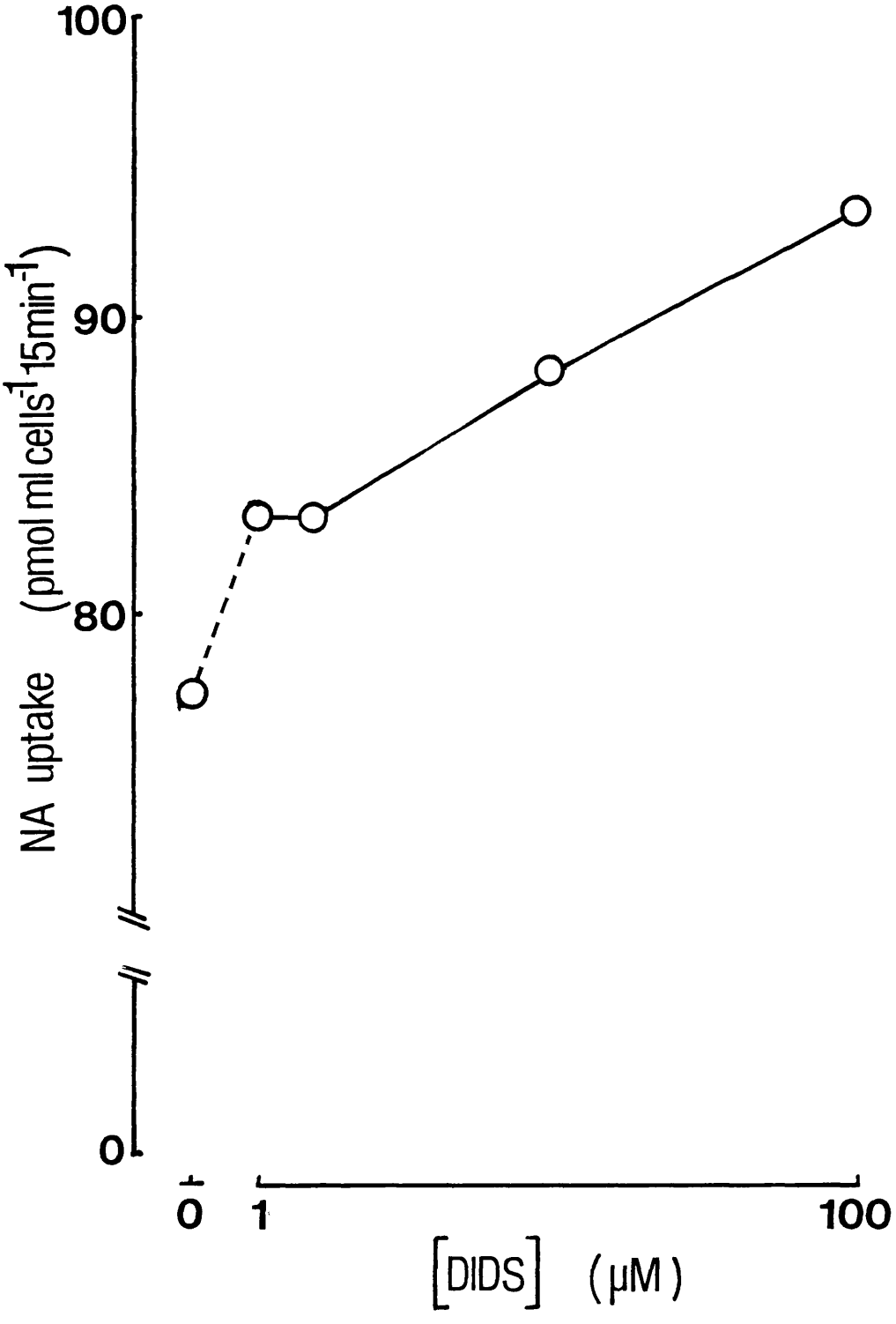

*
*
*
60
50
40
0
NA uptake (pmol ml cells^{-1} 15min^{-1})
Control
+ DIDS

Figure 36

The effect of varying [DIDS] on NA uptake by red cells

^{3}H-NA uptake by red cells was measured from media containing 290 nM NA in the presence of 0-100 μM DIDS. Cells were preincubated with or without DIDS for 30 minutes at 37°C prior to uptake measurements, which were performed in triplicate. The data drawn represents one experiment which is typical of four performed.


100
90
80
0
NA uptake (pmol ml cells^{-1} 15min^{-1})
0
1
100
[DIDS] (μM)

be correlated to the cell swelling induced by this treatment, and it has already been noted that cell volume is not a determinant of NA transport (Chapter 1). Since an elevation of [DIDS] beyond concentrations at which it near maximally inhibits anion exchange continues to exert a progressive effect upon NA transport it is suggested that this action is not a direct consequence of reduced anion transport.

THE EFFECT OF pH ON NA ACCUMULATION BY RED CELLS

Tracer studies

A total of 19 experiments were performed in all of which ^{3}H-NA uptake was markedly stimulated by increases in extracellular pH, an effect consistent with the results of Schanker, Nafpliotis and Johnson (1961). The extent of stimulation was such that influx at pH 8 was approximately 4-5 fold greater than that observed at pH 7. All pH values were directly measured in cell suspensions run adjacent to radio-labelled incubations, and a typical experiment is illustrated in Figure 37.

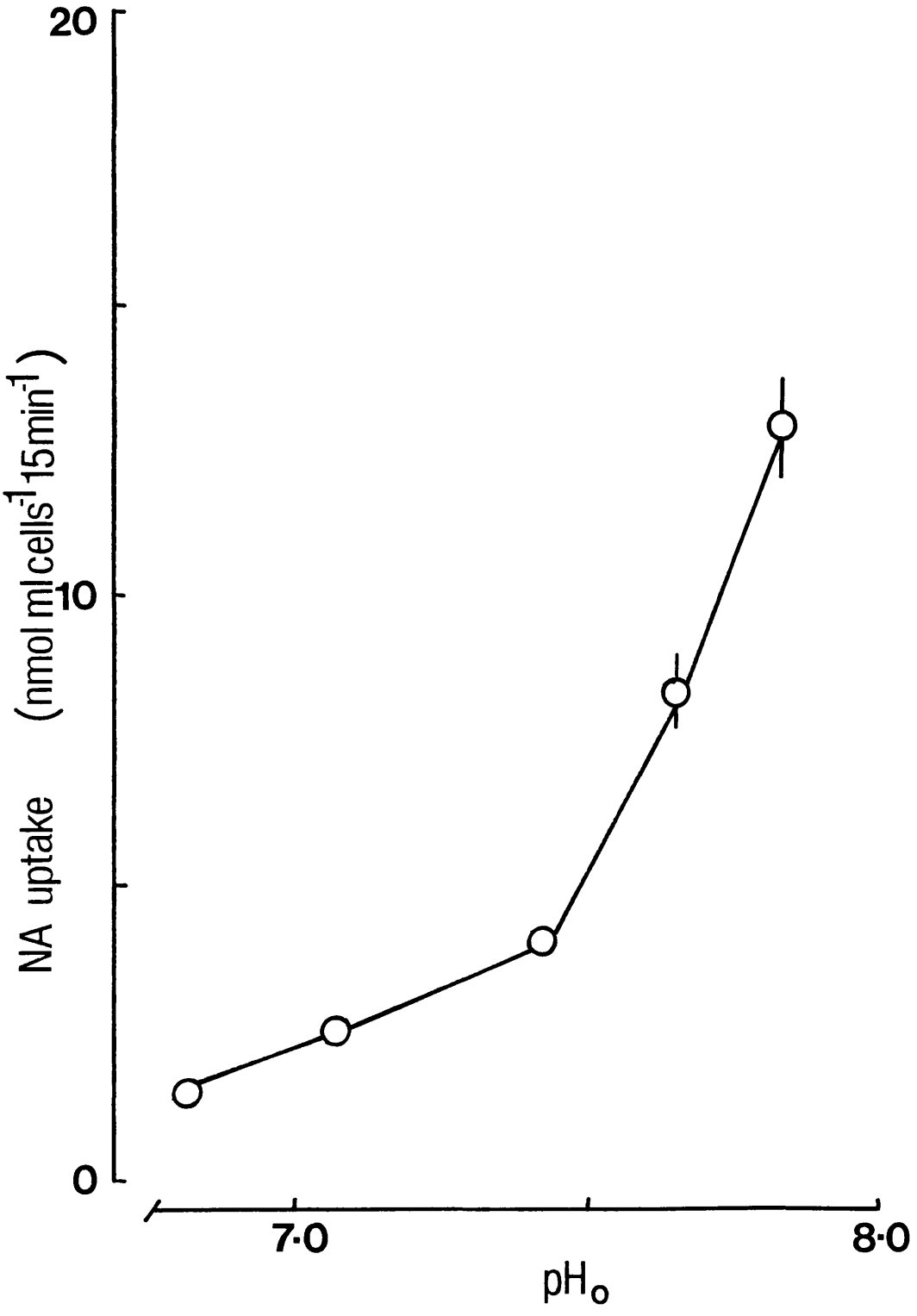
The physiological significance of this result is that under conditions of metabolic stress, e.g. severe exercise, where tissues may become acidotic, the clearance of elevated plasma NA levels associated with such episodes by red cells may become considerably reduced. It is noted that the steepest portion of the pH profile, as illustrated, is in the normal physiological range and therefore inactivation of circulating NA by red cells is optimally sensitive to local variations in acidity.

These results are also consistent with an increased ability of the red cell to accumulate the neutral, lipophilic NA molecule, although it is noted, in contrast to the work of Johnson, Carty and Scarpa (1985), that the steepest portion of Figure 37 (between pH 7.5-8.0) does not fall in the pH range in which the ratio of unionised : ionised NA rises most sharply (between pH 6.6-7.0, or indeed below this level).

Figure 37

The effect of pH on NA uptake by red cells

^{3}H-NA uptake was measured in red cells suspended in media of various pH values. pH was adjusted by adding different quantities of MOPS to buffers containing 10 mM Tris, and was directly measured in supernatants of non-radioactive cell suspensions. The data shows one experiment which is typical of 19 performed. Extracellular NA concentration was 20 µM, and each point drawn is the mean of triplicate determinations.


20
10
0
NA uptake (nmol ml cells^{-1} 15min^{-1})
7·0
8·0
pH$_o$

Net movement experiments

As for the effects of anion substitution upon NA uptake, it was considered of interest to investigate whether an increased unidirectional influx of NA actually yielded elevated intracellular concentrations. This is especially important in view of the work of Raymond and Weinshilboum (1975) pertaining to the pH dependence of COMT activity. Five experiments were performed to compare $[NA]_i$ in red cells incubated for 3 hours in the presence of 100 µM NA in media of either pH 7.4 or pH 7.0. The results are shown in Table 32 and as can be seen the resultant $[NA]_i$ is indeed greater at the higher pH. The ratio of $[NA]_i$ (pH 7.4) to $[NA]_i$ (pH 7.0) is also similar to that which may be calculated from tracer influx studies, although the variability in data obtained from HPLC-ED experiments is considerably larger. It therefore appears that, under the conditions described, tracer influx studies do provide a satisfactory estimation of the relative amounts of free NA within the cell at different pH values. (The possibility that COMT is saturated at these comparatively high $[NA]_o$ seems unlikely, as Axelrod and Cohn (1971) quote a Km of 0.2 mM for the enzymatic inactivation of NA by both human liver and red cell COMT.

The effect of pH upon NA uptake after anion substitution

The stimulation of NA uptake caused by anion substitutions noted earlier in this chapter are relatively small compared to the effects of pH changes, and if extracellular buffering proved to be inadequate, then the possibility might exist that such changes were the result of small deviations in pH caused by disturbances of the transmembrane Cl gradient. (As has previously been mentioned, the distribution of H^+ and Cl^- ions across the red cell membrane are closely linked.) Four experiments were performed to test this hypothesis. As can be seen from Figure 38, the effect of anion replacement is a genuine one at physiological pH, and becomes increasingly more pronounced as pH_o is lowered. For instance, uptake in NO_3 media at pH 6.9 is approximately double that measured in Cl media. Buffering was maintained in each

TABLE 32

THE EFFECT OF pH ON NET UPTAKE OF NA INTO RED CELLS

Experiment	$[NA]_i$ (nM)	
	pH 7.4	pH 7.0
1	109.5	50.7
2	1503.5	1210.1
3	1128.7	664.2
4	1006.2	345.5
5	1898.2	1300.3
MEAN	1129.2	714.2
(SEM)	(298.9)	(241.7)

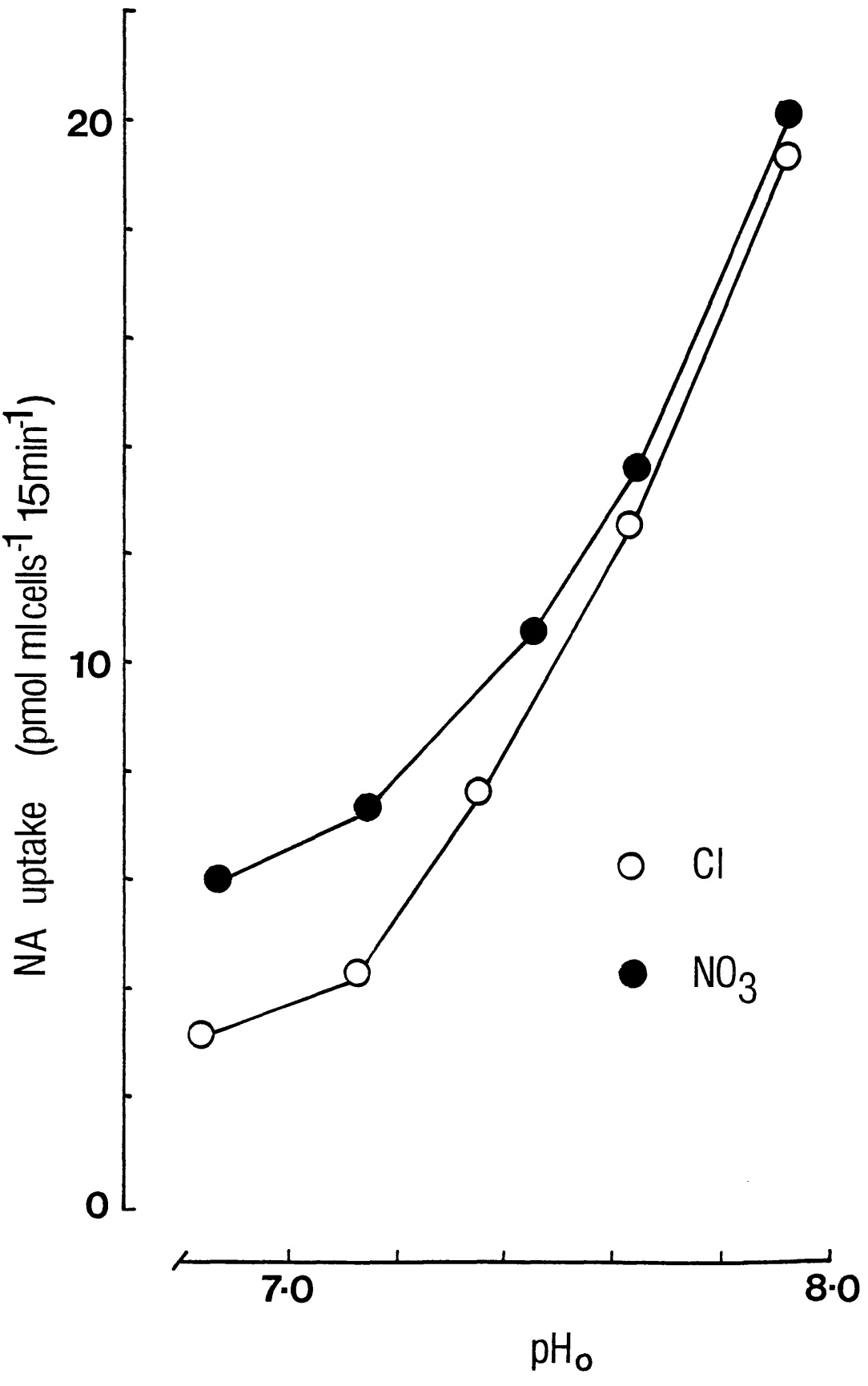
$[NA]_i$ levels were measured after 3 hours incubation at 37°C in the presence of 100 μM NA_o.

The two groups are significantly different ($P<0.05$).

Figure 38

The effect of pH on anion dependency of red cell NA uptake

^{3}H-NA uptake was measured containing either Cl (open symbols) or NO_3 (closed symbols) as the major extracellular anion. The extracellular NA concentration was 40 nM and each point shown is the mean of triplicate determinations. Different pH values were achieved by adding various quantities of MOPS to media containing 10 mM Tris. The resultant pH was then measured in supernatants from isotope free cell suspensions. The figure shows one experiment which is typical of four performed.


20
10
0
NA uptake (pmol mlcells^{-1} 15min^{-1})
Cl
NO_3
7·0
8·0
pH_o

instance using 10 mM Tris-MOPS, and the suitability of this protocol was verified by direct measurement of extracellular pH which remained invariant throughout the 15 minute flux incubations. It should be noted, with reference to previous experiments, that this result is in itself circumstantial evidence for the non-involvement of the DIDS-sensitive anion exchange mechanism in this response, because the activity of the exchanger decreases as pH is lowered. In other words, the exchanger is at its most quiescent when the effects of anion substitution are largest. At "high" pH values, around pH 8, an effect of anion substitution may still be seen, although the stimulation is quantitatively much reduced.

The effect of pH upon NA uptake by ghosts

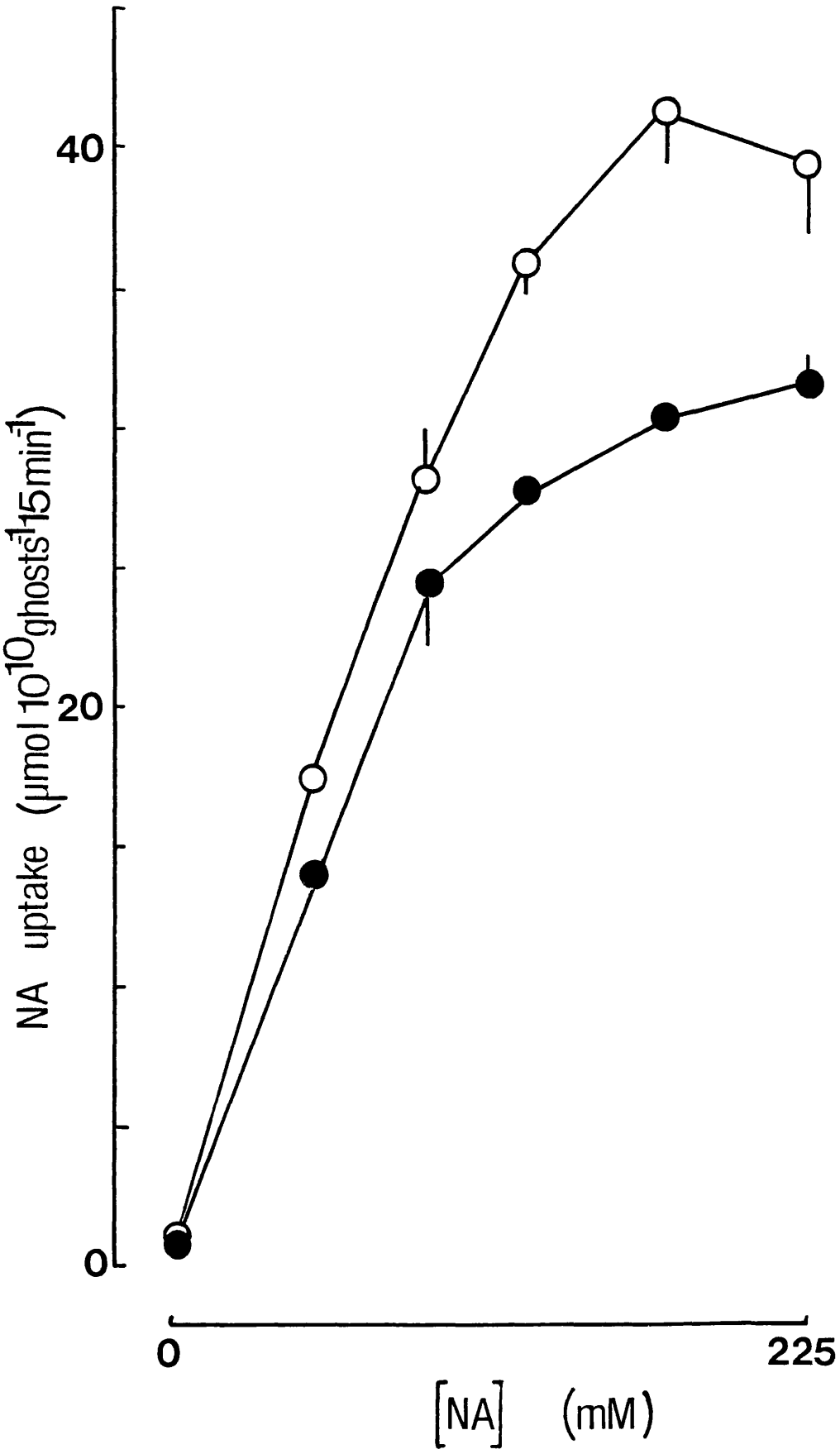
NA uptake into ghosts was measured at pH 7.4 and pH 7.0. Uptake remained pH sensitive and hence, in the absence of metabolic activity and under conditions of vastly reduced intracellular binding capacity, it may be concluded that a considerable portion of the pH sensitivity of uptake resides at the level of the membrane. However, if the ratios of uptake at the two different pH values are taken into consideration, it becomes apparent that the mean gradient of uptake against pH in ghosts is less than that in cells. This may be explained by the reduced internal buffering capacity of the ghost, and the consequent gradual dissipation of the transmembrane pH gradient.

The experiments studying the pH dependence of NA uptake in ghosts (n=3) all included saturating substrate concentrations, so as to allow kinetic inferences to be drawn. A typical experiment is illustrated in Figure 39. The data suggest that the effect of reducing pH is to both increase the apparent affinity of carrier for substrate, but at the same time reduce the maximal velocity of uptake. Km fell from 585 (pH 7.4) to 446 mM (pH 7.0). Vmax fell from 118 (pH 7.4) to 71 μmol 10^{10} ghosts^{-1} 15 min^{-1} (pH 7.0). Thus it would appear that at higher pH values NA is less avidly bound, but the increased rate of carrier translocation ensures that uptake proceeds faster in more alkaline conditions.

Figure 39

The effect of pH on NA uptake by red cell ghosts

^{3}H-NA uptake was measured in ghosts suspended in media buffered either to pH 7.4 (open symbols) or to pH 7.0 (closed symbols). Each point is the mean of triplicate determinations. Ghosts were prepared by the method described by Richards and Eisner (1982). This experiment is one of three performed.


40
20
0
NA uptake (μmol 10^10 ghosts^-1 15min^-1)
0
225
[NA] (mM)

The effect of amiloride on NA transport

As a consequence of a disturbance in extracellular pH, it follows that there is a tendency for intracellular pH to be similarly affected. Many cellular mechanisms depend on a narrow and strictly maintained pH range for optimal activity and therefore the cell possesses mechanisms which operate so as to maintain pH at a stable equilibrium value. This may be achieved either by intracellular buffering or by membrane transport phenomena, one example of which is the electroneutral Na-H exchanger. The Na-H exchanger is found in a wide variety of tissues and is activated by acid loading so as to extrude H^+, simultaneously facilitating the entry of Na^+ by counter-transport. The mechanism is also involved in cell volume regulation, although remains silent in the majority of tissues for most of the time. Exchange of Na for H may be inhibited by the diuretic amiloride, and a variety of recently synthesised amiloride analogues, for example 6-bromo-5-ethylisopropyl amiloride (Friedrich, Sablotri and Burckhardt, 1986).

Originally two experiments were performed to investigate the effects of amiloride (1 mM) on ^{3}H-NA uptake at three pH values: pH 7.4, pH 7.0 and pH 6.6. An effect of amiloride was observed, but this action (a modest inhibition of influx) was greatest at pH 7.4, where it may be assumed that the Na-H exchanger is least functional. Additional experiments were performed, at further elevated pH values, and it was observed that the inhibition attributed to amiloride constituted an increasingly large percentage of total uptake, and became statistically significant ($P<0.01$ at the highest pH point investigated, pH 8.2). The data is illustrated in Table 33.

It seems unlikely that the results could be explained in terms of inhibition of Na-H exchange, and it is worth noting that other actions have also been attributed to amiloride, including inhibition of Na-Ca exchange and inhibition of passive Na permeability in various epithelia (see Villereal, 1986).

It was therefore considered possible, although unlikely in light of the evidence presented in Chapter 3, that the effect of

TABLE 33

THE EFFECT OF AMILORIDE UPON ^{3}H-NA UPTAKE

Experiment	ORIGINAL pH									
	pH 6.6		pH 7.0		pH 7.4		pH 7.8		pH 8.2	
	-	+	-	+	-	+	-	+	-	+
1					3.76	3.32	5.66	4.84	8.46	8.20
2					3.70	3.67	6.34	4.99	9.22	7.36
3			2.32	2.28	4.04	3.45	7.00	4.42	11.11	4.34
4			2.46	2.20	4.26	3.44	7.64	4.30	13.89	2.47
5			3.25	3.00	4.03	4.94	7.42	6.54	10.18	6.97
6			2.83	3.05	4.49	5.10	7.35	6.59	15.79	5.94
7	1.56	1.72	2.60	2.14	4.12	3.18	8.29	7.00	12.85	8.20
8	2.53	2.32	4.43	5.00	9.22	7.58	13.97	8.92	20.41	13.25
MEAN	2.05	2.02	2.98	2.94	4.70	4.34	7.96	5.95	12.74	7.09
SEM			0.32	0.44	0.65	0.53	0.90	0.57	1.40	1.10

- denotes control conditions (amiloride absent).

+ denotes the presence of amiloride (1 mM).

Units of ^{3}H-NA influx: nmol ml cells^{-1} 15 min^{-1}.

$[NA]_o$ was 20 μM throughout.

amiloride might be related to an inhibition of the passive Na entry stimulated at high pH. Accordingly, two experiments were performed in which cells were suspended with and without both Na and amiloride. The experiments were performed at 20 μM $[NA]_o$ using NMDG to isosmotically replace Na. The results indicated that effect of amiloride at high pH levels was apparently not influenced by the identity of the major extracellular cation (Figure 40). Hence, the action of amiloride upon NA transport is apparently Na independent.

Further evidence for this apparently non specific action of amiloride was obtained from experiments designed to consider the pH dependent action on NA uptake of another inhibitor of Na-H exchange, N,N-dicyclohexylcarbodiimide (DCCD) (Friedrich, Sablotri and Burckhardt, 1986). DCCD (5 mM) was also observed to affect NA uptake in a pH dependent fashion. In direct contrast to the action of amiloride, however, DCCD was shown to further enhance the already stimulated uptake of NA at higher pH values (Table 34). Accordingly, NA uptake at pH 7.4 from a medium containing 20 μM NA rose from 3.6 ± 0.06 to 4.99 ± 0.44 nmol ml $cells^{-1}$ 15 min^{-1} (n=6, $P<0.02$), a mean increase of 37.1%. Mean uptake at pH 7.8 rose by 61.9% ($P<0.0001$), and by 55.6% at pH 8.2 ($P<0.0001$). It therefore seems likely that neither amiloride or DCCD exert their effects upon NA transport via the Na-H exchange mechanism. The precise pathways of action of these drugs in this context remains obscure.

Figure 40

The effect of amiloride on NA uptake by red cells

^{3}H-NA accumulation by red cells was measured in the presence (squares) and absence (circles) of 1 mM amiloride, at various extracellular pH values. Influx was measured in triplicate from Na containing media (filled symbols) and from media in which Na had been isosmotically replaced by NMDG (open symbols). Measurements of pH were made directly from the supernatants of non-radioactive cell suspensions. The extracellular NA concentration was 20 μM. The experiment illustrated is one of two performed.

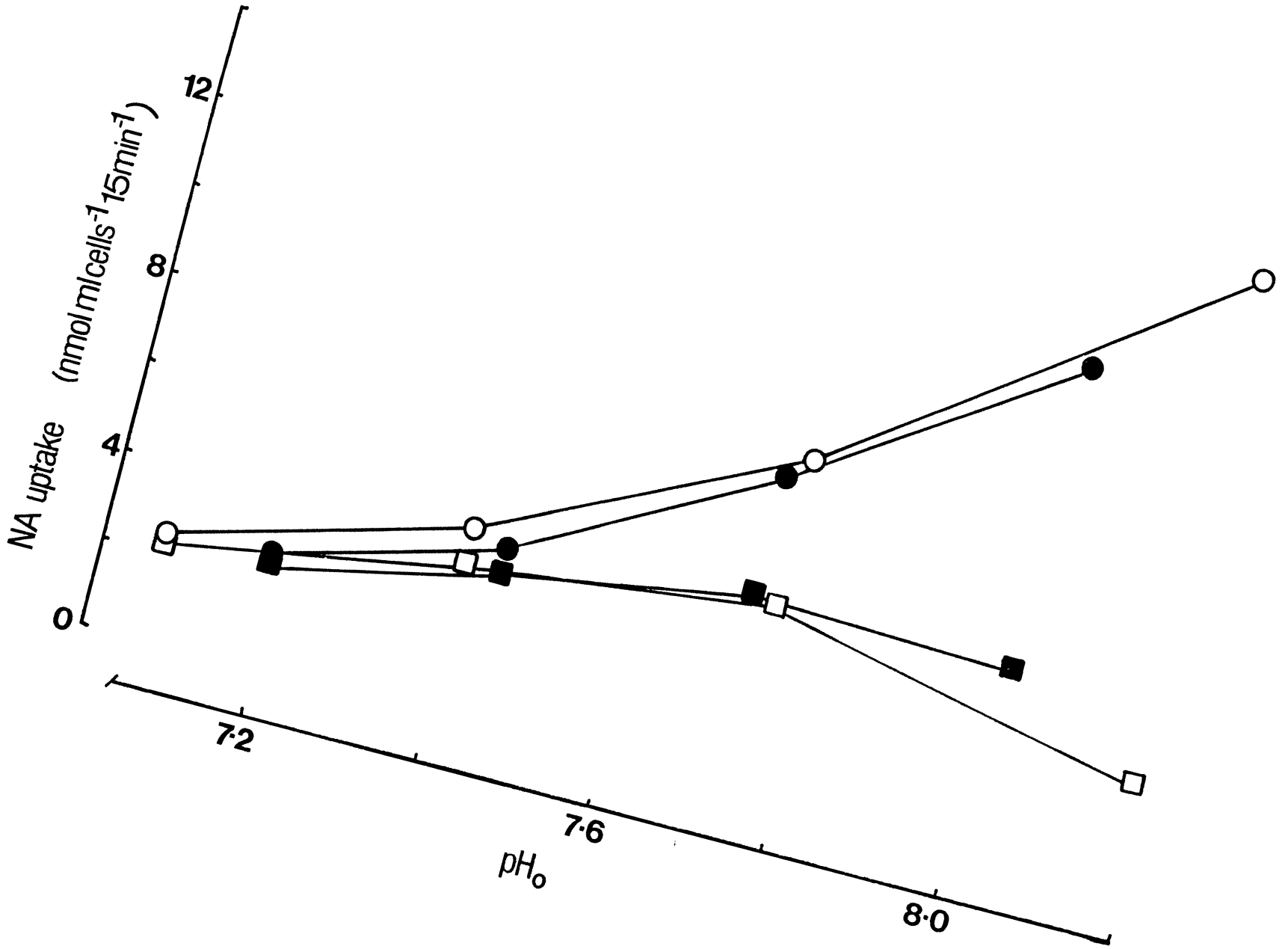

NA uptake (nmol ml cells^{-1} 15min^{-1})
0
4
8
12
7·2
7·6
8·0
pH_o

TABLE 34

THE EFFECT OF DCCD UPON ^{3}H-NA UPTAKE IN RED CELLS

Experiment	ORIGINAL pH					
	pH 7.4		pH 7.8		pH 8.2	
	Control	DCCD	Control	DCCD	Control	DCCD
1	3.58	5.18	5.47	10.34	8.41	12.55
2	3.58	4.75	6.39	10.34	8.62	12.21
3	3.80	3.64	6.05	7.09	8.18	11.88
4	3.39	4.02	5.55	8.19	8.37	13.48
5	3.76	5.92	5.66	11.13	8.46	13.23
6	3.70	6.44	6.34	10.34	9.22	16.40
Mean	3.64	4.99	5.91	9.57	8.54	13.29
(SEM)	(0.06)	(0.44)	(0.17)	(0.64)	(0.15)	(0.67)
	P<0.02		P<0.0001		P<0.0001	

$[NA]_o$ = 20 μM.
[DCCD] = 5 mM.
Units of flux: nmol ml cells^{-1} 15 min^{-1}.

DISCUSSION

The effects of anion substitution upon NA uptake

Effects of Cl replacement upon NA uptake in the human red cell have not previously been described. The results presented in this chapter suggest that there is, however, some dependence of uptake upon the anionic composition of the extracellular medium. The question is therefore posed: in what manner might anion replacement affect accumulation of NA by red cells?

The loss of effect of anion substitution on ghosting suggests that the effect may be mediated by a component of the cell which is lost in the course of ghost preparation. One candidate for this role is the catabolic activity of intracellular COMT, and support for this hypothesis comes from the observation that the effects of anion substitution are partly negated when an inhibitor of COMT activity, tropolone, is added to an intact red cell suspension. It is possible that incubation of red cells in the presence of increased concentrations of tropolone, or for greater periods of time, may have further enhanced the effects observed by allowing increased incorporation of tropolone. This hypothesis is substantiated by the results presented in Chapter 1 where it was noted that tropolone markedly affected net NA uptake. Similar experiments involving anion substitutions were not performed, and the permeability of the red cell membrane to tropolone remains unknown. It would be of interest to directly assay the comparative activity of COMT in media containing Cl, NO_3 and $MeSO_4$. COMT may be purified in the laboratory (see Raymond and Weinshilboum, 1975), and was shown by these authors to be activated maximally by Mg (1 mM), and inhibited by Ca.

Further circumstantial evidence for an effect of anion substitution upon catecholamine catabolism comes from the net transport experiments performed in which removal of intracellular NA appeared markedly retarded in NO_3 media, such that the resultant haemolysate concentrations rose 300-400% above control (see Figure 31). This value is far in excess of the NO_3 induced stimulation of unidirectional ^{3}H-NA influx noted. A further significant result was that the uptake of guanethidine, a compound

which has a prolonged half-life (about 5 days) in vivo and thus, by implication is not rapidly metabolised, does not seem to be significantly affected by anion substitution. To draw firm conclusions from these latter experiments would be dangerous however, as it has already been shown that NA and guanethidine do not cross the red cell membrane by identical routes. The data on NA uptake remains consistent, however, with the hypothesis that anion substitution may affect catecholamine metabolism and secondarily uptake.

While the evidence from experiments performed using ghosts points towards a further link between the metabolism and uptake of NA, it is difficult to reconcile the effects of DIDS in this light. DIDS specifically binds to the cell membrane and is shown in this chapter to somewhat negate the effects of anion substitution. If the effects of anion substitution are manifested both intracellularly, as argued up to this point, and at the membrane level, as suggested by DIDS, then why might the latter component not be observed in the red cell ghost? (Replacement of Cl has no effect on NA transport in ghosts - see Figure 33). A possible explanation might involve the fact that a considerable amount of membrane material is lost during ghost preparation, and that it is part of this fraction that mediates the response to DIDS. This would be consistent with the fact that the effect of DIDS and the consequences of anion substitution are not mediated via the anion exchange mechanism which is known to be effectively reconstituted in resealed red cell ghosts (Funder and Wieth, 1975).

It has been shown that physiological concentrations of HCO_3 (25 mM) stimulate NA uptake, and this result is qualitatively similar to the increased passive ^{22}Na influx observed by other authors in similar circumstances (e.g. Funder and Wieth, 1967). The relationship between ^{22}Na and NA uptake does not hold, however, at the higher concentration of HCO_3 tested (94 mM). Under such conditions it would be expected that ^{22}Na influx would be further enhanced (Wieth, Tosteson and Hoffman, 1978) and indeed results to this effect are presented in Chapter 5. NA uptake

responds differently, however. Uptake of NA at 94 mM HCO_3 is greater than in the absence of HCO_3 but reduced as compared to at 25 mM HCO_3. This result may be explained if, in the presence of 94 mM HCO_3, the interior of the cell becomes marginally more alkaline. Under such conditions, ion-trapping of NA would be reduced, and the tendency towards backflux of the more lipophilic form of amine would also be enhanced. The degree of stimulation of NA uptake might therefore appear retarded.

The effects of pH upon NA uptake

An increase in extracellular pH has been demonstrated to elevate NA uptake by similar amounts in both red cells and ghosts. This result is consistent with the work of Schanker, Nafpliotis and Johnson (1961), and a similar pH dependence for amine transport has also been reported in bovine chromaffin granules (Johnson, Carty and Scarpa, 1985). Net transport experiments also suggest that free NA_i becomes elevated in red cells as a consequence of increased extracellular pH. These results strongly suggest, as discussed, that the effect of pH upon NA transport is primarily mediated at the membrane level and is not secondary to intracellular events. This is because the pH dependence of uptake is clearly seen in red cell ghosts in which both binding and metabolic activity are greatly reduced. The effect of increased extracellular pH appears to be represented primarily as an increase in the maximal rate of activity of the transporter, rather than its affinity for substrate.

More difficult to interpret, but of considerable interest in this section were the results obtained in the presence of both amiloride and DCCD. To consider the former compound, it is apparent from Figure 40 that uptake of NA as pH is elevated in increasingly inhibited by amiloride. Therefore, amiloride sensitive NA uptake is markedly pH sensitive, although the action of the compound can not be related to its role as an inhibitor of Na-H exchange. Due to the relationship described, it seems likely that amiloride acts primarily to inhibit the uptake of neutral NA. Whether amiloride competes with NA for a membrane binding site is,

however, unknown. Kinetic analysis of the action of amiloride was not feasible, as uptake in red cell ghosts at high pH values did not remain linear with time. While the action of amiloride on NA uptake therefore remains poorly understood it seems probable that a protein containing portion of the membrane is involved in the response. This is in light of the many other documented actions of amiloride which are reviewed by Villereal (1986).

The possibility also exists that amiloride exerts its actions from within the cell, as the molecule is readily permeant. In order to further clarify the precise mechanism of action of amiloride, it would be beneficial to consider the actions of the family of structurally related analogues which may now be synthesised (e.g. Benos, 1982).

DCCD is also a relatively non-specific inhibitor of membrane transport functions. Friedrich, Sablotri and Burckhardt (1986) demonstrated, in addition to Na-H exchange, that DCCD inhibited the Na-dependent transport systems for SO_4, dicarboxylates and amino acids in membrane vesicles isolated from rat kidney. DCCD also binds to the alpha subunit of the Na-K-ATPase (Pedemonte and Kaplan, 1986). In contrast to these inhibitory effects, it has been shown here that DCCD gives rise to a pH-dependent stimulation of NA uptake. The irreversible nature of DCCD inhibition of Na-H exchange has been explained on the basis that the molecule forms covalent links with the membrane, possibly with a carboxyl group. One possible implication, therefore, might be that such groups normally tend to inhibit the transport of NA. Clearly, the action of DCCD merits further attention.

In conclusion, evidence has been presented which suggests that the pH sensitivity of NA uptake is mediated primarily at the membrane level. Previously unknown actions of the Na-H exchange inhibitors amiloride and DCCD have also been described.

CHAPTER 5

EFFECTS OF NA UPON RED CELL CATION TRANSPORT

INTRODUCTION

It is well documented that catecholamines and a number of other compounds (e.g. prostaglandins and angiotensin) are able to affect cation transport in a variety of mammalian, avian and amphibian tissues (for examples, see Table 35). The available evidence suggests that these effects are often mediated as a result of the stimulation of receptors located on the surface of the membrane. The binding of NA to β-receptors increases adenylate cyclase activity, which in turn leads to elevated intracellular levels of the second messenger compound cyclic AMP. This compound subsequently activates a specific cyclic AMP dependent protein kinase which is thought to promote phosphorylation of membrane components involved in cation transport (e.g. the ouabain sensitive Na-K pump). Such effects have been observed both *in vivo* and *in vitro*, and the physiological significance of the chain of events culminating in Na pump activation is considered to be the clearance of K from the extracellular water space, for instance during exercise (Clausen, 1983). Consequently, β_2-agonists have been shown to be effective therapeutic agents in the treatment of hyperkalaemia. Such responses have been noted in man and in a variety of animals. As is clear from Table 35, β-agonists have also been shown to affect other cation transport pathways, such as the Na-H exchange mechanism of rainbow trout red cells, which is transiently stimulated by isoprenaline (Baroin, Garcia-Romeu, Lamarre & Motais, 1984).

Biochemical evidence is also available which suggests that NA may directly interact with purified preparations of Na-K ATPase. In such studies (e.g. Hexum, 1977), it has been argued that the stimulation of enzyme activity observed may be the result of a reduction, or reversal, of the inhibitory effects of trace amounts of heavy metals such as Fe^{2+}. This theory is consistent

TABLE 35

THE EFFECTS OF CATECHOLAMINES AND RELATED COMPOUNDS ON CATION TRANSPORT

Reference	Tissue	Membrane transport parameter	Agonist	Effect	Cyclic AMP dependent?	β-receptor mediated?
1	Mouse macrophages	Na-K pump	prostaglandins	↑	+	?
1	Human red cells	Na-K pump	prostaglandins	none	none	-
2	Frog skeletal muscle	Na-K pump	adrenaline	↑	?	Yes
3	Rat hepatocytes	Na-K pump	NA, angiotensin, vasopressin	↑	?	No (∝)
4	Toad smooth muscle cells	Na-K pump	isoprenaline	↑	+	Yes
5	Rat soleus muscle	Na-K pump	NA, adrenaline isoprenaline	↑	+	Yes
6	Turkey red cells	Bi-directional K flux	isoproterenol	↑	+	Yes
7	Rabbit/guinea pig arterial muscle	K efflux (channel)	NA, 5-HT histamine	↑	?	No (∝)
8	Trout red cells	Na-H exchange	isoprenaline	↑	+	Yes
9	Cultured arterial muscle cells	Ca efflux	isoproterenol	↓	+	Yes
10	Toad smooth muscle cells	Ca efflux	isoprenaline	↑	+	Yes

REFERENCES: 1)Braquet, Diez & Garay (1985); 2)Kaibara, Akasu, Tokimasa & Koketsu (1985); 3)Berthon, Capiod & Claret (1985); 4)Scheid & Fay (1984a); 5)Clausen & Flatman (1977); 6)Gardner, Mensh, Kiino, & Aurbach (1975); 7)Bolton & Clapp (1984); 8)Baroin, Garcia-Romeu, Lamarre & Motais (1984); 9)Smith (1984); 10)Scheid & Fay (1984b).

with the claims of Colburn and Maas (1965) that catecholamines bind to divalent cations. Kometiani (1978) also described a dose dependent effect of NA on Na-K ATPase, although it was noted that the response varied from tissue to tissue. Some enzyme preparations failed to respond to neurotransmitter. In addition, at low (μM) NA concentrations, Na-K ATPase activity in synaptosomal fractions was stimulated by NA, in agreement with Hexum (1977), while at high NA concentrations (1-5 mM), Na-K ATPase activity was significantly inhibited.

Yoshimura (1973) also showed a stimulation of Na-K ATPase by NA in an extract of rat brain, as did Coffey, Hadden, Hadden and Middleton (1971) in a preparation obtained from human lymphocyte and neutrophil membranes. Conversely Luly, Barnabei and Tria (1972) reported that adrenaline and glucagon inhibited Na-K ATPase in a liver membrane preparation in vitro.

Regulation of red cell cation transport

While Braquet, Diez and Garay (1985), were able to demonstrate a cyclic AMP dependent increase in isolated mouse macrophage Na pump activity after administration of prostaglandins, these authors observed no such effect in human red blood cells. It is known that human red cells possess membrane bound cyclic AMP dependent protein kinase activity, and that this kinase shares many of the properties associated with similar moieties isolated from other mammalian tissues (Rubin, Erlichman & Rosen, 1972). Human red cell membranes also avidly bind cyclic AMP and possess structures with many of the characteristics of β-receptors (Rasmussen, Lake & Allen, 1975). It would appear however, that β-adrenergic agonists are unable to stimulate red cell adenylate cyclase activity, as discussed by Braquet, Diez and Garay (1985), and thus this system represents an incomplete model regarding cyclic AMP metabolism. It may therefore be the case that β-agonists are unable to regulate red cell cation transport via the second messenger pathway described.

It has, however, been suggested by some authors (e.g. Riozzi, Heagerty, Bing, Thurston & Swales, 1984) that some of the

abnormalities of cation transport observed in essential hypertension may be attributed to circulating NA, which may itself be significantly elevated in some hypertensive patients (Goldstein, 1981). Riozzi and co-workers studied cation fluxes in white cells, whilst the majority of authors interested in hypertension have focussed their attention upon the red cell. Some of the red cell cation transport abnormalities reportedly associated with hypertension are described in Table 36. It is argued that abnormalities in Na transport in such individuals may, at the level of the resistance vessels, secondarily influence Na-Ca exchange thus elevating intracellular Ca and promoting constriction of the arterioles. This theory is consistent with the work of Smith (1984), who was able to demonstrate a cyclic AMP dependent β-receptor mediated reduction in Ca efflux from cultured arterial muscle cells, coupled to distinct morphological changes. Abnormalities in cation transport may not, of course, have any physiological significance in tissues such as the red cell, but may yet act as a convenient, and accessible, marker for the disturbance in the vascular bed. The action of circulating regulators of Na transport have also been implicated in other disease states, such as chronic renal failure (Ellory, Fervenza & Hendry, 1988). In light of the available evidence it was therefore decided to investigate the effects of NA upon cation transport in human red cells.

The possible effects of NA upon cation transport in red cells were investigated using a number of different protocols. First, Na transport was measured in red cells obtained from individuals subjected to either cold pressor test or NA infusion. Both protocols were designed to cause transient increases in both plasma NA concentration and the blood pressure of the individual. These conditions therefore in part mimicked the hypertensive state. In addition, Na transport was measured in red cells sampled from unstressed subjects after the addition of various amounts of NA solely *in vitro*.

TABLE 36

ABNORMALITIES OF CATION TRANSPORT IN RED CELLS OF PATIENTS WITH ESSENTIAL HYPERTENSION

Reference	Membrane transport parameter	Abnormality
1	Na-K pump	↑
2	Na-K pump	↓
3	Na-K pump	↑
3	Co-transport	↑
4	Co-transport	↓
5	Li-Na counter-transport	↑
6	Co-transport	↑
7	Co-transport	↑ (Caucasian)
7	Co-transport	↓ (Black)
8	Li-Na counter-transport	↑

References:

1) Woods, Beevers & West (1981).
2) Postnov, Orlov, Schevchenko & Adler (1977).
3) Bin Talib, Chipperfield & Semple (1984).
4) Garay, Elghozi, Dagher & Meyer (1980).
5) Canessa, Adragna, Solomon, Connolly & Tosteson (1980).
6) Canessa, Bize, Solomon, Adragna, Tosteson, Dagher, Garay & Meyer (1981).
7) Tuck, Gross, Maxwell, Brickman, Krasnoshtein & Mayes (1984).
8) Brugnara, Corrocher, Foroni, Steinmayr, Bonfanti, & De Sandre (1983).

Clinical use of the cold pressor test

The cold pressor test (CPT) has been established as a clinical tool for approximately 50 years (see, for example, Hines & Brown, 1936). These authors performed CPT upon a number of individuals, classified as either hypertensive or normotensive. Among the normotensive group, two clear subpopulations became evident: "normoreactors" and "hyper-reactors". In hyper-reactors CPT elicited significantly greater increases in blood pressure than in the normal group. Repeated measurements indicated that these responses appeared to be a genuine characteristic of the individual, such that there was an average variation in range of response of only 10%.

As a result of following the responses of subjects to CPT over a number of years, Hines and Brown noted that certain of the hyper-reactors eventually developed essential hypertension. In contrast, the normoreactors all remained normotensive. It was therefore suggested that CPT might serve as a diagnostic tool in the detection of essential hypertension prior to onset. Some credence to this theory has more recently been supplied by the work of Wood, Sheps, Elveback and Schirger (1984) in which individuals were intermittently subjected to CPT over the course of 50 years. Essential hypertension was seen to develop in 71% of hyper-reactors, as opposed to only 19% of normoreactors. As Horwitz (1984) points out, however, it proved impossible to assess every individual for the whole course of the study. In addition, some normotensive individuals were originally classified normoreactors at the time of the first CPT (1934), but as hyper-reactors at a later date (1961). The interpretation of the data of Wood, Sheps, Elveback and Schirger is thus open to some speculation.

The CPT has thus evolved as a tool in the prediction of future essential hypertension, while its clinical usefulness in this respect remains unproven. It is clear, however, that the test is a reliable and easy method of inducing short term elevations of both blood pressure and NA in 99% of people. It should be noted that it has not been assumed that CPT acts to elevate circulating

NA levels alone. This point is expanded upon later in this chapter. The other protocol that has been employed, NA infusion, may also effect the plasma concentrations of a number of other circulating agents in man. Alongside CPT however, NA infusion too is recognised as a reliable technique for the simultaneous elevation of both blood pressure and peripheral NA concentration.

CPT and NA infusions have therefore been utilised in this study in an attempt to discern whether increased NA levels in vivo may affect red cell ^{22}Na transport, in conjunction with more closely controlled in vitro studies.

RESULTS

COLD PRESSOR TESTS

Cold pressor tests (CPT) were performed upon nine male volunteers using the protocol described in Methods. Prior to flux measurements, red cells were resuspended in buffered media containing 25% (v/v) plasma which had been stored in sealed containers at 4°C since CPT. ^{22}Na transport was therefore determined under conditions designed to closely simulate the environment during CPT.

EFFECTS OF COLD PRESSOR TEST

1. Blood Pressure

The effects of CPT upon blood pressure were marked. Blood pressures were measured at the start of the experiment (t_0) and after a 20 minute period during which each subject was asked to relax (t_{20}). These two values were not significantly different (Table 37). Systolic blood pressure (SBP) fell slightly, from an initial value of 126 ± 5 to 119 ± 4 mm Hg (mean ± SEM), while diastolic blood pressure (DBP) remained stable at 71 ± 4 (t_0) and 72 ± 3 mm Hg (t_{20}).

Immersion of one hand in an ice-water mixture, however, led to significant increases in both measured parameters, as assessed by Student's t-test. SBP rose from 119 ± 4 to 140 ± 6 mm Hg ($P<0.01$), while DBP rose from 72 ± 3 to 99 ± 3 mm Hg ($P<0.0001$). Consequently, mean blood pressure was also significantly elevated from 88 ± 2 to 113 ± 3 mm Hg ($P<0.0001$).

2. Plasma catecholamines

Plasma NA and adrenaline were both measured at t_0, t_{20} and during CPT. The measured results are presented in Table 37. It was observed that in some samples the concentration of adrenaline present fell below the limits of detection of the HPLC system used, about 0.5 nM (Walker, 1987), and in these instances it was assumed that adrenaline was present only in negligible amounts.

As with SBP, plasma NA fell slightly, but not significantly, during the 20 minute period of acclimatisation: from 2.50 ± 0.27

TABLE 37

COLD PRESSOR TESTS

	t_0	t_{20}	CPT	
Systolic BP (mm Hg)	126 (5)	119 (4)	140 (6)	P<0.01
Diastolic BP (mm Hg)	71 (4)	72 (3)	99 (3)	P<0.0001
Mean BP (mm Hg)	90 (3)	88 (2)	113 (3)	P<0.0001
Plasma NA (nM)	2.50 (0.27)	1.97 (0.26)	3.20 (0.49)	P<0.05
Plasma (nM) adrenaline	0.97 (0.49)	0.81 (0.36)	0.99 (0.30)	ns
RBC $[Na]_i$ (mmol 1 $cells^{-1}$)	-	6.43 (0.39)	6.45 (0.38)	ns
Total JNa_{out}	-	2.49 (0.15)	2.28 (0.09)	ns
Pump JNa_{out}	-	1.58 (0.07)	1.36 (0.08)	ns
Co-T JNa_{out}	-	0.22 (0.04)	0.23 (0.03)	ns
Residual JNa_{out}	-	0.70 (0.07)	0.69 (0.07)	ns

Units of ^{22}Na efflux: μmol ml $cells^{-1}$ hr^{-1}.

All results are presented as mean (±SEM), n=9.

Statistical analysis was performed using Student's paired t-test.

to 1.97 ± 0.26 nM. These values are typical of normal resting plasma NA concentrations in healthy adult humans. (Pluto, Burger & Weicker, 1986). Subsequently CPT significantly stimulated plasma NA concentrations, which rose approximately 1.6 fold to reach a new level of 3.20 ± 0.49 nM ($P<0.05$, compared to t_{20}). Plasma adrenaline concentrations followed a similar trend, falling from 0.97 ± 0.49 nM at t_0 to 0.81 ± 0.36 nM at t_{20}. During CPT circulating adrenaline was estimated at 0.99 ± 0.30 nM. None of these values were statistically different.

There was no correlation observed between plasma NA concentrations and blood pressure.

3.Intracellular Na and ^{22}Na transport

Intracellular Na concentrations, $[Na]_i$, and ^{22}Na transport were only measured in red cells obtained from blood sampled at t_{20} and during CPT. Red cell $[Na]_i$ was not affected by CPT, and was estimated as 6.43 ± 0.39 mM (t_{20}) and 6.45 ± 0.38 mM (CPT) (Table 37). In addition, no significant change in ^{22}Na transport was elicited by CPT. Total ^{22}Na efflux fell slightly from an initial value of 2.49 ± 0.15 to 2.28 ± 0.09 μmol ml cells^{-1} hr^{-1}. Bumetanide-sensitive and residual (ouabain plus bumetanide-insensitive) components of efflux were not affected by CPT. The results are presented in Table 37.

It may be argued that the effect of CPT upon Na pump activity might have become significant if further experiments had been performed. It would still remain impossible to ascribe any such effect solely to the action of NA, however (see Discussion). It was therefore decided to design a second series of experiments in which red cell ^{22}Na transport could be measured both before and during NA infusion. The advantages offered by this protocol were threefold:

1) Higher concentrations of plasma NA could be achieved in vivo, such that any effects of NA upon ^{22}Na transport would therefore become more pronounced.
2) These elevated concentrations could be more

carefully standardised between volunteers by administering doses calculated on the basis of body weight.

3) NA infusions could be maintained for periods of 1-2 hours while CPT lasted for less than five minutes. It was therefore possible to expose red cells to greater amounts of NA for longer periods of time.

NA INFUSIONS

Na infusions were performed as described in Methods. Two doses were administered, the one ascribed low comprising 50 ng NA kg body wt^{-1} min^{-1}; while a second higher dose of 100 ng NA kg body wt^{-1} min^{-1} was also given to most subjects. Ten male volunteers received the low dose and eight of these ten went on to receive the high dose. Blood pressure, plasma NA and red cell ^{22}Na transport were measured before and during each infusion, as for CPT. In addition, ^{22}Na transport measurements were repeated in plasma free media.

EFFECTS OF NA INFUSION

1.Blood pressure

A significant rise in both systolic and diastolic blood pressures was observed as the result of both high and low dose infusions (Table 38 & 39). During the low dose infusion SBP rose from 119 ± 4 to 131 ± 4 mm Hg ($P<0.05$), and during the high dose from 115 ± 4 to 143 ± 5 mm Hg ($P<0.0001$). Greater elevations of SBP were noted as a result of the high dose, and analysis revealed that this effect was significant in subjects who underwent both infusions on the same day: (SBP low dose, 131 ± 4 mm Hg; high dose, 143 ± 5 mm Hg; $P<0.05$). During the low dose infusion DBP rose from 75 ± 3 to 84 ± 2 mm Hg ($P<0.02$), and during the high dose from 74 ± 3 to 90 ± 3 mm Hg ($P<0.002$).

Comparison of blood pressure data with that obtained during CPT suggests that infusion of NA elicited approximately equal

TABLE 38

LOW DOSE NA INFUSIONS

	CONTROL	INFUSION	
Systolic blood pressure (mm Hg)	119 (4)	131 (4)	P<0.05
Diastolic blood pressure (mm Hg)	75 (3)	84 (2)	P<0.02
Mean blood pressure (mm Hg)	89 (2)	100 (2)	P<0.002
Plasma NA (nM)	1.51 (0.21)	7.23 (0.81)	P<0.0001

	Physiological salt solution		+ Addition of plasma	
	CONTROL	INFUSION	CONTROL	INFUSION
RBC $[Na]_i$ (mmol l cells^{-1})	6.58 (0.36)	6.69 (0.40)	6.58 (0.36)	6.69 (0.40)
Total JNa_{out}	2.32 (0.19)	2.34 (0.20)	2.54 (0.15)	2.49 (0.16)
Pump JNa_{out}	1.50 (0.15)	1.52 (0.14)	1.53 (0.10)	1.51 (0.10)
Co-T JNa_{out}	0.24 (0.04)	0.23 (0.05)	0.19 (0.04)	0.22 (0.04)
Residual	0.58 (0.07)	0.59 (0.07)	0.81 (0.13)	0.76 (0.07)

Units of flux: μmol ml cells^{-1} hr^{-1}.
All values are mean (±SEM).
All values for $[Na]_i$ and ^{22}Na transport were not significantly different.

TABLE 39

HIGH DOSE NA INFUSIONS

	CONTROL	INFUSION	
Systolic blood pressure (mm Hg)	115 (4)	143 (5)	P<0.0001
Diastolic blood pressure (mm Hg)	74 (3)	90 (3)	P<0.001
Mean blood pressure (mm Hg)	88 (3)	107 (3)	P<0.0001
Plasma NA (nM)	1.54 (0.30)	11.60 (1.50)	P<0.0001

	Physiological salt solution		+ Addition of plasma	
	CONTROL	INFUSION	CONTROL	INFUSION
RBC $[Na]_i$ (mmol l cells^{-1})	7.01 (0.41)	6.83 (0.42)	7.01 (0.41)	6.83 (0.42)
Total JNa_{out}	2.37 (0.19)	2.26 (0.20)	2.56 (0.15)	2.38 (0.22)
Pump JNa_{out}	1.43 (0.17)	1.33 (0.19)	1.51 (0.11)	1.31 (0.18)
Co-T JNa_{out}	0.28 (0.07)	0.29 (0.05)	0.22 (0.05)	0.23 (0.04)
Residual	0.66 (0.08)	0.65 (0.08)	0.83 (0.07)	0.83 (0.09)

Units of flux: μmol ml cells^{-1} hr^{-1}.
All values are mean (±SEM).
All values for $[Na]_i$ and ^{22}Na transport were not significantly different.

pressor responses to those induced by the short term immersion of an extremity in an ice water bath.

2.Plasma NA

Both infusion protocols significantly elevated plasma NA concentration. At the low dose, concentrations achieved were approximately five fold greater than resting values, rising from 1.51 ± 0.21 to 7.23 ± 0.81 nM ($P<0.0001$) (Table 38). This effect was enhanced during the high dose infusion, as NA rose from 1.54 ± 0.30 to 11.60 ± 1.50 nM ($P<0.0001$). In the case of the subjects who received both infusions during the same session, the plasma NA levels achieved during the high dose were significantly greater than those attained at the lower dose: 12.82 ± 1.10 compared to 6.66 ± 0.91 nM ($P<0.002$).

3.Intracellular Na and ^{22}Na transport

Intracellular Na was unaffected by NA infusion (Tables 38 & 39). In the low dose experiments the mean starting red cell Na concentration was 6.58 ± 0.36 mmol 1 cells^{-1}, as compared to 6.69 ± 0.40 mmol 1 cells^{-1} during infusion. At the higher dose, the mean initial value was 7.01 ± 0.41 mmol 1 cells^{-1}, compared to 6.83 ± 0.42 mmol 1 cells^{-1} during infusion.

Furthermore, there were no significant changes in ^{22}Na transport brought about by NA infusion or by the addition of plasma to the medium. All the measured parameters were closely comparable both before and during NA infusion at both the low and high doses in the presence and absence of plasma. The data are presented in Tables 38 and 39. It was noted, however, that as a result of the higher dose, a slight reduction in Na transport reflecting a small inhibition in Na pump activity could be observed in incubations performed in the presence of plasma. As previously stated, this change was not significant and may therefore be false. It was noted, however, that this result is qualitatively similar to that observed during CPT and thus presents three possibilities:

1) It is conceivable that NA may require a

plasma borne co-factor, which is not present in the incubation medium, to exert an effect upon ^{22}Na transport. It is further possible that this compound may decompose in plasma stored at 4°C, thus explaining why the "effects" observed are small and insignificant.

2) It may be the case that there is some circulating agent present in plasma, and stimulated in vivo by CPT or NA infusion, which may itself affect ^{22}Na transport.

3) When cells are re-incubated in the presence of plasma for ^{22}Na transport measurements, they once again become exposed to NA, and/or other compounds, which are not present in the incubation medium. It may, therefore, be this period of re-exposure which tends to decrease Na pump activity.

The results therefore remain ambiguous, and it is clearly not possible to demonstrate an effect of NA on ^{22}Na transport on the basis of the work previously described. Therefore, a third series of experiments was performed in which red cells were exposed, in vitro, to NA alone in the absence of plasma.

THE EFFECT OF NA APPLIED IN VITRO ON ^{22}Na TRANSPORT

The data from CPT and NA infusions suggest that small alterations in red cell ^{22}Na transport may occur hand in hand with coupled elevations of plasma NA and blood pressure, although it is not possible to discern any direct links between the three variables. One reason for this is that both CPT and NA infusion may affect circulating levels of substances other than NA (see Discussion). It is therefore possible that NA could markedly affect ^{22}Na transport in red cells, while other compounds elicited simultaneously by the two experimental manoeuvres might mask such an effect by acting in the opposite direction. Results from these studies must therefore remain ambiguous.

Therefore, in an attempt to more clearly define the possible effects of NA on red cell ^{22}Na transport, it was decided to investigate the action of known amounts of the amine applied solely in vitro. In addition, the effects of NA were investigated with relation to both the influx and efflux of ^{22}Na across the red cell membrane. It was thereby hoped to ascertain whether any effect upon ^{22}Na exit from the cell might be secondary to a changed rate of uptake, rather than a direct effect upon the Na-K pump itself.

In a preliminary series of experiments (data not presented) it became apparent that no effects of NA, applied in vitro, upon ^{22}Na movements in red cells could be detected in standard Tris-MOPS buffered media. Hence, to maximise the likelihood of detecting any response to NA, the experiments to be described were performed in a buffered medium containing phosphate and HCO_3, which were incorporated at the expense of Cl. In ^{22}Na efflux studies, HCO_3 was present at a concentration of 25 mM. In ^{22}Na influx experiments this concentration was sometimes raised to 94 mM, as indicated in the text and legends. Phosphate (HPO_4 + H_2PO_4) remained constant at 11 mM, with Cl comprising the balance to make each solution isotonic. pH was maintained by constant gassing with suitable mixtures of CO_2/O_2.

The HCO_3 containing flux medium was selected in light of the work of Funder and Wieth (1967), and later Funder, Tosteson and Wieth (1978). Funder and Wieth (1967) demonstrated that the effects of HCO_3 upon ^{22}Na uptake was a significant stimulation. Na uptake rose linearly with increasing HCO_3 concentration to 250% above control values at 120 mM HCO_3. The effects of HCO_3 upon ^{22}Na transport in this work are discussed later in this chapter.

^{22}Na efflux experiments

^{22}Na efflux experiments were primarily performed in order to evaluate the effects of NA upon Na-K pump activity. Other pathways of ^{22}Na transport were examined by measuring ^{22}Na influx. The experiments involved a study of ^{22}Na efflux at three concentrations of NA. The lowest, 4 nM, was chosen so as to be

just within the limits of the expected plasma values for a healthy, resting adult human. Similar concentrations of NA have been suggested to affect ^{22}Na transport in the leucocyte (Riozzi, Heagerty, Bing, Thurston & Swales, 1984), where a dose dependent reduction of ouabain sensitive Na efflux rate constant by NA was reported. The authors suggested that the effect, in common with most of the work cited in the introduction to this chapter (see Table 35), was mediated by stimulation of β-adrenoceptors, which are also found on the red cell membrane (Rasmussen, Lake & Allen, 1975).

The two higher concentrations studied here were in excess of those considered by Riozzi, Heagerty, Bing, Thurston and Swales (1984). The intermediate concentration, 12 nM, was chosen so as to approximate to the steady state plasma levels achieved during the high dose infusions (Table 39); the highest concentration, 48 nM, was comparable to plasma levels often found in patients suffering from catecholamine secreting tumours. When present, NA was added to the cell suspension immediately prior to the start of the flux incubation.

The effects of NA upon ^{22}Na efflux rate constant (ERC) are summarised in Table 40. No significant disturbances in either ouabain sensitive or insensitive ^{22}Na transport were elicited by NA, although at the two higher concentrations (12 nM and 48 nM), there was a slight reduction in mean ouabain sensitive ERC coupled to a small increase in ouabain insensitive ERC. Pump ^{22}Na ERC fell from 0.141 ± 0.025 to 0.108 ± 0.014 $hour^{-1}$ in the presence of 12 nM NA, and from 0.152 ± 0.030 to 0.133 ± 0.017 $hour^{-1}$ at the higher NA concentration. Ouabain insensitive ^{22}Na ERC values (control vs. NA) were respectively 0.139 ± 0.014 as opposed to 0.153 ± 0.011 $hour^{-1}$ (12 nM NA) and 0.140 ± 0.019 as opposed to 0.162 ± 0.014 $hour^{-1}$ (48 nM NA). Examination of the original data revealed that these effects were not consistent from one experiment to another. Furthermore, when repeat experiments were performed upon red cells sampled from the original seven donors to reexamine the effects of 12 nM NA (such that n=14), the differences between the control and the test condition diminished.

TABLE 40

THE EFFECT OF NORADRENALINE UPON ^{22}Na EFFLUX

$[NA]_o$	Condition	Efflux rate constant ($hour^{-1}$)		
		Total	+ ouabain	ouabain sensitive
4nM (n=6)	Control	0.259 (0.041)	0.138 (0.018)	0.121 (0.024)
4nM (n=6)	Test	0.256 (0.031)	0.127 (0.014)	0.129 (0.021)
12 nM (n=7)	Control	0.280 (0.034)	0.139 (0.014)	0.141 (0.025)
12 nM (n=7)	Test	0.261 (0.022)	0.153 (0.011)	0.108 (0.014)
48 nM (n=5)	Control	0.293 (0.042)	0.140 (0.019)	0.152 (0.030)
48 nm (n=5)	Test	0.295 (0.025)	0.162 (0.014)	0.133 (0.017)

All values are mean ± SEM.

In each case results (test vs. control) were not significantly different.

Ouabain sensitive ERC fell from 0.141 ± 0.017 (control) to 0.117 ± 0.011 $hour^{-1}$ (12 nM NA). The results remained statistically insignificant.

It was therefore concluded that under the conditions described NA does not appear to affect the rate of ^{22}Na efflux from human red cells. The red cell therefore remains distinct from a variety of other tissues in which Na-K homeostasis is profoundly influenced by catecholamines. This result is in agreement with the work published by Braquet, Diez and Garay (1985). It would also seem to be the case therefore, that NA is unlikely to be the substance responsible for the abnormalities in red cell cation transport observed in hypertension.

^{22}Na influx studies

The effects of HCO_3 upon ^{22}Na uptake

The uptake of ^{22}Na was studied in media prepared to contain one of three concentrations of HCO_3: zero (control), 25 mM and 94 mM. As previously mentioned, when present, HCO_3 was added to media to replace isosmotic quantities of Cl. Control incubations were buffered with 10 mM Tris-MOPS (as were the majority of experiments investigating NA transport in Chapters 1-4). In the presence of HCO_3 a CO_2/O_2 gas mixture was used to maintain a constant extracellular pH.

^{22}Na influx was markedly affected by extracellular HCO_3 in a manner similar to that previously described by Funder and Wieth (1967). The presence of HCO_3 stimulated ^{22}Na uptake, and the results are shown in Figure 41. Total uptake was significantly enhanced in the presence of 25 mM compared to zero HCO_3, and rose from 2.43 ± 0.11 to 3.83 ± 0.23 μmol ml $cells^{-1}$ $hour^{-1}$ (n=10, $P<0.0001$). In the presence of 94 mM HCO_3, as compared to media containing 25 mM HCO_3, total uptake was further stimulated and increased from 4.37 ± 0.34 to 7.90 ± 0.36 μmol ml $cells^{-1}$ $hour^{-1}$ (n=7, $P<0.0001$). Residual influx, measured in the presence of both ouabain and bumetanide, was also enhanced in HCO_3 containing media, ($P<0.0001$ for control vs. 25 mM HCO_3, and 25 mM HCO_3 vs. 94 mM HCO_3). Diuretic sensitive ^{22}Na transport was also

significantly elevated in high HCO_3 media and rose from 0.70 ± 0.13 to 1.65 ± 0.30 μmol ml cells^{-1} hour^{-1}, (25 mM HCO_3 vs. 94 mM HCO_3: n=7, P<0.02).

A significant portion of the HCO_3 stimulated flux was abolished when cells were preincubated with 0.25 mM DIDS, in agreement with the work of Funder, Tosteson and Wieth (1978), (Table 41). DIDS binds to an integral membrane protein involved in red cell anion exchange, and this result suggests that Na and HCO_3 could be co-transported by the anion exchange mechanism, possibly in the form of $NaCO_3^-$ ions. Table 41 suggests, however, that the HCO_3 stimulated transport of Na is also partially mediated via a bumetanide sensitive route, thus implying the participation of the Na-K-2Cl transporter in the response. This might suggest that this pathway is capable of transporting HCO_3 ions. The anion dependence of diuretic sensitive Na-K co-transport has previously been studied by Chipperfield (1981, 1985) and it is now established that Na-K co-transport is not a specifically Cl dependent pathway. Bromide, for instance, will support transport. The effects of HCO_3 substitutions were not considered in either of these papers.

The results presented here also suggest that DIDS is able to interact with the Na-K co-transport system. The possibility of such an effect was also considered by Chipperfield (1985). He also noted a reduction of co-transport activity in the presence of DIDS, but this effect failed to achieve significance. This discrepancy may represent a combination of two factors:

1) Chipperfield (1985) studied the effect of DIDS in HCO_3 free media, and
2) The concentration of DIDS employed in these studies (250 μM) was greater than that employed by Chipperfield (50 μM).

It would appear that at the concentrations utilised in these experiments DIDS is able to inhibit ion transport pathways other than Cl/HCO_3 exchange; (see also the effects of DIDS on NA transport, Chapter 4).

Figure 41

The effect of HCO_3 upon red cell ^{22}Na transport

Red cell ^{22}Na uptake was measured from media containing zero HCO_3 (open bars), 25 mM HCO_3 (hatched bars) or 94 mM HCO_3 (cross hatched bars). Appropriate mixtures of CO_2/O_2 were supplied to maintain an extracellular pH of 7.5 in HCO_3 containing media, while in HCO_3 free solutions, pH was buffered to 7.5 using 10 mM Tris-MOPS. All media contained 145 mM Na (Cl + HCO_3) and 5 mM KCl. Ouabain (10^{-4} M) was added to obviate the need to correct for backflux. Total influx (A) was significantly stimulated by increasing concentrations of HCO_3. Residual influx (B), measured in the presence of bumetanide, was also significantly stimulated. Bumetanide sensitive ^{22}Na uptake (C), representing Na+K+Cl co-transport, was also stimulated in HCO_3 media, but only significantly so at 94 mM HCO_3. The data are means of 10 or 7 experiments, as indicated in text, ± standard error. Significance was assessed using Student's paired t-test (see text).

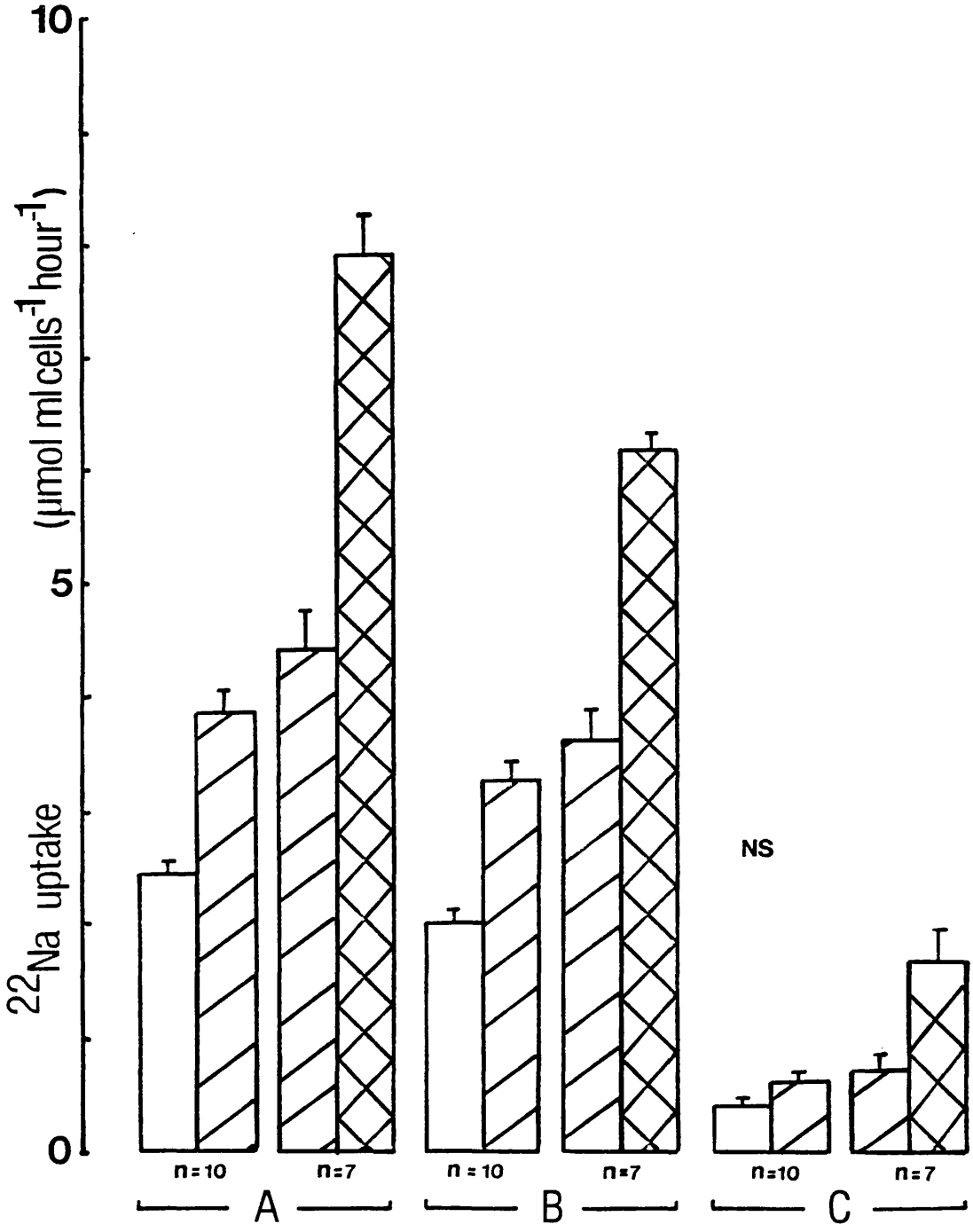

10
5
0
^{22}Na uptake (µmol ml cells^{-1} hour^{-1})
NS
n=10
n=7
n=10
n=7
n=10
n=7
A
B
C

TABLE 41

THE EFFECT OF DIDS ON ^{22}Na INFLUX IN THE PRESENCE OF 25 mM HCO_3

	TOTAL INFLUX	RESIDUAL INFLUX	CO-TRANSPORT INFLUX
Control	3.99 (0.20)	3.34 (0.12)	0.65 (0.13)
+ 250 μM DIDS	3.16 (0.23)	3.06 (0.24)	0.10 (0.10)
	P<0.02	ns	P<0.01

Units of flux: μmol ml cells^{-1} hour^{-1}.

All values are mean (± SEM), n=7.

The effect of NA upon ^{22}Na influx

The effect of two concentrations of NA (10 nM and 1 μM) upon ^{22}Na uptake were studied in freshly obtained red cells suspended in media containing either 25 mM or 94 mM HCO_3. The parameters measured were total influx (determined in the presence of ouabain, so as to avoid having to correct for simultaneous tracer efflux), and influx in the presence of ouabain plus bumetanide. The latter condition more closely defines the leakiness of the cell membrane to Na. That fraction of influx mediated by diuretic sensitive co-transport was calculated as the difference between the two components.

Experiments in media containing 25 mM HCO_3

The results of 23 experiments (6 at 10 nM NA, 17 at 1 μM NA) are presented in Table 42. The data suggest that neither concentration of the catecholamine affects any of the components of ^{22}Na influx measured. Total ^{22}Na uptake was 4.11 ± 0.18 as compared to 4.20 ± 0.14 μmol ml cells^{-1} hour^{-1} in the presence of 1 μM NA_o. Of this total Na-K co-transport constituted approximately 15% and was measured as 0.66 ± 0.07 and 0.63 ± 0.06 μmol ml cells^{-1} hour^{-1} in the absence and presence, respectively, of 1 μM NA_o. Residual ^{22}Na uptake was 3.43 ± 0.13 μmol ml cells^{-1} hour^{-1} in control conditions and 3.57 ± 0.12 μmol ml cells^{-1} hour^{-1} when amine was added to the medium. The results at the lower concentration of NA were qualitatively similar.

Experiments in media containing 94 mM HCO_3

Due to the lack of effect of NA upon ^{22}Na transport in the preceding section, only one concentration of amine was utilised in experiments conducted using media containing 94 mM HCO_3. Therefore, a total of 5 experiments were performed in which ^{22}Na movements were measured in the presence and absence of 1 μM NA_o. The results are shown in Table 43.

In the presence of 94 mM HCO_3 ^{22}Na uptake was stimulated approximately four fold above control values (Figure 41). Under

these conditions it still remained impossible, however, to observe any consistent effect of NA upon ^{22}Na transport. Total influx was slightly, but insignificantly, reduced in the presence of NA and fell from 8.01 ± 0.50 to 7.43 ± 0.42 μmol ml cells^{-1} hour^{-1}. This corresponded to a decline in Na-K co-transport activity which fell from 1.76 ± 0.39 to 1.08 ± 0.25 μmol ml cells^{-1} hour^{-1}. Again, this result was not significant and there was considerable inter-individual variation, both in the magnitude of resting co-transport activity and in the 'response' to NA. Residual ^{22}Na uptake remained unaltered at 6.14 ± 0.22 (control) and 6.20 ± 0.47 μmol ml cells^{-1} hour^{-1} (+NA).

The results would therefore tend to support the hypothesis that ^{22}Na transport in human red cells is not regulated by NA.

TABLE 42

THE EFFECT OF NA ON ^{22}Na INFLUX IN THE PRESENCE OF 25 mM HCO_3

	TOTAL INFLUX	RESIDUAL INFLUX	CO-TRANSPORT INFLUX
Control	3.56 (0.18)	3.06 (0.17)	0.49 (0.04)
+ 10 nM NA	3.67 (0.21)	3.11 (0.16)	0.57 (0.06)
(n=6)	ns	ns	ns
Control	4.11 (0.18)	3.43 (0.13)	0.66 (0.07)
+ 1 μM NA	4.20 (0.14)	3.57 (0.12)	0.63 (0.06)
(n=17)	ns	ns	ns

Units of flux: μmol ml cells^{-1} hour^{-1}.

All values are mean (± SEM).

TABLE 43

THE EFFECT OF NA ON ^{22}Na INFLUX IN THE PRESENCE OF 94 mM HCO_3

	TOTAL INFLUX	RESIDUAL INFLUX	CO-TRANSPORT INFLUX
Control	8.01 (0.50)	6.14 (0.22)	1.76 (0.39)
+ 1 μM NA	7.43 (0.42)	6.20 (0.47)	1.08 (0.25)
(n=5)	ns	ns	ns

Units of flux: μmol ml cells^{-1} hour^{-1}.

All values are mean (± SEM).

DISCUSSION

CPT and NA infusions

CPT and NA infusions have been utilised in this series of experiments to produce consistent and significant elevations in both blood pressure and circulating NA concentration. It has been demonstrated, however, that ^{22}Na transport in red cells obtained from volunteers subjected to these protocols is not significantly different to measurements made under control conditions, both in the presence and absence of plasma. This is in contrast to the findings of Riozzi, Heagerty, Bing, Thurston and Swales (1987), who observed significant increases in ouabain resistant ^{22}Na efflux rate constants in leucocytes obtained from relatives of hypertensive subjects.

One of the problems with this type of approach is, however, that CPT and NA infusion both affect a number of variables other than those measured in this study. For instance, prolonged cold immersion of man has been shown to increase metabolic rate approximately three fold (Johnson, Hayward, Jacobs, Collis, Eckerson & Williams, 1977). The consequent elevation of catecholamines caused by reduced skin temperature may also secondarily affect lipolysis, ketogenesis, glycogenolysis, gluconeogenesis, proteolysis and mineral metabolism, thus influencing plasma concentrations of a wide variety of compounds (for review, see MacDonald, Bennett & Fellows, 1975). It is therefore naive to make the assumption that CPT solely affects the metabolism and disposition of catecholamines.

In man infusion of NA has also been shown to elevate circulating concentrations of other substances including glucose, glycerol, β-hydroxybutyrate and acetoacetate. At the same time, plasma levels of insulin, lactate, pyruvate and alanine were seen to be significantly depressed (Silverberg, Shah, Haymond & Cryer, 1978). These authors used comparable doses and infusion times to those used in this work. It is therefore conceivable that both CPT and infusion may elicit changes in the plasma levels of a number of compounds which could all potentially affect transmembrane ^{22}Na movements. It has been shown, for example, in toad bladder, that

insulin may stimulate active ^{22}Na transport both by the unmasking of latent Na-K pump units and by a mechanism involving the de novo synthesis of protein (Wiesmann, Sinha & Klahr, 1976).

One is forced, therefore, to conclude that to study the effects of NA upon ^{22}Na transport in human red cells it is necessary to expose isolated red cells to NA in vitro. The CPT and infusion experiments do suggest, however, that if ^{22}Na transport is impaired as a result of hypertension, then such defects do not develop in the short periods over which these protocols lasted (up to 2 hours). As previously described however, it would perhaps seem more likely that defects in ^{22}Na transport might be influential in the development of hypertension, rather than vice versa, with enhanced Na efflux leading to elevated Ca_i, and hence constriction of resistance vessels.

Proponents of this theory, which is described by Bing, Heagerty, Thurston and Swales (1986) suggest that such defects might arise as a consequence of some global membrane abnormality, rather than due to the action of an endogenous inhibitor of ion transport. It has further been proposed that such abnormalities might be observed in the cells of pre-hypertensive individuals, and thus act as a marker for the future onset of high blood pressure (e.g. Canessa, Bize, Solomon, Adragna, Tosteson, Dagher, Garay & Meyer, 1981). Thus it has been argued in some quarters that measurements of red cell ^{22}Na transport may be of use in predicting which members of the community may be later at risk from hypertension.

Certain workers have also proposed that first degree relatives of established hypertensives may exhibit abnormal cation transport, suggesting the involvement of genetic influences in this multifactorial condition. The value of such assertions in the prediction of future hypertension naturally remains in question, as it is not possible to say at the time experiments are performed, whether or not these healthy normotensive subjects will indeed go on to suffer from high blood pressure in later life.

In vitro studies

While the literature concerning ^{22}Na transport in relation to hypertension is in some instances confusing and contradictory, the evidence from the in vitro studies presented here is clear. It appears that there is no evidence for the regulation of red cell ^{22}Na transport by NA at concentrations ranging from 4 nM to 1 μM. This result is consistent with the the findings of Braquet, Diez and Garay (1985) and may be explained on the basis of an impairment of red cell adenylate cyclase : cyclic AMP coupling, which is an integral factor in mediating the response in most other tissues (see Table 35). In this study, both influx and efflux of ^{22}Na were unaffected by NA even when ^{22}Na transport was artificially magnified by performing incubations in media containing HCO_3. It would therefore seem likely that the abnormalities observed in essential hypertension are not mediated by this amine. The in vitro experiments also suggest that intracellular NA metabolites (e.g. normetadrenaline) are unlikely to affect ^{22}Na transport in this tissue. As has been shown, NA is rapidly metabolised within human red cells (Chapter 1) and it is likely that, during flux incubations performed in the presence of 1 μM NA, considerable amounts of these metabolites would have accrued.

In conclusion, therefore, while considerable amounts of NA may be accumulated by the human red cell, the presence of the compound has been shown to have no effect upon a number of well defined cation transport pathways.

DISCUSSION

A study has been performed of the transport of NA across the red cell membrane. In addition, the effects of NA upon other membrane transport phenomena have been investigated. The results presented have addressed important questions which have not previously been investigated, and have therefore enhanced the current level of understanding of red cell catecholamine physiology. In addition, the work has pointed the way forward for future studies (see Conclusions). The significance of this research may relate to important therapeutic areas in which catecholamine metabolism is known to be affected, such as hypertension. There follows a summary and discussion of the work performed to date.

The link between uptake and metabolism: evidence for carrier mediated NA transport

Many authors have suggested that the transport of NA may be mediated solely by the process of simple diffusion (e.g. Schanker, Nafpliotis & Johnson (1961); Banaschak & Bluth (1978). These authors therefore, took few, if any, steps to further elucidate the mechanism of transmembrane NA movements with regard to possible dependence upon ionic species or other factors. More recent work, however (e.g. McQuitty & Nicol, 1979), has brought to light the possibility that, while NA accumulation may not be active in that it is not strictly dependent upon a source of metabolic energy, a facilitated diffusion mechanism may exist. The criteria upon which facilitated diffusion may be recognised are described in Table 1.

Preliminary results (Chapter 1) demonstrated kinetics for NA uptake by red cells that were consistent with simple diffusion. This conclusion was reached on the basis that as $[NA]_o$ was elevated, uptake remained linear with concentration. Saturation of uptake could not therefore, be demonstrated and accordingly a

maximal rate of transport of substrate was not evident. Support for the existence of carrier mediated NA accumulation could not be shown. The maximum $[NA]_o$ investigated in these experiments was 250 mM, which is some 10^8 times in excess of the average normal plasma [NA] measured in the peripheral circulation of a healthy resting adult human (Pluto, Burger & Weicker, 1986). It was decided not to measure uptake of amine at concentrations in excess of 250 mM as uptake ceased to remain linear with time at these high [NA] levels. The results are therefore clearly inconsistent with those of McQuitty and Nicol (1979), who reported a Km for (-)NA uptake by human red cells of 2.1 mM.

In experiments performed at the same time it was demonstrated that intracellular NA was subject to catabolic degradation, presumably as a result of the action of intracellular COMT, the major enzyme responsible for O-methylation of NA and other related compounds in this tissue. While it was not possible to measure all the breakdown products formed within the red cell, due to the limitation of the HPLC assay procedure utilised, significant quantities of DHPG could be detected. It is probable that the alternative radio-enzymatic technique employed by Alexander, Velasquez, DeCuir and Maronde (1980), amongst others, would have revealed in addition large amounts of normetadrenaline, which is the major red cell NA metabolite in man. Regardless of this omission, however, it was clear that the amounts of free NA retained by red cells fell well short of the concentrations which may be calculated from radio-isotope experiments.

It therefore seems likely, in the first instance, that the human red cell may act as a 'sink' for plasma NA. Presumably by virtue of abundance, the red cell mass may also have a high capacity for NA uptake and metabolism. It follows, under such circumstances, that removal of free NA from the intracellular pool by COMT activity will provide a constantly favourable downhill electrochemical gradient for NA transport into the cell, which accordingly will have the capacity for accumulation of ever increasing amounts of substrate. Intracellular binding may also serve to reduce free $[NA]_i$.

It was therefore considered important to ascertain whether the catabolic activity and binding capacity of the red cell may in some way affect the accumulation of NA. In other words, can a direct link between amine uptake and metabolism be demonstrated?

These factors have not previously been investigated in detail in the red cell and it was felt that they may be of some relevance to the discrepancies presented in the literature. The interrelationship between uptake and metabolism was assessed by measuring influx of NA under conditions in which both metabolism and binding of amine were minimised. This was achieved by the use of red cell ghosts which could be depleted of approximately 95% of their initial haemoglobin content, and furthermore, resealed to contain tropolone, a known inhibitor of COMT activity (Belleau & Burba, 1963). Under these conditions, at comparatively low NA concentrations, uptake was observed to be of approximately the same magnitude as that in intact red cells. However, as $[NA]_o$ was increased a discrepancy arose such that accumulation by ghosts became significantly less than by cells. Uptake therefore seemed to be impaired at high substrate concentrations, if catabolic activity was suppressed.

It was also clear that NA accumulation differed qualitatively between ghosts and red cells. Uptake was in fact shown to be saturable and obeyed Michaelis-Menten kinetics with a Km of 154 mM and Vmax of 54.2 μmol 10^{10} ghosts^{-1} 15 min^{-1}. On the basis of these findings it is thus possible to ascribe one of the prerequisites for carrier mediated uptake to the NA transport system in human red cells. It was noted, however, that the apparent affinity for uptake is considerably less than that quoted by both McQuitty and Nicol (1979) and Danon and Sapira (1972). One possible reason for this difference could be that these authors used different experimental media and radio-labelled isomers. Both of these later pieces of work and the results contained in this thesis make it clear, however, that the facilitated transport of NA into red blood cells *in vivo* would never become saturated due to low plasma NA concentrations. The mechanism is a classical high capacity, low affinity system.

Further circumstantial evidence for the carrier mediated uptake of NA and related compounds was supplied by experiments which focussed attention on the influx of guanethidine. In these experiments, instead of considering the accumulation of a relatively unstable and rapidly metabolised substrate (NA) into an inert intracellular space, the uptake of a very slowly metabolised compound functionally related to NA (guanethidine) was measured in freely metabolising cells. Guanethidine, which has a half life in vivo of 4-5 days, was also shown to enter red cells by a saturable mechanism which had a Km of 19.41 mM and a Vmax of 9.56 μmol ml cells^{-1} 15 min^{-1}.

Rates of uptake of guanethidine were similar in magnitude to those measured for NA and, importantly, it was also demonstrated that guanethidine could inhibit NA uptake in a concentration dependent fashion. This observation suggests that the two compounds at least partially share a common route of entry. Subsequent experiments, however, unearthed minor dissimilarities in the ionic requirements for NA and guanethidine uptake and, on the basis of these findings it would seem that these compounds may enter red cells by more than one pathway.

Another link between the uptake and metabolism of catecholamines was suggested by experiments designed to investigate the effects of tropolone on NA accumulation in intact red cells. The results of these experiments, in which intracellular NA concentrations were directly measured after prolonged (3 hours) incubations, suggested that inhibition of COMT again led to a decline in intracellular NA levels, as measured by HPLC.

It therefore appears that an inhibition of NA metabolism might directly inhibit uptake. Otherwise, it would be expected that a reduction in catabolic activity coupled to unaltered transport would, in fact, lead to elevated red cell amine levels. The effect of tropolone upon ^{3}H-NA uptake after 15 minutes was minimal, which suggests a gradual inhibition of COMT with time. This delay probably represents the interval required for tropolone itself to cross the red cell membrane, and supports the notion

that the effect observed is indeed mediated from within the cell. Tropolone is known to cross biological membranes (e.g. rat kidney) reaching maximal intracellular concentrations after approximately 30 minutes (Broch, 1973). Red cell tropolone uptake remains to be systematically studied.

The results presented thus far would therefore seem to suggest that carrier mediated transport of NA may occur across the red cell membrane. In general, two different forms of carrier mediated activity might operate:

1) A directly energy dependent process, in which the transport of NA would be clearly linked to the splitting of ATP, and said to be active; or
2) Facilitated diffusion, in which NA transport would not be directly dependent upon cellular energy.

It is proposed that this latter phenomenon may adequately account for the data obtained, on the basis of three pieces of information. First, NA uptake was shown not to be affected when cells were exposed to a variety of metabolic poisons. These agents included 2,4-dinitrophenol, ouabain and Na nitroprusside and the results (Chapters 1 & 3) are in agreement with those, for instance, of Danon and Sapira (1972). Secondly, NA uptake is similarly unaffected when red cells are depleted of ATP by various methods, and thirdly, NA accumulation occurs in red cell ghosts which contain neither ATP nor sufficient cellular apparatus to generate ATP de novo. Active transport may therefore be discounted as the process by which NA crosses the red cell membrane. Facilitated diffusion appears to be the most likely means by which red cells accumulate NA.

It would therefore appear that red cell NA transport is similar in some respects to the carriers present in certain other tissues (Uptake I and Uptake II). It is possible to discount the theory that uptake is mediated via either of these mechanisms, however, due to evidence obtained from experiments performed to investigate the effects of cocaine and reserpine upon NA influx. These compounds are well characterised inhibitors of the NA transport systems present in neuronal and extraneuronal tissues,

but have no effect upon the corresponding pathway in red cells. It would therefore appear that at least three distinct routes of transmembrane NA movement may exist in vivo. At this stage, the nature of the red cell NA binding site remains unknown but is clearly not a typical β-adrenergic locus as blocking agents such as propranolol and timolol fail to affect uptake. Human red cells are known to possess β-receptor like structures, although in comparison to other cell types (e.g. rat red cells), catecholamine stimulation fails to increase adenylate cyclase activity and therefore does not affect intracellular [cAMP] (Rasmussen, Lake & Allen, 1975). β-receptor populations are also thought to decline as human reticulocytes differentiate to form mature erythrocytes (Tucker & Young, 1982).

Inhibition studies

Further evidence consistent with the existence of carrier mediated NA uptake is provided by the studies involving other amines detailed in Chapter 2. It has been shown that a number of compounds structurally related to NA may inhibit NA uptake, and these include adrenaline, metaraminol, histamine and 5-HT. It was noted, however, that not all amines produced such effects. A notable example of a compound which does not appear to inhibit NA influx is dopamine, although it is known that this substance is able to enter red cells. (Bryon & Bischoff, 1970). There is no obvious reason why dopamine should differ in its behaviour from other amines tested.

The results also show that extracellular histamine and metaraminol are able to inhibit NA transport in ghosts as well as in intact red cells. The fact that inhibition of uptake persists in ghosts, and is of a comparable magnitude to that noted in cells, suggests that amine interactions are probably localised to the level of the membrane, and are not secondary to intracellular events such as metabolism and binding. Indeed, 5-HT and guanethidine both inhibit NA uptake in intact red cells but are only slowly, if at all broken down by such cells.

Inhibition studies subjected to kinetic analysis

(Dixon, 1953) suggested a non-competitive pattern of amine interaction. A non-competitive inhibitor (I) may bind both to the carrier (C) and the carrier-substrate complex (CS). In the latter case the product CSI is formed. It is assumed that CSI is subsequently unable to break down to yield the end product, and hence S (i.e. NA) is unable to traverse the red cell membrane.

The high concentrations of inhibitors required to affect NA uptake also once again points towards the existence of a relatively non-specific carrier, with a low affinity for its chosen substrates. The Ki for inhibition of NA uptake in intact red cells by 5-HT was 15.07 ± 1.83 mM while histamine proved slightly more effective at blocking transport with a Ki of 4.50 ± 0.79 mM. Both values interestingly are in the same range as the Km for NA uptake quoted by McQuitty and Nicol (1979) and Danon and Sapira (1972).

In experiments utilising ghosts it was also attempted to show the effects of amines incorporated within the cell interior, and indeed it could be shown that both intra-ghost 5-HT and histamine inhibited the uptake of NA. If the nature of inhibition at the internal and external surfaces is the same and therefore apparently non-competitive, it is not surprising that counter-transport (noted in rabbit red cells by Blakeley & Nicol, 1978) was not observed in these experiments. It is suggested that either histamine or 5-HT may bind to C or CS at both the inner and outer facing surface of the membrane, and hence inhibit inward movement of NA in both cases as described. As each experiment proceeds it is argued that free inhibitor leaches from the internal compartment into the external medium. Hence, intra-ghost [inhibitor] falls, resulting in a gradual dissociation of I from C or CS, assuming that binding is reversible. Free carriers may therefore become available, in time, to transport increasing amounts of NA into ghosts, as is observed. External inhibitor effects in these experiments are ruled out on the grounds that [5-HT] and [histamine] would become greatly diluted upon leaving the ghost.

It would therefore appear:

1) That the mechanism for human red cell NA uptake does not display counter-transport behaviour under the conditions studied, as predicted; but,
2) There apparently remains, however, some means of communication between the inner and outer facing surfaces of the membrane.

The cation dependence of NA uptake

Many transport processes may be characterised by their dependence upon, and sometimes co-transport of simple inorganic ions. The cation and anion dependence of NA transport in human red cells and ghosts was unknown and has accordingly been investigated in this study.

Of all ionic species, the Na^+ ion is probably the cation most frequently involved in the carrier mediated transport of other compounds. Evidence exists to support the involvement of Na in glucose transport (e.g. in small intestine; see Schultz, 1977), the translocation of certain amino acids (see Eddy, 1987), and even in the movement of other ions. It has recently, for example, been suggested that Cl/HCO_3 exchange in some tissues may be Na dependent (Kondo & Fromter, 1988). The effect of Na replacement upon NA influx was shown to be more complex than in many systems, however, in that it appeared to be both dependent upon the choice of replacement cation and the concentration of NA in the medium.

The majority of experiments were performed using N-methyl-D-glucamine (NMDG) to isosmotically substitute for Na and it could be shown that both in ghosts and in intact red cells at high $[NA]_o$ (1 - 250 mM) that, in Na-free media, amine accumulation was significantly, although not substantially, diminished. This reduction, which amounted to no more than about 15%, appeared to be the result of a lower affinity of the transporter for NA. It is quite clear however, that since the majority of NA uptake under these conditions persists, the Na dependent portion may be relatively unimportant. Interestingly, similar experiments performed using guanethidine suggested that the uptake of this

compound is in no way Na dependent. This result suggests that two pathways of NA accumulation may occur, only one of which, the Na independent route, may be shared by guanethidine. It would be interesting to measure the Na dependent NA uptake in the presence of a variety of inhibitors in order to ascertain whether the presence of such compounds (e.g. histamine & 5-HT) causes a greater portion of NA transport to proceed via the Na dependent route. It would also be of value to characterise the cation dependence of uptake of these related amines to see how many exhibit Na dependent uptake characteristics (as for NA), and how many are Na independent (as for guanethidine).

Another interesting result was discovered upon investigating NA uptake at substrate concentrations nearer to the physiological range. Here, the effect of replacing Na paradoxically stimulated influx, a result which is not easily interpreted. One possible explanation for this paradoxical effect could be that some Na dependent efflux of NA occurs in the experiments at elevated $[NA]_o$, in which $[NA]_i$ may significantly rise. Elevation of $[NA]_i$ is unlikely to occur in experiments performed at physiological $[NA]_o$: indeed it has been shown to be impossible to measure NA_i in red cells incubated in NA containing solutions of less than 50-100 μM. Furthermore, to detect any appreciable NA_i it is necessary to incubate cells under these conditions for periods of 2-3 hours to load them with amine. Therefore, since one substrate (NA_i) is effectively absent, a cation dependent efflux pathway could not operate, resulting in a an apparent stimulation of net uptake. This hypothesis remains to be tested and remains speculative in that it assumes a high dependency upon Na, or possibly K, for NA transport out of the red cell. In contrast, experimental evidence suggests that transport in the opposite direction is not significantly Na or K dependent. It would have to be argued, therefore, that the red cell NA transporter was asymmetric in this respect.

In the early stages of experimentation it was felt possible that the complicated nature of the results might represent some artefact, caused for example by the specific choice of NMDG as a

Na substitute. In other words, did the results truly represent the effects of reduced $[Na]_o$, or effects caused by NMDG. This point was of particular concern as NMDG has been shown to influence the cation permeability properties of red cell membranes under certain experimental conditions (Blackstock & Stewart, 1986). To some extent the choice of substitute is indeed relevant, as replacement of Na_o by choline yielded identical results to NMDG, while the use of K as replacement under otherwise identical conditions failed to affect NA uptake. Variations in intracellular Na and K concentrations were also achieved, using the ionophore nystatin, and similarly failed to affect uptake.

It therefore seems possible that NA uptake has a small component which is dependent upon the presence of inorganic cations and that organic cations appear unable to fulfil this requirement. The likelihood is that this requirement is specifically for monovalent cations, as inclusion in and omission from experimental media of Ca^{2+} and Mg^{2+} (2.5 mM and 1 mM respective final concentrations), or a combination of both, was without effect upon NA transport (see Chapter 3). In addition, the inclusion of EDTA, a weak chelator of divalent cations (X^{2+}), into nominally X^{2+} free media also failed to affect NA transport.

Further links between Na, K and NA handling, however, remain elusive. One approach tested was to investigate the effects of a number of cation transport inhibitors upon NA uptake, but it was observed that inhibition of the ouabain sensitive Na-K pump, bumetanide sensitive Na-K-2Cl co-transport and the phloretin sensitive Na-Na exchange all failed to affect NA accumulation; neither did simultaneous inhibition of combinations of these transport pathways affect NA movements (Chapter 3).

In addition, manoeuvres which were shown to greatly elevate the membrane permeability to Na did not reveal any effect upon NA uptake. Varying degrees of leakiness to Na and K were encountered in nystatin treated red cells, and also in ghosts produced both by the method of Richards and Eisner (1982) and by that of Bodemann and Passow (1972). Despite increases in passive Na influx of up to ten fold in some preparations, NA uptake remained within what was

considered to be the normal range. It may therefore be concluded that the rates of NA and Na uptake are not closely linked, and that NA, despite having cationic characteristics at physiological pH, does not flow rapidly through the same leak pathways as the Na ion. This may of course represent to some degree the larger size of the amine.

In conclusion there does appear to be evidence for the first time, however, to suggest that Na dependent NA transport may be measured in vitro in human red cells. It is equally clear that the characteristics of this Na dependence differ markedly from those described in various other tissues.

The anion dependence of NA uptake

The effects of anion substitution upon NA uptake are clearer to appreciate in that only one pattern of response has been observed - a small but significant stimulation of uptake in Cl free media, which persists over the whole range of NA concentrations tested. Furthermore, the effect of anion substitution (i.e. Cl replacement) could only be observed in one preparation - the stimulation of NA uptake caused by replacement of Cl by either NO_3 or $MeSO_4$ was only seen in intact red cells and was not evident in red cell ghosts. This observation may suggest, in direct contrast to the effects of cation substitution, that the consequences of anion replacement are not necessarily confined to the level of the membrane.

If anion replacement effects are not entirely mediated at the membrane, it may be argued that the effects observed could be the result of some disturbance of catecholamine metabolism. Furthermore, since tropolone is known to inhibit COMT activity and at the same time retard NA uptake, it would have to be argued that replacement of Cl by NO_3 or $MeSO_4$ leads to stimulated catecholamine catabolism. It should be pointed out, of course, that this is a situation unlikely to be encountered physiologically. If the hypothesis is valid, then the effects of anion substitution and of treatment of red cells with tropolone should be antagonistic, and indeed this has been shown to be the

case. Tropolone does at least partly retard the effects of NO_3 and $MeSO_4$ upon NA uptake.

The fact that tropolone only partially retards the effects of anion substitution may have two possible implications:

1) That, over the time course of the experiments concerned, tropolone was not able to exert its maximal effect (due, perhaps, to a slow rate of incorporation into red cells); or
2) That there does indeed exist some membrane mediated component of the response to anion substitution, which is lost during ghost preparation.

Evidence which remains consistent with the latter suggestion is supplied by work designed to investigate the effects of DIDS on NA uptake. In these experiments it was clearly shown that DIDS was also able to partially retard the effects of anion replacement upon NA uptake in red cells (see Chapter 4). Unfortunately the combined effects of DIDS and tropolone were not investigated. Neither was the action of DIDS upon NA accumulation in red cell ghosts considered, simply because there was no effect of anion substitution measured in the first instance. The concentration dependence of the action of DIDS suggests that the effect upon NA influx noted is not mediated by the band 3 anion exchange protein, a supposition which is substantiated by the fact that red cell ghosts retain the capacity for DIDS sensitive Cl transport (Funder & Wieth, 1976), and yet no effect of Cl replacement is seen in this preparation. It is suggested that the action of DIDS arises by virtue of its relatively low binding specificity, and that the portion of the membrane which mediates this response may be shed during ghost preparation.

It is possible that Cl substitution may affect membrane surface charges. Since, at physiological pH values, the molecule has some degree of cationic character, any increase in surface negativity would potentially act to attract NA, and possibly as a consequence stimulate uptake. It is interesting to note that the passive uptake of the Na ion is also enhanced when Cl_o is replaced by NO_3 , albeit to a greater extent than occurs with NA (Brand &

Whittam, 1984). The relative degrees of stimulation may, however, simply represent the greater charge associated with the Na ion.

Finally, it was again possible to demonstrate that the ionic requirements for guanethidine uptake differed from those for NA transport. Guanethidine accumulation into red cells failed to respond in any way to the replacement of Cl_o by $MeSO_4$, and thus appears to be an essentially anion independent process. This difference may once again represent the relatively stable nature of the guanethidine molecule, in that, unlike NA uptake, that of guanethidine is not related to intracellular metabolism. Alternatively, the difference may arise due to a difference between the relative cationic characters of the two molecules and therefore between the charges associated with each of them.

It is interesting to speculate that, since guanethidine fails to respond to both anion and cation replacements, it is only the cation dependent portion of amine uptake that is influenced by anion substitutions. Since cation dependent NA uptake is a relatively small component of the total, this would explain why the effects of anion substitutions are similarly small. It would be of interest to pursue this line of thought by investigating the effects upon NA transport of Cl substitution in Na free media (e.g. using NMDG NO_3).

In conclusion, evidence has been presented for the first time to suggest that the uptake of NA by human red cells is dependent upon the anionic composition of the extracellular medium. This is not a question that has been addressed in detail in other tissues and hence it is not possible to draw any comparisons. In the one system in which anion dependent NA transport has been studied however, the PC12 clonal cell line, virtually all uptake could be eliminated when Cl was removed from experimental solutions (Friedrich & Bonisch, 1986). The results described remain consistent therefore with the notion that red cell NA transport is clearly distinct from amine uptake mechanisms characterised in other tissues of the body.

The pH dependence of NA uptake

It has been known for many years that the accumulation of NA by human red cells is a pH dependent process. The results presented in this thesis have elaborated upon these observations by once again studying NA uptake in the presence and absence of COMT activity at various extracellular pH values.

It was observed that as pH_o was raised, NA uptake became enhanced. This suggests that the degree of stimulation was such that an increase in pH of approximately one unit caused an elevation of uptake of some five fold. It was also noted that the sensitivity of uptake to pH_o was greatest in the physiological range. It was further evident that the response and its magnitude were similar both in red cells and ghosts. The existing evidence would therefore tend to suggest that the stimulation caused upon alkalinisation of the medium is mediated at the membrane level, and that the preferred form of substrate for transport is the lipophilic, neutral NA molecule. These findings, taken in isolation, would clearly be consistent with the suggestion that NA enters the human red cell by the process of simple diffusion, and similar evidence prompted Schanker, Nafpliotis and Johnson (1961) to reach just such a conclusion. How then may these results be reconciled with the evidence which has already been presented which suggests that NA transport is carrier mediated?

One series of observations which would be more difficult to explain in terms of simple diffusion are the observed effects of amiloride. Amiloride (1 mM) has been shown to inhibit NA uptake and this action was noted to become increasingly pronounced as pH_o was raised. It is important to stress that the effect of amiloride in this context is distinct from its well known action as an inhibitor of Na-H exchange, because:

1) The ability of amiloride to retard NA uptake increases as Na-H exchange activity would be expected to decline (i.e. with increased extracellular pH); and
2) Another compound known to inhibit Na-H exchange, dicyclohexylcarbodiimide (DCCD) has the opposite

effect on NA transport to amiloride. DCCD was shown to further stimulate the already elevated NA uptake measured at high pH_o.

It has been well documented that amiloride may act as a relatively non-specific inhibitor of a variety of membrane mediated phenomena (e.g. Villereal, 1986), and it is suggested that the results described in this section represent a further addition to this list. It thus seems possible that amiloride may act to at least partially inhibit a membrane bound NA carrier. Since the effect of amiloride increases as pH_o is elevated it is further proposed that this carrier may preferentially bind neutral, as opposed to cationic, NA and becomes increasingly active under alkaline conditions. Such a preference for lipophilic NA molecules has previously been demonstrated in bovine chromaffin granules in which transmembrane H^+ gradients are reportedly closely coupled to NA transport (Johnson, Carty & Scarpa, 1985).

The physiological significance of this finding is not clear, although a possible coupling between metabolism and uptake may once again be invoked as COMT activity in the red cell also increases with pH in the range studied (pH 7.0 - 8.0). It is again stressed, however, that uptake into non-metabolising ghosts was also pH dependent. Another possibility is that such a pH dependent uptake process may enhance the actions of catecholamines under stressful conditions, such as exercise. Strenuous exercise may induce a transient and mild acidosis in the bloodstream, due to unbuffered lactic acid. Plasma pH may drop by 0.1-0.2 pH units. Simultaneously, catecholamines may become increasingly important in mobilising energy supplies. For instance, β-adrenergic stimulation increases hepatic glucose output. Catecholamines may therefore be of reduced usefulness if accumulated and inactivated by red cells. As has been seen, a small elevation in pH may profoundly affect red cell NA movements, and acidosis will tend to maintain elevated plasma concentrations.

The effect of NA on red cell cation permeability

Many investigators have described effects of catecholamines upon cation transport in a variety of tissues, such as frog skin (Watlington, 1968). It did not therefore seem unreasonable to search for similar relationships in the human red cell. In addition, a number of clinical research workers have described deviations in Na transport in pathological disturbances such as essential hypertension, a condition which, amongst other factors, may sometimes be characterised by an elevation of the circulating levels of NA (e.g. Imai, Wang, Yoshiue & Tamura, 1973). A causal link between increased plasma NA and abnormal Na handling by red blood cells has been speculated upon but never proven.

In the course of this work, cation transport in red blood cells was measured under three conditions:

1) After a transient local elevation of NA levels in vivo, caused by cold pressor test.
2) After prolonged exposure of red cells to elevated NA levels in vivo, as the result of NA infusion.
3) After exposure of red cells to NA solely in vitro. This last protocol enabled red cells to come into contact with concentrations of up to two orders of magnitude greater than might be encountered in vivo.

In all cases it was shown NA transport remained unaffected by variations in NA concentration, although after cold pressor test there was a small but insignificant depression of Na pump activity noted. Naturally however, to attribute any such effect after either CPT or NA infusion to NA alone would be foolish, as it would be expected that both manoeuvres might drastically affect many circulating factors. These experiments are made additionally complicated, because once blood has been sampled from the volunteer, many of these factors may decay during isolation and treatment of the red blood cells. The precise environment of the red cell at the time of the experiment is thus not strictly defined. The experiments performed solely in vitro however, in which known amounts of NA were added to red cell suspensions,

would seem to offer no conclusion other than NA is unable to affect Na transport in this tissue.

CONCLUSIONS

During these studies an attempt has been made to further characterise the mechanism of red cell NA transport. It is felt that the studies allow a fuller picture to be painted of a facilitated diffusion pathway which may operate *in vivo* to remove considerable quantities of amine from the plasma for subsequent inactivation. The experiments performed here have suggested further areas of study. Certain questions remain unanswered, therefore, and some appear worthy of further investigations.

Uptake of amines by human red cells

While it is clear that many other amines may bind to the same membrane receptor as that which mediates NA transport, it is equally clear that there exists some evidence that amine accumulation may proceed via a number of routes. It would be of interest to elucidate these pathways in greater detail.

Uptake of NA by other red cells

It is known that rat and rabbit red cells, to name just two species, are able to accumulate and metabolise NA. Some differences already appear to exist between the mechanisms of uptake of NA by the red cells of the rabbit and man, although further detailed investigations are required in order to ascertain exactly how diverse the systems are.

Efflux of NA from human red cells

All of the studies presented in this thesis have concerned measurements of NA influx into red cells or right side out ghosts. There thus exists no kinetic evidence pertaining to the mechanism of efflux of NA from red cells. Initially, NA efflux studies were not undertaken due to the problems posed by intracellular NA catabolism. Preparation of inside out vesicles would preclude such a problem, and would also facilitate a study of the possible mechanisms of efflux of NA metabolites such as normetanephrine.

Pathophysiological conditions

Evidence exists to suggest that NA uptake into human red cells may be affected by disorders of catecholamine metabolism, such as phaeochromocytoma. It would also be of interest to investigate NA transport in essential hypertension which, as has been stated, is also often characterised by elevated plasma NA levels. The question therefore arises as to whether increased plasma NA is a consequence of increased sympathetic overflow or reduced red cell NA uptake, or a combination of both. If the latter is a relevant factor, then is such a reduction secondary to disturbances in intracellular metabolism, or purely mediated at the membrane level?

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ABSTRACT

THE UPTAKE OF NORADRENALINE BY HUMAN RED BLOOD CELLS AND GHOSTS

by C.R. DUNK

A study has been made of the transport of noradrenaline into human red cells and resealed ghosts. In cells uptake appeared to obey the kinetics of simple diffusion, whilst in metabolically inert ghosts, uptake was identified as a low affinity high capacity saturable transport mechanism. Uptake was markedly temperature sensitive but not dependent upon cellular metabolism, consistent with facilitated diffusion rather than active transport of noradrenaline. Non-competitive inhibition of uptake was achieved by a variety of structurally related compounds when present at either the inner or outer membrane surface.

The ionic requirements for noradrenaline transport by red cells and ghosts have been examined. When external sodium was replaced isosmotically by N-methyl-D-glucamine the apparent affinity for uptake by ghosts was modestly inhibited. Replacement of external sodium by potassium was ineffective, suggesting a requirement for both sodium and/or potassium. Specific sodium transport inhibitors were without effect and it was shown that the mechanism has no requirement for calcium or magnesium.

Replacement of external chloride by either nitrate or methylsulphate stimulated red cell noradrenaline accumulation, but was ineffective in ghosts. It is suggested that anion substitution may act secondarily on transport by affecting binding and/or catecholamine metabolism.

Noradrenaline uptake was inversely proportional to external hydrogen ion concentration, suggesting that lipophilic substrate is favoured for transport.

It is concluded that noradrenaline transport does not occur via the "uptake 1" or "uptake 2" pathways characterised in other tissues.

It has been shown that the slowly metabolised noradrenaline analogue, guanethidine, is accumulated by red cells. Guanethidine transport is saturable, sodium and chloride independent, and inhibition studies reveal separate routes of entry for this compound and noradrenaline.

Noradrenaline has no effect upon red cell cation transport. Therefore, abnormalities reported in clinical disorders, such as essential hypertension, are not attributable to increased plasma noradrenaline concentration.

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STATEMENT

The accompanying thesis/~~dissertation~~* submitted for the degree of PhD entitled

THE UPTAKE OF NORADRENALINE BY HUMAN RED BLOOD CELLS AND GHOSTS

is based on work conducted by the author in the Department of MEDICINE of the University of Leicester mainly during the period between AUGUST 1984 and AUGUST 1987

All the work recorded in this thesis/~~dissertation~~* is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University/~~part of this work has been submitted for another degree indicated in the University of~~*

Signed: Chris Hunt ..... Date: 1/6/89.....

* delete what is inapplicable