Transport of Adenine Nucleotides into Secretory Vesicles of the Adrenal Medulla.

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This thesis was composed by myself and all the results therein are based entirely on my own work.

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ABBREVIATIONS.

ATPase	Adenosine 5'-triphosphatase
BSA	Bovine serum albumin
PEP	Phosphoenolpyruvate
serotonin	5-Hydroxytryptamine
Bicine	N,N-bis(2-hydroxy-ethyl)glycine
Hepes	4-(2-hydroxymethyl)-1-piperazine-ethane sulphonic acid
Tris	Tris-(hydroxymethyl)-aminomethane
DMSO	Dimethylsulphoxide
DTT	Threo-1,4-dimercapto-2,3-butanediol (Cleland's Reagent)
SDS	Sodium dodecylsulphate
ΔpH	Transmembrane pH-gradient
$\Delta\Psi$	Electrical membrane potential
c.p.m.	Counts per minute
d.p.m.	Disintegrations per minute
r.p.m.	Revolutions per minute
mol.wt.	Molecular weight
n.m.r.	Nuclear magnetic resonance

ABSTRACT.

The secretory vesicles of the adrenal medulla, chromaffin granules, store high concentrations of catecholamines, adenine nucleotides and acidic protein. These organelles release their contents into the bloodstream upon a neural stimulus of the gland.

The high concentration of catecholamines is achieved and maintained by a specific transport mechanism within the granule membrane which is energized by an electrochemical membrane potential established by a proton-pumping ATPase. It is less clear how adenine nucleotides are accumulated and their concentration gradient maintained.

The aim of this investigation was to characterize how the adenine nucleotides are transported through the granule membrane. Lysed and resealed chromaffin granules, 'ghosts', were used because they are essentially devoid of soluble matrix components and reflect therefore the situation during biogenesis, where nascent immature granules are first filled with nucleotides and then with catecholamines.

Kinetic experiments showed that several adenine nucleotides and also phosphoenolpyruvate are transported into the ghost interior in a temperature dependent process. Transport of serotonin, a substrate for the catecholamine transporter, was monitored concomittantly in dual isotope experiments and served as a control for the energization and integrity of the ghosts.

Uptake of the adenine nucleotides proceeds for about two hours at 37° C, to a final concentration gradient of about

two-fold. At this steady state a high rate of influx of nucleotides is balanced by an equal efflux. The uptake process cannot be saturated, but is inhibited by low concentrations of cibacron blue, and partly by atractyloside, protein modifying agents and substances which discharge the membrane potential. It is concluded that in ghosts only a small proportion of the uptake process is energy-dependent, and that the adenine nucleotides do not equilibrate with the membrane potential, as has been proposed for intact granules. It seems unlikely that nascent chromaffin granules accumulate their high concentration of nucleotides uniquely via a membrane potential-dependent transport process: diffusion may play an important role as well.

To label proteins of the chromaffin granule membrane, the radioactive adenine nucleotide analogues γ-(^{32}P)azidoATP, dialdehyde (^{3}H)adenine nucleotides and fluoro—sulphonyl-benzoyl-(^{3}H)adenosine and a less specific label, (^{14}C)chloro nitrobenzofurazan, were used. After labelling under various conditions labelled proteins were analyzed by SDS polyacryl-amide gel-electrophoresis. For an additional characteri-sation of the labelled proteins a separation technique into two dimensions was used.

Each substance labelled several proteins and at least four proteins were labelled by all analogues. These proteins are likely candidates for ATP-requiring enzymes in the granule membrane, amongst them the putative nucleotide permease. These labelling experiments aid a further characterisation and classification of the many proteins of the chromaffin granule membranes. Two-dimensional separation

revealed minor proteins reacting strongly with some labels. Analogous labelling experiments with the soluble proteins of the granule matrix were also performed.

It is concluded that there is probably more than one way by which nucleotides pass through the chromaffin granule membrane: active uptake and diffusion (with subsequent binding to matrix components) might both be important in vivo. Several proteins which react with adenine nucleotides could be shown in the membrane as well as the matrix of chromaffin granules but a further characterisation will be needed to show their individual function.

BACKGROUND TO THE PROJECT.

Catecholamine storing cells in the adrenal medulla.

The mammalian adrenal gland consists of two parts: the medulla and the cortex. These two morphologically different tissues can be observed in a sectioned adrenal gland. The inner pink, vascularized medulla (about 10% of the tissue) is surrounded by a 3-5mm thick brown cortex. Embryologically the cortex is mesodermal tissue, whereas the medulla is ectodermal, deriving from the cells in the neuronal crest, the same origin as the cells of the adrenergic sympathetic nervous system. The neuroanatomy and the physiology of the adrenal gland is extensively reviewed in the Handbook of Physiology (1975).

The adrenal medulla consists predominantly of catecholamine secreting cells, also called chromaffin cells because they can be stained with chromium salts. Most of these cells store adrenaline: a smaller number store noradrenaline, but the proportion varies from one species to another. The chromaffin cell contains a large number of small electron-dense vesicles which store and secrete catecholamines (see Benedeczky & Smith, 1972, for electron micrographs). These vesicles are called chromaffin granules. Noradrenaline and adrenaline storing cells within the adrenal medulla can be distinguished in electron micrographs because the noradrenaline-storing vesicles

appear more electron-dense than adrenaline storing vesicles. This is due to an electron — dense complex which noradrenaline forms with the fixing agent osmiumtetroxide.

In the sympathetic nerve cell, which is of similar embryological origin to the chromaffin cell, the synaptic vesicles are developed in the Golgi apparatus in the cell body and are then moved along the axon to their release site. The vesicles filled with the neurotransmitter noradrenaline release their contents in response to an action-potential which is itself caused by a depolarisation of the nerve cell membrane. The same electrical mechanism causes catecholamine release in the chromaffin cell. The splanchnic nerve inervating the adrenal medulla stimulates by releasing acetylcholine, and this causes a depolarisation of the chromaffin cell. The depolarisation is accompanied by an influx of calcium, and this is the immediate trigger for the release of the contents of the secretory granules into the blood stream, which happens most likely via an exocytotic process (Benedeczky & Smith, 1972). Because of these close similarities the catecholamine storing cells from the adrenal medulla are used as a model for sympathetic nerve cells in elucidating questions of bioenergetics and biogenesis of the cells, and because chromaffin granules can be isolated and purified relatively easily in quantities necessary for biochemical studies they have been used as a model system for the study of processes which may well occur in noradrenaline and acetylcholine — containing synaptic vesicles, as well as other biogenic amine storage vesicles like the serotonin storing vesicles in platelets or the

histamine-containing granules of mast cells.

Biogenesis of chromaffin granules.

In electron micrographs of adrenal medullary cells the electron-dense chromaffin granules, which contain most of the cell's catecholamines, are seen to be distributed throughout the cytoplasm. Somewhat more granules are observed away from the nucleus and the Golgi apparatus, towards the sites of exocytosis bordering the blood capillary, but this polarity is likely to depend on the state of the cell (Elfvin, 1965, 1967)

The proteins of the chromaffin granules are synthesized within the rough endoplasmic reticulum as is the case for other secretory proteins. This was shown in experiments in which animals were treated with reserpine which depleted the cells of their chromaffin granules. In the recovery phase the endoplasmic reticulum was swollen and contained electron-dense material, indicative of an increased protein synthesis (Abrahams & Holtzman, 1973). Further ultrastructural studies revealed that the packing of the newly synthesized proteins then occurs in the Golgi apparatus and vesicles are released to the cytoplasm from the ends of the Golgi lamellae in a "budding — off" process (Holtzman et al.,1973). The newly formed vesicles appeared uneven and less dense because of their low content of catecholamines. Due to their different shape from mature granules, and their unfilled state, these vesicles have been termed presecretory vesicles. Their sometimes uneven shape

has been suggested to result from fusion with clathrin-coated vesicles.

These coated vesicles may act as a shuttle between the rough endoplasmic reticulum and the presecretory vesicles, supplying them with newly synthesized chromogranins and other soluble proteins. The Golgi region may either be by-passed by the vesicle or interposed between two shuttle systems, one providing the transport from the rough endoplasmic reticulum to the Golgi, the other from the Golgi to the presecretory granules.

Coated vesicles might also be involved in retrieving membrane components from discharged granules for re-use. Studies on synthesis rates of chromaffin granule membrane proteins and soluble matrix proteins suggested that membrane proteins are synthesized at a rate which is five times slower than the rate of synthesis of the soluble secretory proteins (Winkler, 1977). This much higher turnover of soluble proteins compared with membrane proteins suggests that the membrane is not synthesized together with the secretory proteins and that the membranes or individual components of it are reused a number of times with a proportion (20%) being lost and presumably digested by lysosomes in every secretory cycle. An analogous situation which suggests the reutilisation of empty secretory vesicles has been observed in sympathetic nerves. In a process known as retrograde axoplasmic transport, synaptic vesicle membrane fragments flow from the nerve-endings through the axon back into the cell body, in opposite direction to the

ordinary axoplasmic transport of synaptic vesicles (Fillenz et al.,1976).

Upon neural stimulus of the secretory cell, chromaffin granules discharge their entire soluble contents catecholamines, ATP, soluble DBH, encephalines and chromogranin into the blood stream. If membranes are retrieved, they have to be refilled with the small molecules catecholamines and ATP as well as soluble protein. Coated vesicles might be involved in membrane retrieval, by fusing with vesicles budding off from the Golgi to form presecretory vesicles containing soluble proteins. But how are these vesicles then filled with ATP and catecholamines?

In the recovery phase after the administration of insulin or reserpine which depletes the adrenal cells of chromaffin granules, newly formed granules showed a much lower catecholamine/ATP ratio than found normally (Slotkin & Kirshner, 1973). This suggested that ATP enters granules before catecholamines. By analogy, during the ontogenic development of adrenal glands in rats, the ratio of catecholamines to ATP was smaller than one before birth and increased up to ten in the adult animal (O'Brien et al.,1972). This lead to the idea that the soluble proteins and ATP form a core to which the catecholamines bind. Since then a mechanism of active catecholamine transport which is independent of matrix components has been well characterized (Apps, 1982). The mechanism of the import of ATP into the granules which apparently happens after the filling with soluble proteins but before the accumulation of

catecholamines is less well understood. It is this mechanism of ATP transport into chromaffin granules this thesis deals with mainly.

Composition of the chromaffin granule membrane and matrix.

Like most other cell organelles, chromaffin granules consist basically of a membrane which surrounds a matrix space. In electron micrographs they are seen as membrane limited vesicles which contain in their mature stage an electron-dense interior, the chromaffin granule matrix. For a molecular characterisation it is useful to assess the composition of the membrane and the matrix individually. A separate preparation of the two phases is obtained by lysing purified intact chromaffin granules in a hypotonic medium and separate the membrane from the soluble content by centrifugation. The composition and the structure of the membrane and the matrix have been reviewed by several authors (Winkler, 1976; Njus & Radda, 1978; Phillips & Apps, 1979; Winkler & Westhead, 1980) using mainly data on the bovine chromaffin granule; a list of the components is given in Table 1.

The membrane.

The bovine chromaffin granule membrane contains lipids and proteins in a dry weight ratio of 2 to 1, for comparison in the inner mitochondrial membrane about 75% of the dry weight is protein. The lipids are mainly phospholipids with about 30 mol % cholesterol. Several enzymatic activities

Table 1: Composition of bovine chromaffin granules.

Component	% of granule dry weight	% of fraction
Membrane:		
Cholesterol	4.5	
Phospholipids	15.0	
Protein	9.7	
Dopamine β-hydroxylase		approx. 20% of total membrane protein
Mg^{++}-ATPase		
Cytochrome b561		
Phosphatidylinositolkinase		
Catecholamine carrier		
Matrix:		
Catecholamines	19.1	
Adrenalin		72
Noradrenalin		27
Dopamine		1
Nucleotides	16.8	
ATP		72
ADP		10
AMP		3
GTP		9.5
GDP, UDP etc.		5.5
Calcium	0.12	
Ascorbate	0.06	
Protein	32.3	
Chromogranin A		40
Dopamine β-hydroxylase		4
Mucopolysaccharide	0.6-2.2	
Encephalins		

have been associated with the membrane and some of these have been characterized as membrane proteins.

DBH. About 20% of the total membrane protein is dopamine-β-hydroxylase (DBH, E.C.1.14.17.1). This enzyme catalyzes the conversion of dopamine to noradrenaline in the biosynthesis of the catecholamines, and is the only enzyme of that pathway in the chromaffin granule, all others being located in the cytoplasm. DBH is a tetramer of 280,000 molecular weight and contains four copper atoms and the four subunits are thought to be identical. Its enzymatic activity is located on the matrix side of the membrane and the protein probably does not span the membrane. It is also present in a soluble form within the matrix, and in bovine chromaffin granules the enzymatic activities are distributed about equally between the membrane and the matrix. DBH is a glycoprotein and its carbohydrate chains face the matrix side as well. It is probable that the largest part of the enzyme projects into the matrix and that it is only anchored in the membrane with a small hydrophobic tail, which is probably the only difference between the membrane- bound and the soluble species. The two proteins isolated from the two sources have a very similar amino acid composition and a similar electrophoretic mobility (Bjerrum et al., 1979).

DBH is a mixed function oxidase. It splits oxygen, one oxygen atom appearing in the noradrenaline product, the other being reduced to water. Due to the localization of the enzyme, the substrate dopamine first has to be taken up by the granule before it becomes hydroxylated to

noradrenaline. Since the next enzyme in the pathway of the adrenaline synthesis, phenylethylamine-N-methyltransferase (E.C.2.1.1.28), is located in the cytoplasm, noradrenaline then seems to have to leave the granule again to become methylated to adrenaline which in turn is taken up again. It remains unclear why the chromaffin granule uses a complex compmartmentalized route for the synthesis of adrenaline.

ATPase. The membrane contains a Mg^{++}-dependent ATPase (E.C.3.6.1.4) which appears to be closely similar to the F_1F_0-ATPase complex from mitochondria (Apps & Schatz, 1979). The whole enzyme-complex has a molecular weight of 400,000, the three largest subunits α, β and γ show the same electrophoretic mobility as the subunits of the mitochondrial enzyme with molecular weights of 51,000, 50,000 and 28,000, respectively. The subunits from the chromaffin granule enzyme have been shown to cross react, although less well, with antibodies directed against the mitochondrial enzyme. A DCCD-binding proteolipid which probably derives from the H^+-conducting membrane segment of the granule enzyme has been isolated and was about 10% smaller and its amino acid composition more hydrophilic than the corresponding protein isolated from bovine mitochondria (Sutton & Apps, 1981).

The enzymes from both sources couple the hydrolysis of ATP with the pumping of protons across the membrane. In contrast to mitochondria, in chromaffin granules the enzyme's active site faces the cytoplasmic side of the

membrane, pumping protons into the interior of the granule. When an artificial proton electrochemical gradient was imposed across lysed and resealed chromaffin granule membrane vesicles, the enzyme was able to generate ATP from ADP and phosphate (Roisin et al.,1980). In vivo however, the main task of the enzyme is to energize the granule membrane via ATP hydrolysis whereas in aerobic mitochondria the energized membrane powers the synthesis of ATP. The chromaffin granule enzyme is inhibited by agents which typically inhibit proton pumping enzymes like Nbf-Cl, trialkyltin derivatives and DCCD (Apps et al., 1980a). It is however not inhibited by oligomycin, which specifically inhibits the mitochondrial enzyme by interacting with its F_0-portion, nor affected by inhibitors acting at other types of ATPases, like vanadate and ouabain. The F_1-ATPase is only a minor protein in the chromaffin granule with perhaps as little as 10 copies of the enzyme present per granule, compared with about 200 DBH and 1750 cytochrome b561 molecules per granule (Winkler & Westhead, 1980).

The chromaffin granule has a very low passive proton permeability (Johnson & Scarpa, 1976a). No leakage of K^+ from intact granules is observed without addition of nigericin (which catalyzes a H^+-K^+-antiport) or a combination of valinomycin (to catalyze an electrogenic uniport for K^+) with an uncoupler (to transport protons). Therefore the proton-translocating ATPase generates a transmembrane pH-gradient. Such a gradient has been demonstrated by the methylamine distribution technique. With this method the internal pH was shown to be 5.5 and a

further drop of 0.2 to 0.5 pH units was observed when ATP was added to the suspension of intact granules (Casey et al.,1977). In the same investigation it was shown that the decrease in the internal pH was larger in the presence of chloride, a permeant anion. This is because the chromaffin granule has a high internal buffering capacity which leads to an inside positive membrane potential, instead of a pH-gradient, when the ATPase pumps protons, but any potential eventually becomes large enough to oppose a further proton influx. If the membrane potential is neutralized by chloride uptake or movements of other permeant ions it cannot work against a further influx of protons. Therefore concomittant to the chloride uptake a swelling of the granules is observed which eventually leads to lysis.

Cytochrome b561. Cytochrome b561 is a major membrane component with a reported molecular weight of 22,000 (Apps et al.,1980b). It is likely to be the only hemoprotein in the chromaffin granule (Flatmark et al., 1971). No biological function has so far been ascribed to it, but since the protein probably spans the membrane it might be involved in an electron transfer chain. DBH, a mixed-function oxygenase, has a requirement for electrons but no interaction with the cytochrome could be shown so far, and the cytochrome seems not to be able to feed electrons to DBH (Grouselle & Phillips, 1981). The cytochrome is however oxidized by external ferricyanide and reduced by dithionite or ascorbate. In intact granules the cytochrome is in its reduced form which gives the granules

their pink colour. This reduced environment could be of importance in preventing oxidation of the stored catecholamines.

Further enzymatic activities which were shown to be associated with chromaffin granule membranes but which so far have not been identified with any protein are a phosphatidylinositolkinase (Phillips, 1973) and an ATP/ADP isotope exchange activity (Apps & Reid, 1977).

Transport activities. The granules contain several activities for transporting small molecules across the membrane into the matrix. The main activities, the transport of catecholamines and adenine nucleotides will be discussed below. Other transport activities include a mechanism in which Ca^{++} is transported across the membrane in exchange for 2 Na^{+} (Phillips, 1981). This uptake of Ca^{++} is not energy dependent and may have a role in depleting high cytoplasmic Ca^{++} levels after the depolarisation-induced Ca^{++} entry through the cytoplasmic membrane of the adrenal cell.

An activity for the uptake of dehydroascorbate was also shown (Tirell & Westhead, 1979), its activity was however 50 times lower (0.07nmol/min/mg protein) than that of catecholamine uptake (3.5nmol/min/mg) and ATP uptake (0.26nmol/min/mg). Dehydroascorbate itself seems to have no function in the chromaffin granule; in its reduced form as ascorbate however, it can act as an electron donor for DBH. A possible role for cytochrome b_{561} could therefore be to reduce transported dehydroascorbate to ascorbate inside the matrix.

Structural proteins. Another minor component of the membrane is α- actinin, 97,000 mol.wt. (Aunis et al.,1980). It is probably inserted in the outside of the membrane and serving as an anchorage for actin, 42,000 mol.wt., which itself is associated with granules but might not be in direct contact with the membrane. These two proteins interact probably with the cytoskeleton and might be necessary for the movement of the granules towards their sites of exocytosis.

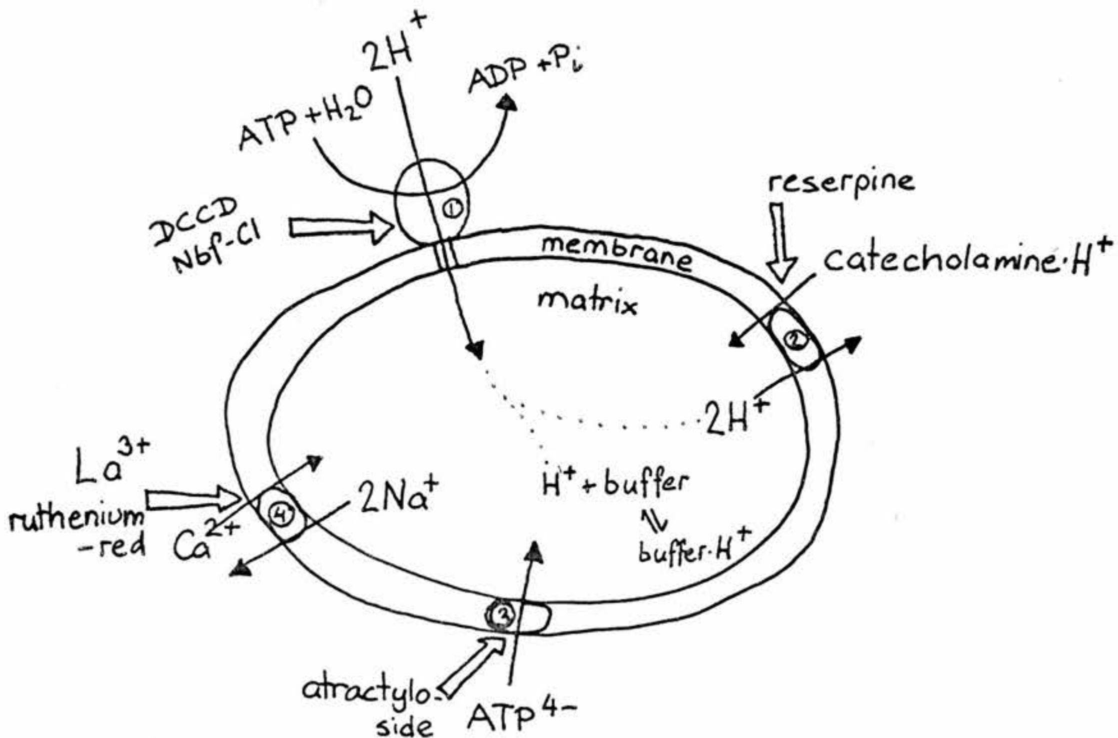
The matrix.

When chromaffin granules are suspended in a hypoosmotic medium they burst and release their soluble contents. This lysate then contains most of the catecholamines and adenine nucleotides which were present in the matrix in intact chromaffin granules. A major part of the total granule protein is soluble as well: its exact amount depends somewhat on the procedure which is used to wash the remaining membrane fraction, but generally varies between 60 and 80%. Table 2 lists the components present in the soluble matrix. The protein concentration in the matrix is about 200mg/ml of which DBH is, in contrast to its abundance in membranes only a minor component (about 4% of the total matrix protein). The main component in the matrix is the acidic protein chromogranin A (CGA), which accounts for about 40% of all the matrix protein. It has a similar molecular weight (about 72,000) to the subunits of DBH and is probably the biggest and most abundant protein of a

Table 2: Composition of granule matrix.

Catecholamines	650mM
Nucleotides	160mM
Calcium	25mM
Magnesium	6mM
Ascorbate	20mM
Protein	200 mg/ml
pH	5.5
Volume (newest)	4.6 ul/mg
Size of whole granule	200-300 nm

Scheme of chromaffin granule transport activities:



1. Mg^{++}-dependent ATPase, proton pumping
2. Catecholamine transporting protein
3. putative ATP carrier as proposed by Winkler's group
4. Ca^{++}/Na^{+} exchange protein

The white arrows indicate specific inhibitors.

larger group of acidic proteins, generally termed chromogranins, with isoelectric points between pH 4.9 and 5.6 (Apps et al.,1980b). It is their buffering capacity at a low pH which is mainly responsible for the acidic interior (pH 5.5) of chromaffin granules.

Recent reports also suggests that the chromaffin granules contain encephalins and their precursors, which are like the catecholamines released into the blood stream after exocytosis. The presence of a large precursor protein of about 50,000 mol.wt. in the granule matrix, containing sequences for both leu — and met — encephalins has been reported (Lewis et al.,1980). Furthermore an acidic copper-containing protein of unknown function has been reported (Gregoryan et al., 1981).

The total catecholamine concentration in the granules is in the range of 650mM and as discussed above, granules either store adrenaline or noradrenaline depending on the cell they derive from. The content of catecholamines varies in a given granule population, its average being 2.5μmol/mg total granule protein. The content of ATP is about 150mM. Published molar ratios of catecholamine/ATP vary between 4.2 and 4.85 (Winkler, 1976). Other nucleotides which include ADP, AMP, GTP and UTP are present as minor components. Heavier granules representing about 30% of the population might contain 3.5μmol/mg catecholamine with a ratio to ATP of 10-12 (Terland et al., 1979). Other small molecules in the matrix include magnesium, calcium and ascorbate.

Chromaffin granules are stable in the cytoplasm which

can be assumed to have an osmolarity of 300m . They are also stable when isolated and suspended in 0.3M sucrose. From these observations it has to be concluded that the matrix components interact somehow in order to reduce the total osmolarity of the matrix by a factor of three. The likeliest way of reducing the activities seemed to be via charge interactions between the catecholamines, which are protonated at the matrix pH of 5.5, and ATP which carries between 3 and 4 negatve charges at that pH. This seemed also to agree roughly with the molar ratio of these components in the matrix. This model was supported by observations made in vitro (Berneis et al.,1970), where physiological concentrations of ATP and catecholamines in presence of Ca^{++} yielded in a separation of two liquid phases of which the denser one consisted of a high molecular weight complex formed by ATP and catecholamines at a ratio of 3.6. However these complexes were heat labile and were not observed at physiological temperatures; furthermore, they were strongly dependent on optimal amounts of divalent cations. These studies did not normally take into account the high concentrations of the acidic protein also present in the matrix.

In n.m.r. studies it was shown that there were some interactions between metal ions, ATP and catecholamines with chromogranin A serving as a stabilizing agent by forming complexes with ATP and adrenaline (Daniels et al.,1978). It was emphasized that the interactions were dynamic and weak but rendered the solution more viscous, thus lowering the activity enough to make the granules osmotically stable.

Further studies confirmed the view that no structured phase containing catecholamines was present in the matrix and that therefore the catecholamine-protein interactions were of a non — ordered nature, i.e. no high molecular complex or gel was formed. Molecular interactions between ATP and catecholamines or electrostatic interactions between the small molecules and chromogranin molecules in random coil conformation were possible, as revealed by n.m.r.studies (Sen et al.,1979). It was also pointed out that positively charged amino acids on chromogranin A might interact with ATP thus increasing the amount of negative charges on the protein. This polyanion would then form a loose mesh, serving as a counterion matrix for charge interactions with catecholamines.

From the fact that during the biogenesis ATP and protein but little or no catecholamine is present in the granules, and that granules exist whose ATP to catecholamine ratio is larger than 4.5, indicates that a rigid stochiometric complex is unlikely. Also only weak interactions of the catecholamines with other molecules in the matrix might ensure that upon release into the blood stream the catecholamines are immediately available as free molecules for their pharmacological action, and that they do not remain complexed and therefore inactive for any length of time.

Since there seems to be no strong binding of small molecules, most certainly not of catecholamines, within the matrix, the question arises how such a high concentration

gradient of these molecules is achieved and maintained. Obviously there have to be several active transport activities for small molecules and these are discussed below.

Transport of catecholamines into chromaffin granules.

Intact chromaffin granules contain catecholamines at a concentration of 650mM of which perhaps 200mM is free, if the activity coefficient is about 1/3 (see above). Compared with the concentrations in the cytoplasm which are probably no higher than 20μM this represents a gradient of at least 10,000 – fold. How can such a concentration gradient be achieved and maintained? Some accumulation of catecholamines could be obtained by binding to the matrix side of the granule membrane or to soluble proteins within the matrix. But as discussed before, the majority of the catecholamines seemed to be free in solution in the matrix and only weak interactions between soluble molecules were present.

Already in 1962 Kirshner had observed that radioactive adrenaline was transported into chromaffin granules in a process requiring ATP and Mg^{++}. This transport could be inhibited by the rauwolfia alkaloid reserpine or by replacing Mg^{++} by EDTA. There was no uptake observed when ATP was replaced by ADP or AMP. Mg^{++} could be substituted by Mn^{++} but not by Ca^{++}. Although the requirement for MgATP and the inhibitorsensitivity suggested an energy dependent transport, it was also argued that the only requirement for Mg^{++} and ATP was to form with catecholamines a high molecular weight complex inside the matrix. The formation of such a structure would remove the catecholamine molecules quantitatively from the soluble phase thereby accumulating

them inside the granules.

From experiments with intact granules it was not clear whether the observed catecholamine transport represented a net uptake or an exchange of radiolabelled catecholamines with unlabelled molecules from within the granules. To overcome this problem Phillips (1974a) used a preparation of chromaffin granules which had been lysed in a hypotonic medium. These chromaffin granule ghosts were prepared from purified chromaffin granules applied to a Sephadex G-50 column, which was eluted with a hypotonic medium. The membranes were resealed by adjusting the osmolarity with concentrated sucrose. The preparation yielded chromaffin granule membrane vesicles which had lost most of their soluble contents. The catecholamine content was reduced from 2.8µmol/mg to 0.1µmol/mg protein. These resealed vesicles, or ghosts as they were called, in analogy to lysed red blood cells, had retained the ability to accumulate catecholamines in an energy-dependent process which could be inhibited by reserpine. Phillips (1974b) also showed that the transport mechanism had an affinity for several biogenic amines, such as noradrenaline, adrenaline, tyramine and 5-hydroxytryptamine (serotonin) with apparent Km values between 8 and 18µM.

Subsequently the mechanism of catecholamine transport into both, ghosts and intact granules was characterized by several groups. Bashford et al.(1975, 1976) showed that mitochondrial uncouplers not only stimulated a chromaffin granule ATPase activity but also inhibited the transport of

catecholamines. This was a first indication that the enzyme which transported catecholamines into chromaffin granules might not hydrolyze ATP itself but that the transport and the ATP hydrolysis might be coupled chemiosmotically, via a movement of protons across the membrane.

Further support for this model was obtained by Phillips (1978), who imposed an artificial inside acidic pH gradient across the chromaffin granule ghosts by preincubating them in a medium buffered at a low pH and then resuspending them in a buffer of a higher pH. Radioactive serotonin added to the medium was accumulated by the ghosts and the rate and extent of this uptake was proportional to the size of the imposed gradient. This uptake was also inhibited by reserpine and uncoupling agents.

According to the chemiosmotic theory (Mitchell, 1973), an energy supply and an energy consumption by different enzymatic processes can be coupled via the establishment of a proton electrochemical gradient across a sealed membrane vesicle with the enzymes spanning this membrane but being located at different sites. In the case of chromaffin granules an H^+-ATPase, which may be of the F_1 type (Apps & Schatz, 1979), sits on the outside of the chromaffin granule and pumps protons into the granule interior, thus establishing an electrochemical potential difference of the proton (protonmotive force, Δp). This is given by the equation

$$\Delta p = \Delta \mu_{H^+} / F = \Delta \Psi - Z \times \Delta pH$$

with F = the Faraday constant; ΔP = the electrical membrane potential; Z = 2.30 RT/F (61mV at 37°C); ΔpH = the transmembrane proton gradient, $\Delta \mu_{H^+}$ = membrane potential.

The effect of this protonmotive force on catecholamine transport was then studied in more detail. Isolated chromaffin granules have an internal pH of 5.5, giving a transmembrane proton gradient of about 1.5 pH units, with the cytoplasm assumed to be buffered at neutral pH. The membrane potential established in presence of MgATP is in the order of 50mV, inside positive. By perturbing independently either part of the proton electrochemical gradient, it was shown that the transport of catecholamines depended on both, the proton gradient and the membrane potential (Johnson & Scarpa, 1979). Although a dependence of the catecholamine transport on these two sources of energy was shown, only studies with chromaffin granule ghosts could determine what the actual driving force for the catecholamine uptake was.

Since ghosts lack an internal buffering capacity, pH-gradients and electrical gradients can be established independently by adding ATP in presence of a membrane permeant anion like Cl^- or a permeant weak base like ammonia. In energized ghosts Cl^- will cross the membrane and neutralize the charge difference but not the proton gradient whereas ammonia is protonated inside the ghosts thus depleting ΔpH but not $\Delta \Psi$. Using these two agents Johnson et al. (1979), Knoth et al. (1980) and Apps et al. (1980a) showed that catecholamine transport can be energized

by either the transmembrane proton gradient or the transmembrane potential gradient. A maximal rate of uptake of catecholamines into chromaffin granule ghosts was observed when both elements of the proton motive force were present.

Several mechanisms of proton linked catecholamine uptake were proposed by Njus & Radda (1978). Based on results and discussions from various authors, the mechanism which emerged as the likeliest was one in which catecholamines are transported across the membrane electrogenically in a process which exchanges two protons for one positively charged catecholamine molecule. This model took account of the fact that the uptake could be driven independently by both ΔpH or $\Delta\Psi$. Although the proposed mechanism is electrogenic and coupled to a flux of protons, the transport of catecholamines can be powered formally by $\Delta\Psi$ alone, in which case the ghost interior is depleted of protons and an alkalinisation occurs (Johnson et al., 1979). Since the ghosts normally contain a considerable amount of buffer, a reversed pH gradient is only formed gradually, but it will then stop the uptake of catecholamines.

Although a lot of evidence for this model had come from kinetic studies it is ultimately not possible to prove the stochiometric model of two protons being exchanged for one catecholamine molecule by looking at rates of uptake in kinetic experiments. Phillips & Apps (1980) therefore used a thermodynamic approach considering the concentration gradient of catecholamines established by into ghosts when

the system had reached an equilibrium. The aim was to show that the concentration of catecholamines obtained inside the ghosts depended on the proton electrochemical gradient as predicted by the model when the system was in equilibrium. For this it was necessary to reduce the effects of residual endogenous catecholamines in the ghosts preparation and this was achieved by using very small amounts of ghosts. The highest ratios of catecholamine distribution across the membrane, catecholamines inside / catecholamines outside (ca.i / ca.o), were obtained at very low external catecholamine concentrations; conditions which probably eliminated the increased leakage effects observed at higher concentrations.

For a quantitative assessment of the catecholamine uptake the equation derived from Mitchell et al. (1979) was used which links an established concentration gradient of a metabolite across a membrane with the energy used by the process .

$$Z \cdot \log (ca.i / ca.o) = (m-n) \cdot \Delta\Psi + n \cdot Z \cdot \Delta pH$$

Z is equal to 2.3xRT/F (59mV at 25°C) and converts the concentration ratios into millivolts. n is the number of protons transported in exchange for one metabolite (catecholamine) molecule. m is the charge of the metabolite in its transported form; this makes (m-n) a proportional factor determining to what extent the transport can be driven by the charge difference alone.

To eliminate errors when using this equation the

concentration gradient of the metabolite has to be high. Thus Apps & Phillips (1980) used very low substrate concentrations, which reduced the efflux of accumulated substrate and with which gradients of up to 20,000 fold could be obtained. The obtained values agreed well with the energetic term in the equation which was calculated from ΔpH and $\Delta\Psi$ measured at equilibrium and the stochiometry of one positively charged catecholamine being transported against two protons is therefore currently accepted to be correct. Similar results would be observed if uncharged catecholamines were exchanged with one proton, but there is at present disagreement as to whether the carrier is specific for protonated or unprotonated catecholamines (Sherman & Henry, 1981; Knoth et al., 1981b).

So far, no results are available which correlate the catecholamine transport activity with a specific membrane protein which might be the catecholamine transporter. Granule membranes have been solubilized and reconstituted in lipid vesicles (Maron et al., 1979; Giraudat et al.,1980) and the reconstituted vesicles showed an ATP dependent accumulation of noradrenaline, but the experiments were not done by reconstituting into artificial liposomes a mixture of proteins which had been solubilized and purified individually. In both reports merely a heterogenous mixture of solubilized chromaffin granule proteins was used, showing only that the enzyme activities of the ATPase and catecholamine translocation were still active when reconstituted.

The transport of ATP into chromaffin granules.

The total concentrations of nucleotides in chromaffin granules is about 160mM, of which the main compound is ATP at 120mM (Winkler, 1976). Investigations into the biogenesis have shown that ATP accumulates into presecretory vesicles, the immature chromaffin granules which have budded off from the Golgi apparatus before the filling with catecholamines. The concentration of ATP inside the granules is lower than that of adrenaline or noradrenaline, whereas the cytoplasmic level of ATP, at a few millimolar is higher than that of the catecholamines which is in the micromolar range. This means that the granule will have to establish and maintain an ATP gradient which is likely to be smaller than 100-fold compared with a catecholamine gradient which is nearer 10,000-fold. The localisation of the catecholamine biosynthetic enzyme DBH also suggests that the catecholamines have to pass the membrane several times and in either direction whereas adenine nucleotides are only accumulated once. Therefore the ATP transporting facility might not be as abundant and active as the catecholamine transport mechanism. Nevertheless, ATP at the neutral pH of the cytoplasm, carries about four negative charges, which makes it very polar. It is therefore difficult to envisage how it could penetrate the membrane by simple diffusion, without some protein facilitating the transport.

In contrast to the literature on catecholamine transport, rather little is known about the transport of adenine nucleotides into chromaffin granules. Only

Winkler's group seems to have made any advances in the field and has published a number of reports based on experiments performed with purified intact granules (Peer et al.,1976; Kostron et al.,1977; Aberer et al.,1978; Weber & Winkler, 1981).

The original observation made by Peer et al. (1976) was that radioactive ATP accumulated in chromaffin granules when perfused bovine adrenal glands were labelled with radioactive adenosine or phosphate. The uptake into the granules occured with a considerable time lag. Radioactive ATP did not appear in the granules before at least 30 min. had elapsed after the administration of labelled adenosine to the perfused glands. Similar results were obtained when radioactive phosphate was administrated. Inhibitors of oxidative phosphorylation prevented the appearance of radioactive ATP in the chromaffin granules. From the kinetics of the labelling and from the fact that no radioactive ADP or AMP couldbeshown to be taken up by the chromaffin granules it was concluded that ATP was synthesized outside the granules, most likely in the mitochondria, and only then transported into chromafin granules. However no label accumulated within mitochondria in an intermediate phase. When the mitochondria were poisoned with cyanide, the granules seemed to take up some adenosine, this was probably due to a diffusion process due to elevated cytoplasmic levels of adenosine since cyanide also inhibited the phosphorylation of adenine in the cytoplasm.

To investigate the transport of ATP into the granules further, in vitro studies with isolated chromaffin granules were performed by Kostron et al. (1977). Partly purified bovine chromaffin granules were incubated with (^{3}H)ATP under various conditions and the labelled granules were then purified from mitochondria and other contaminants on density gradients and the associated radiolabel was measured. It was shown that ATP was taken up by the granules in a temperature dependent process. More than 60% of the incorporated radioactivity was ATP. The rate of uptake was constant for the first 10 min. when incubated at 37°C. The rates of uptake depended on the substrate concentration and started to level off above 2mM ATP. At this concentration the uptake rate was 0.268nmol ATP/min/mg protein. Both, uptake of ATP and noradrenaline were activated by Mg^{++} and partly inhibited by NEM or CCCP, whereas reserpine specifically inhibited the amine uptake only. Atractyloside, a substance which inhibits the mitochondrial adenine nucleotide exchange protein inhibited the uptake of ATP but not that of catecholamines into the granules. It was therefore suggested that the carrier in the granules might be similar to that in mitochondria.

Although originally no uptake of ADP or AMP had been observed in perfused glands, an affinity of the transport mechanism for ADP and AMP was shown by Aberer et al. (1978). ATP and ADP had the same apparent Km of 1.4mM, the affinity for AMP being lower with an apparent Km of 2.9mM. The rates of uptake were lower for ADP and AMP than for ATP. Atractyloside inhibited ATP uptake competitively whereas

carboxyatractyloside was non — competitive. Activation by Mg^{++} and inhibition by EDTA suggested that the ATP uptake depended on the same proton electrochemical gradient established by the ATPase as the catecholamine transport mechanism. Further studies were done with substances like nigericin or NH_4^+ which affected only the formation of the pH gradient but not of the membrane potential. This inhibited the uptake of noradrenaline but did not affect the transport of ATP. It was concluded therefore that the nucleotide uptake depended only on the electrical component, $\Delta\Psi$, of the proton electrochemical gradient, in contrast to the catecholamine transport which also depends on the chemical part, ΔpH.

It was also shown that the nucleotides were incorporated into the soluble interior of the granules rather than bound to the membrane, since a hypoosmotic shock released 98% of the accumulated radioactivity. Data on uptake and leakage of adenine nucleotides at 0°C suggested that AMP and ADP cross the membrane by diffusion more easily than ATP, probably because of the reduced charge of these molecules. Aberer et al. (1978) concluded from their data that ATP is transported into chromaffin granules by a different carrier than catecholamines and that the two uptake systems use different parts of the same driving force.

These studies were carried further by Weber & Winkler (1981). They showed that at substrate concentrations above 2mM the ATP import into granules had a diffusion-mediated component additional to the active uptake. The active part

of the uptake at these higher substrate concentrations was determined from measurements made in absence or presence of the lipid permeable anion thiocyanate (SCN^-) which inhibited only the active uptake of adenine nucleotides (powered by $\Delta\Psi$) but not the passive diffusion, since it abolishes $\Delta\Psi$. They also showed that the substrate for the transport was the free and not Mg^{++}-complexed nucleotide. To overcome the problem of measuring an uptake of free ATP which is powered by a Mg^{++}-requiring ATPase the ATP uptake was monitored at concentrations of free ATP at which the driving force and therefore catecholamine uptake was constant. The observation of a catecholamine transport at all was probably due to a small amount of endogenous Mg^{++} present in the granule preparation.

The apparent Km for the uptake of ATP in presence of Mg^{++} was 2.9mM and in its absence 0.9mM. The Km in absence of Mg^{++} had previously been determined by the same group as 1.4mM (Aberer et al.,1978). The relative high Km of the mechanism compared with the high affinity of the mitochondrial adenine nucleotide exchange protein was suggested to reflect the millimolar cocentrations of nucleotides in the cytoplasm (2.7mM for ATP in liver cytoplasm, Akerboom et al., 1979).

Weber & Winkler (1981) also showed that the minor nucleotides of the granule matrix, GTP (9%) and UTP (5% of the total nucleotide pool), were transported into the granules by the same mechanism as the adenine nucleotides with Km's for the active uptake of 0.3mM for GTP and 0.7mM

for UTP. They interpreted this finding as indicating that the nucleotide carrier in the chromaffin granules was of broad specificity with the nucleotides within the granules reflecting the nucleotide composition in the cytoplasm.

Transport of adenine nucleotides into other cell-organelles.

Chromaffin granules are probably the best characterized vesicles storing high concentrations of both adenine nucleotides and neurotransmitters, but they are not unique and similar vesicles are present in other cells, such as acetylcholine — and noradrenaline-containing synaptic vesicles and serotonin granules from blood platelets and histamine granules of mast cells (Johnson et al., 1980). Additionally mitochondria have a well established mechanism for the exchange of adenine nucleotides. Some of the aspects of these organelles are discussed below in view of the mechanism of adenine nucleotide accumulation in chromaffin granules.

Dense granules. The serotonin storing granules, also called dense granules, from blood platelets have been isolated and shown to accumulate serotonin in response to a transmembrane proton gradient (Wilkins & Salganicoff, 1981). The mechanism resembled that of chromaffin granules closely. Dense granule ghosts established a proton gradient across the membrane in a process linked to the hydrolysis of MgATP which drove the uptake of neurotransmitter. This uptake could also be driven by an artificially imposed proton gradient. The concentration of adenine nucleotides stored within these

granules amount to several millimolar, but they could not be detected by ^{32}P n.m.r. when whole human platelets were analyzed (Ugurbil et al., 1979). Some resonances were observed when the content of granules from pigs was analyzed at higher temperatures. This suggested that the nucleotides in dense vesicles might be stored in some aggregated form within the matrix, and be perhaps in a less soluble state than in chromaffin granules.

It is unclear how the nucleotides are accumulated into the granules in the first place. It was shown that the metabolically active, cytoplasmic pool of adenine nucleotides in human platelets was labelled quickly after the incubation of the cells with radioactive adenine or adenosine. The nucleotides within the granules however, which represented 60% of the cells nucleotides, only became labelled very slowly and the radioactivity between the cytoplasm and the granules had only reached equilibrium after one day. About 1-2% of the label in the cytoplasm was transferred into the granules per hour. This was probably due to radioactivity being incorporated into newly formed granules (Reimers et al.,1977). For comparison the rate of serotonin transport into the granules was 300 times higher. Obviously ATP, which is the major nucleotide in the granules, is in a rather inacessable pool and little uptake or exchange of adenine nucleotides in already existing granules takes place.

Synaptic vesicles. The cholinergic synaptic vesicle is related in its structure and function to the chromaffin

granule. With a diameter of 80nm it is smaller than the chromaffin granule but of similar size to the dense granules. It contains 520mM acetylcholine (8nmol/mg protein), 170mM ATP (2.8nmol/mg protein) and 20mM GTP. The nucleotide content in the granule is thought to reflect the content of the cytoplasm (Wagner et al.,1978).

The transport of nucleotides into these vesicles has recently been described by Luqmani (1981), who also presented some evidence for a carrier mediated transport. The mechanism he described does in many ways exhibit similar characteristics to the adenine nucleotide transport into chromaffin granules described by Winkler's group. Uptake experiments were done by incubating purified whole granules for 30 min. with radioactive ATP. The uptake was temperature-dependent and at 26°C saturation kinetics was observed. The rates of uptake were determined from incubations done for 30 min. After 30 min. a sharp efflux of ATP out of the granules was observed, which could not be assigned to lysis, and no explanation for this observation was given. The apparent Km-values were for ATP 1.15mM, ADP 1.22mM and AMP 3.32mM. This decrease of the affinity of the transport mechanism for the monophosphate had also been observed in chromaffin granules (Aberer et al., 1978). These Km – values were somewhat lower than the ones measured in chromaffin granules. No results were presented on the influence of Mg++ on the uptake but the opinion was expressed that both ATP and acetylcholine uptake were not energy dependent (Giompres & Luqmani, 1980). The amount of (^{3}H)ATP accumulated into the granules exceeded the

radioactivity in the medium 20-25 fold and was equivalent to 20-50% of the initial endogenous amount of ATP present in the vesicles. With a substrate concentration of 200μM ATP, 100μM atractyloside inhibited the ATP uptake by one third but had no effect on the acetylcholine transport. At a much higher concentration of atractyloside (1mM), the ATP uptake was still observed at one third of the rate.

Luqmani (1981) concluded that a transport mechanism of broad specificity, as in chromaffin granules, was present in the synaptic vesicles, and speculated that one of the predominant membrane proteins of the synaptic vesicle, with an apparent molecular weight of 34,000, might be a candidate for the adenine nucleotide carrier.

Mitochondria. When considering the transport of adenine nucleotides across biological membranes it is obviously necessary to discuss the ADP/ATP translocating protein of the inner mitochondrial membrane. Knowledge of the protein facilitating this transport and of its mechanism is advanced compared with the mechanisms discussed so far and it might be therefore used as a source of ideas for less well understood systems. The details for the following discussion have been mainly taken from Klingenberg (1980).

The mitochondrial ADP/ATP translocator is a protein with a strict exchange mechanism. It transports ATP or ADP but not AMP across the membrane in exchange for either ATP or ADP, a mechanism which includes the non-productive exchanges of ATP with ATP or ADP with ADP. The exchange mechanism is basically not energy dependent. In deenergized or uncoupled

mitochondria the affinity for ADP and ATP are equal in both directions. In energized mitochondria however the uptake of ADP is preferred over that of ATP. In a mixture of 8% ADP and 92% ATP both nucleotides contribute 50% to the influx which is equivalent to an affinity for ADP which is more than 10 times larger than that for ATP. Consequently the efflux rates in energized mitochondria are higher for ATP than for ADP. The transport in energized conditions is therefore mainly ADP into, and ATP out of the mitochondria, which seems obvious in view of the physiological role of the mitochondria as producers of ATP for the cell's needs.

In deenergized mitochondria no preference of the nucleotide transported is observed. The reason for this has been explained by an electrogenic mechanism for the exchange process. The adenine nucleotides are transported in either way as free anions rather than as Mg^{++}-complexes. Under these conditions ADP carries three negative charges, and ATP four. In a model which assumes that the translocating protein carries three positive charges the transport of ADP is electroneutral and that of ATP electrogenic. This results in the translocation of one negative charge in the direction in which ATP is transported. The exchange of the two nucleotides is sequential, i.e. only one molecule is transported at a time.

The preference of an export of ATP and an import of ADP over any other type of exchange can be explained by the way the mitochondrial membrane is energized. In the energized state an electrical potential which is positive on the

cytoplasmic side is present across the membrane. This potential is established by the respiratory chain and is partially used by the ATPase to synthesize ATP. The translocase loaded with ATP, giving it a total of one positive charge will therefore tend to transport this charge outwards in response to the electric field across the membrane, whereas the transport of ADP will not be influenced by it. This electrophoretic influence on the polarity of the exchange mechanism could also be shown in artificially energized liposomes containing the purified translocase. The protein has a molecular weight of 30,000 in beef heart mitochondria and the active form is a dimer of two identical subunits. The transport is specifically inhibited by atractyloside and bonkrekate. Both inhibitors bind to the substrate site of the carrier. Atractyloside displaces ADP, which means that it binds from the cytoplasmic side. Bonkrekate binds to the translocase from the matrix side of the mitochondrion and eventually traps all carrier proteins there.

As outlined above, the transport mechanism of adenine nucleotides in mitochondria is sequential and strictly exchange process; and although the dependence on the membrane potential and the susceptability to atractyloside are characteristics in common with the adenine nucleotide transport in chromaffin granules the mitochondrial model cannot explain how the high concentrations of nucleotides found in the granules are obtained initially.

INTRODUCTION TO THE PROJECT.

The aim of this project was to study and characterize the mechanism by which chromaffin granules acquire and maintain the high ATP concentration found in the matrix in vivo. Lysed and resealed chromaffin granule vesicles, so called ghosts were used to study kinetically the uptake of radioactive adenine nucleotides. Ghosts have already been sucessfully used to characterize the transport mechanism for catecholamines in the vesicle membrane and are an accepted model for membrane functions of intact granules (Apps, 1982). Because contamination of ghosts by mitochondrial membranes is slight (Apps et al., 1980b) their use simplifies the performance and interpretation of kinetic experiments. The incorporation of radioactive substrate molecules into the vesicles can be followed in time courses with filter assays (Phillips, 1974b). This is an exact and quick method compared with measurements of the transport into intact granules, which have to be further purified after the incubation with the transported substrate because they are contaminated with mitochondria. This time-consuming procedure restricts kinetic studies and often only a single time point can be conveniently taken, providing an unsatisfactory basis for the calculation of initial uptake rates.

Ghosts are largely free of matrix components like proteins (chromogranins) to which adenine nucleotides might bind, and of endogenous adenine nucleotides which might exchange with radioactive material during the uptake experiments. It was hoped that by the use of ghosts, it

would be possible to interprete the accumulation of radioactivity inside the vesicles as actual uptake rather than exchange. This seems to be crucial since the problem in question is how the granules acquire adenine nucleotides initially, during the biogenesis, when they do not contain any nucleotides yet.

Some studies on the transport of ATP into ghosts had already been reported from this laboratory when this project was started (Phillips & Morton, 1978). It was shown qualitatively that ATP is transported into ghosts, presumably in a similar process as reported for intact granules (Kostron et al., 1977). Lysis-sensitive and -insensitive uptake was measured and the difference was considered to be the actual uptake and not binding to membrane components. The individual time-points in the kinetic experiments scattered considerably, therefore mean values from the results of several experiments were determined. The lysis-sensitive uptake appeared to be a saturable process with an apparent Km of about 1mM. Since the measurements were done in presence of Mg-ions the authors were unable to conclude unambiguously that the observed saturation was that of the nucleotide transport and not of another ATP-requiring process like the energisation of the ghosts by the ATPase, especially since they had also shown that the ATP-uptake was energy-dependent and could be driven in response to an artificially imposed potential gradient. The actual amounts of nucleotides taken up in these experiments were however extremly low and it was therefore not clear wether this was the mechanism used by the granules in vivo.

The results also showed that the catecholamine- and ATP-transport are two distinct processes which can be independently inhibited by reserpine and atractyloside respectively. Inhibitor studies further suggested that the catecholamine uptake takes place in response to a pH-gradient, whereas the ATP uptake in response to the potential gradient ($\Delta\Psi$). Although a clear distinction between these two processes was made, the characterisation of the ATP uptake was only preliminary though in accordance with the observations made for intact granules by Kostron et al. (1977).

The purpose of the further kinetic studies presented in this work was to investigate these original observations of ATP uptake into ghosts in more detail and to correlate the results with the observations made for intact granules. Phillips and Morton (1978) had used ATP concentrations of less than 1mM which is probably lower than the concentration in the cytoplasm. For these studies concentrations up to several millimolar were used, to represent the concentration found in vivo.

During the present investigations on the ATP uptake more became known on the mechanism of the catecholamine transport activity of the chromaffin granule membrane, as a result of work in several laboratories. Mechanistic models were proposed by those groups and their results are discussed and compared with the findings made for the ATP transport in the following chapters.

Kinetic experiments were also to be used to test the inhibitory effect of various substances on adenine nucleotide uptake. In particular, protein modifying agents

were tested which would not only inhibit ATP uptake but also covalently label the putative ATP-transporting protein.

In the second part of the investigations it was planned to use some of these inhibitors to label chromaffin granule membrane proteins radioactively. If some of the proteins became specifically labelled, this would then permit their identification within the mixture of all membrane proteins, using electrophoretic techniques. Of particular interest were affinity labels which were already known to inhibit other ATP-requiring enzymatic processes. Some of these labels show affinity because their structure resembles parts of the ATP molecules. To obtain those structural analogues in a radioactive form, it was planned to synthesize them from radioactive precursors.

Using these affinity labels it would ideally be possible to identify one single protein in the membrane as the nucleotide transporter. As an example, in brown adipose tissue-mitochondria, Heaton et al. (1978) have been able to identify two distinct proteins, the adenine nucleotide exchange protein and the regulatory site of the energy-dissipating ion uniport, using the radioactive photoaffinity label 8-azido-(γ-^{32}P)ATP. For the chromaffin granule membrane however, it was expected that several proteins would become labelled. It would then be necessary to investigate whether the labelling conditions can be altered to limit the labelling of some of the proteins.

A comparison of the results obtained under the various labelling conditions and with the various labels could then give new information on the identity of some of the chromaffin granule membrane proteins.

Some results of the presented investigations have been published in abstract form (Grueninger et al., 1980; Grueninger et al., 1981) and are submitted as a full paper (Grueninger et al., 1982).

MATERIALS AND METHODS.

Materials.

All chemicals used were of analytical grade or highest chemical purity available and supplied by either BDH Chemicals Ltd. or Sigma Chemical Company both at Poole, Dorset, UK. Enzymes were from Boehringer, Mannheim, Germany; Triton X-100, PPO and POPOP from Koch-Light Laboratories, Colnbrook, Bucks., UK; and deuteriumoxide from Norsk Hydro, Oslo, Norway. All radiolabelled substances were from The Radiochemical Centre, Amersham, UK, except (^{14}C)Nbf-Cl which was from Comissariat à l'Energie Atomique, Gif-sur-Yvette, France and (^{32}P)azido-ATP which was from New England Nuclear, Southampton, UK. Cibacron Blue was a gift from Ciba-Geigy Ltd., UK. Sources of other important items are stated within the text.

<u>Preparation of chromaffin granules, resealed ghosts, granule membranes and lysate from bovine adrenal glands.</u>

The preparation of resealed chromaffin granule ghosts and granule membranes was essentially performed as described by Apps et al. (1980a). 15-30 Bovine adrenal glands were obtained from the local slaughterhouse and transported on ice to the laboratory. The time between the death of the animals and the start of the preparation was normally 2-3 hours. The glands were trimmed free of fat, dissected into halves, and the pink medulla was scraped off the orange-brown cortex with the aid of a scalpel. The medullae were placed in 200-400 ml ice cold buffered sucrose: 0.3M sucrose, 10mM NaHepes (1M Hepes is adjusted to pH 7.4 with 4N NaOH, dilution to 10mM gives a pH of 7.2), and passed through a small mincer with holes of 2mm diameter.

Portions of 40ml of the suspended mince were given three passes in a glass homogenizer with a loosely fitting teflon pestle at 600 r.p.m. Nuclei, intact cells, connective tisue and blood cells etc. were centrifuged from the homogenate in 2-4 250ml buckets in a Beckman JA14 rotor at 4000 r.p.m. (2500x*g* max) for 5 min. All centrifugation steps were performed at 4°C, and added buffer was always ice-cold. The pellet was discarded and the supernatant was centrifuged in the same rotor at 14,000 r.p.m. (30,000x*g* max) for 30 min. The supernatant containing the soluble cytoplasm was discarded. The pellet contained mitochondria in an upper

brown and chromaffin granules in a lower pink layer. Some purification of granules was obtained by gently washing off the top layer of mitochondria with buffered sucrose. The rest of the mitochondria and the chromaffin granules were resuspended in buffer. The suspension was gently homogenized at low speed with the same homogenizer as above and spun in 2-4 40ml tubes in a Beckman JA20 rotor at 15,000 r.p.m. (27,000x*g* max) for 20 min. More granules could be obtained from the washed off mitochondria by resuspending and spinning them in the same way. The new pellet consisted again of two layers; the mitochondrial layer was washed off and still contained enough granules to be used for preparing chromaffin granule membranes (see below). The lower pink granule layer of the pellet was resuspended in 10ml buffered sucrose to give a thick suspension. This preparation of crude granules was then used to prepare either chromaffin granule membranes or lysed and resealed membrane — vesicles (i.e.'ghosts').

For experiments with intact granules the preparation was further purified from mitochondria on sucrose density step gradients. In thick walled, 70ml polycarbonate tubes 20ml of resuspended crude granules were layered over 50ml 1.6 or 1.8M sucrose in 10mM NaHepes, pH 7.2. Purified granules were collected as a pellet after centrifugation in a Beckman Ti45 rotor at 45,000 r.p.m. (240,000x*g* max) for 60 min. and purified adrenal mitochondria could be harvested from the step gradient interface. Yield and purity of the two organelle preparations depends on the density of the sucrose solution, granules of a higher purity but lower yield are

obtained with a cushion of 1.8M sucrose.

Ghosts. Ghosts were prepared from the thick suspension of crude granules. About 10ml of the suspension were added dropwise to 300ml 10mM NaHepes, pH 7.2, which was stirred slowly on ice. The granules lysed immediately in the hypotonic buffer as seen by the change of colour from the pink of the granules to the white of the lysed vesicles. The suspension was left stirring for further 10 min, after which 54ml 2M sucrose was added dropwise to restore the osmotic pressure. The resealed ghosts were centrifuged in a Beckman Ti45 rotor at 23,000 r.p.m. (62,000x*g* max) for 30 min. The supernatant, which contains the matrix components of the chromaffin granules, was dialyzed and concentrated as described below to give chromaffin granule lysate.

The pellet again showed two layers, the upper (pink and bigger), chromaffin granule ghosts, and the lower brown one, mitochondria, probably in the form of submitochondrial particles because of the hypoosmotic shock. The ghosts were carefully scraped off the mitochondrial layer with a spatula and resuspended by gentle homogenisation in buffered sucrose to a final volume of about 20ml. The remaining mitochondrial contamination was then removed from these ghosts on a density step gradient. Four cellulose nitrate tubes for the Beckman SW41 rotor were filled with 4.5ml of 0.4M sucrose, 10mM NaHepes, pH 7.2, and a cushion of 3ml 0.4M sucrose in D_2O was underlayed with a long needled syringe. 5ml of the ghosts suspension were then put on the top of this gradient. After centrifugation at 40,000 r.p.m.

(200,000xg av) for 30 min the purified ghosts were collected from the H_2O/D_2O interface. Separated mitochondria could be seen as a thin brown pellet. The pooled yield from the four gradients was about 6ml of ghosts at a protein concentration between 3 and 5mg/ml. The mitochondrial contamination as assessed by cytochrome oxidase activity was in average 1-2% w/w. The catecholamine concentration was in the range of 150-300 nmol/mg protein.

Membranes. Purified chromaffin granules were suspended in 10mM NaHepes, pH 7.2, by homogenisation, and diluted further to a volume of 200ml to ensure total lysis. Lysed granule membranes were centrifuged from the lysate containing matrix components in a Beckman Ti45 rotor at 45,000 r.p.m. (240,000xg max) for 20 min. The supernatant was used to prepare the lysate fraction. Pelleted membranes were further purified from remaining mitochondrial contamination on a sucrose density step gradient. In thick walled 70ml polycarbonate tubes 20ml aliquots of resuspended membranes in 10mM NaHepes were layered over a cushion of 50ml 1M sucrose in 10mM NaHepes, pH 7.2. After centrifugation in a Beckman Ti45 rotor at 45,000 r.p.m. (240,000xg max) for 30 min, the purified granule membranes were collected from the interface. A typical recovery was 3ml of membranes at 8mg/ml protein concentration. The mitochondrial contamination was between 1 and 2.5% w/w.

Membranes and ghosts were stored at -20°C before use. No difference in adenine nucleotide uptake was observed between ghosts which had been freshly prepared or frozen

once. The extent of serotonin uptake was about 20% higher in freshly prepared than unfrozen and thawed ghosts. For experiments which included isoelectric focussing only fresh membranes could be used because frozen material did not solubilize well enough in the focussing sample buffer.

Lysate. The lysate fraction containing soluble chromaffin granule matrix components was obtained from the lysis of the intact granules. It was dialysed and concentrated under pressure using a negative pressure micro protein dialysis concentrater (Micro-ProDiCon, Bio-Molecular Dynamics, Beaverton, Oregon, USA). A dialysis bag with an exclusion limit of 10,000 mol. wt. was used and the dialysis buffer was 4mM NaHepes, pH 7.2. Dialysis and concentration were performed at 4°C for 24 hours with one change of buffer. This reduced the volume of the lysate 50 to 100-fold and the final protein concentration was 10-20 mg/ml. Samples were kept at -20°C.

Content of adenine nucleotides in the ghost preparation.

The amount of adenine nucleotides present in the preparation of ghosts was 123±23nmols of ADP/mg protein (mean ± S.D. for six determinations on three preparations), and 23±11nmoles AMP/mg protein; but no ATP was detectable. These determinations were done in presence of 0.03% Triton X-100; in its absence the adenine nucleotide concentration was about 30% less, regardless of whether ghosts were osmotically buffered or not. This indicates that maximally 30% of the measured adenine nucleotides are associated with

the ghosts and the rest is in the outside buffer. This is equivalent to an internal concentration of 10mM.

Transport assays.

Transport of adenine nucleotides, phosphoenolpyruvate and serotonin into resealed chromaffin granule ghosts was measured by incubating ghosts in a medium containing the radiolabelled substrate and filtering samples taken in time intervals to separate the ghosts from the medium. The standard incubation medium to monitor ATP uptake contained 10 to 30mM NaHepes, pH 7.2, 0.3M sucrose, 10mM KCl, 1 to 2.5mM $MgSO_4$, 5mM (^{3}H)ATP (2mCi/mmol) and 0.5mg/ml ghosts. In some experiments 10mM phosphoenolpyruvate (PEP) and 2U/ml pyruvate kinase (PK) were present, to serve as an ATP regenerating system. For dual labelling experiments 25μM (^{14}C)serotonin (0.42mCi/mmol) were additionally present in the medium. Accumulation of (^{3}H)ADP or (^{3}H)AMP (2mCi/mmol) and of (^{14}C)phosphoenolpyruvate (2.5mCi/mmol) was measured in similar media. For PEP transport studies its concentration was 200μM and no KCl was present in the medium because it did not have any stimulatory effect. Details of composition of the various incubation media are given in the individual experiments. When the concentration of a radioactive substrate was altered, its specific radioactivity was altered accordingly thus keeping the actual amount of radioactivity per volume incubation medium constant. Under these conditions unspecific binding to filters etc. was constant in terms of radioactive counts.

Time courses of substrate uptake were started by adding the ghosts to the rest of the incubation medium and incubating the mixture at the appropriate temperature. For experiments performed at 37°C a water bath was used. Prewarming media as well as protein at 37°C before mixing had little effect on observed uptake rates, because the media reached 37°C within less than 30 seconds. For some experiments ghosts were preincubated with various substances. This was done at 37°C and is indicated in the individual cases.

For time courses samples of 100µl were withdrawn with a Gilson adjustable sampling pipette. There was no need to change the tips between the individual samples of one time course. Samples were removed at intervals indicated in the figures and diluted immediately into 4ml of a quench solution, consisting of 10mM NaHepes, 10mM pyrophosphate, 0.3M sucrose, pH 7.2. The pipette tip was carefully rinsed twice in this buffer. The diluted sample was mixed quickly on a vortex mixer and filtered immediately on a single milipore filter unit which was connected to a fully open water pump. Cellulose nitrate filters presoaked in quench solution were used which had been cut to an exact size of 25x25mm from larger sheets (Sartorius Membranfilter, pore size 0.45µm, Sartorius, Goettingen, Germany). The filters were washed with two portions of 2ml 10mM NaHepes, pH 7.2, 0.3M sucrose each, and dissolved in scintillation fluid.

With the above procedure which was carried out at room temperature no leakage of accumulated ATP could be observed

when compared with experiments where all steps were performed in the cold room or on ice. Presoaking of the filters in the quench solution for 30-60 min. reduced the scattering of the measurements. Pyrophosphate instead of unlabelled ATP was used in the quench solution, but had the same effect of reducing unspecific binding of (^{3}H)ATP. Addition of pyrophosphate to the washing buffer did not further reduce unspecific binding of label to the filters nor did increased amounts of washing buffer. With the above conditions unspecific binding of label to the filters was between 1900 and 2000 (^{3}H) d.p.m. in all experiments. This was measured by filtering apropriate amounts of media which contained no protein. Unspecific binding of PEP was around 240 (^{14}C) d.p.m. and was negligible for serotonin. These values were always subtracted from the results.

When lysis-sensitive uptake was investigated, samples were removed from the incubation medium as above and diluted into 4ml of a quench solution containing 10mM NaHepes and 10mM pyrophosphate, pH 7.2, but no sucrose. The samples were mixed and left at room temperature for 10 min, after which enough sucrose was added to give 0.3M. The samples were then filtered and washed as above.

Scintillation counting.

To measure the radioactivity trapped on the filters, they were dissolved in 2.5ml of a toluene based scintillation fluid containing 33% Triton X-100 (5g 2,5-diphenyloxazole (PPO), 0.3g 1,4-di-(2-(5-

phenyloxazolyl))-benzene (POPOP) (Koch-Light Laboratories) in 1l toluene and mixed with 500ml Triton X-100).

Clear samples were obtained by adding 90µl water (Pande, 1976), leaving them one hour at room temperature and then shaking them vigorously until the filters were fully dissolved. Glass mini vials (12x50mm) were used and they were put into ordinary 22.5ml glass vials for scintillation counting. The larger vials were not capped additionally. Samples were counted overnight in a programmable refrigerated scintillation counter (Nuclear Chicago Mark III) for 10 min or until 10,000 (^3H) c.p.m. had been accumulated. A program was used which corrects for individual quenching and calculates automatically d.p.m from the measured c.p.m. The counting efficiency was 26% for (^3H) and 54% for (^{14}C). For dual isotope experiments a program was used which also corrected for spillover. In control experiments no (^{14}C) counts could be detected in the tritium channel and vice versa. To standardize the counts measured with the actual amount of substances they represent, the radioactivity of 10µl whole incubation medium was measured. The reason for dissolving the filters rather than suspending them in scintillation fluid without Triton was to minimize quenching of the low – energy tritium radioactivity. The scintillation fluid containing Triton was also used to measure the radioactivity of water-based fluid samples, whereas for samples soluble in organic solvents Triton was omitted.

Separation of incubated ghosts from the medium, and preparation of re-lysed ghosts and lysate.

For various experiments it was necessary to remove the incubated ghosts from the medium. This was done by passing the mixture down a Sephadex G25 or G50 column. The portions containing the ghosts were assessed by their turbidity and normally only the first half of the portions were pooled for further use. The radioactivity of those fractions was retained by filters by 50-80%, compared with the mixture applied to ther column which contained only 0.1% radioactivity retained by filters.

For preparative lysis by hypoosmotic shock, ghosts separated as above were suspended in at least 20 volumes ice cold 10mM NaHepes, pH 7.2, and allowed to stand on ice for 10 min. Membrane fragments were pelleted by spinning at 50,000 r.p.m. in Beckman rotors Ti50 (240,000x*g* max) or Ti50.2 (310,000x*g* max) for 15-30 min.

Preparation of sucrose density gradients.

Linear gradients of 0.4-1.5M sucrose in 10mM NaHepes, pH 7.2, were poured into cellulose nitrate centrifuge tubes for the Beckman SW41 rotor. The total volume of the tubes is 12.8ml and gradients of 11.5ml volume were poured to leave room for a sample volume of 1ml. A gradient mixer with two connected chambers of the same volume was used. The sucrose was pumped from the chamber with the higher concentration onto the forming gradient, slowly enough to allow the buffer from the other chamber to flow after. One gradient was formed within 15-20 min and stored at 4°C until used, when

necessary overnight. Volumes and concentrations of sucrose in 10mM NaHepes, pH 7.2, for the two mixing chambers were calculated with the following formula:

Cb = Ca - Vt/Vg x (C bottom - C top)

Ca = higher sucrose concentration, equal to the concentration at the bottom of the gradient. Cb = lower sucrose concentration, Vt = sum of volumes of both mixing chambers, 0.5xVt is to be filled into each of the chambers. Vg = total volume of the gradient. C bottom = concentration at the bottom, C top = concentrationat the top of the gradient.

The sample in 0.3M sucrose was carefully overlayed immediately before spinning the gradient to equilibrium, 40,000 r.p.m. (200,000x*g* av) for 3 hours. To collect the gradient, the tube was pierced at the bottom with a needle and the gradient was pumped off from the bottom into 20-40 fractions within 30 min. These steps were performed in the cold room. The fractions were assayed for enzyme activity and radioactivity. Sucrose densities were determined from measurements with a refractometer (Abbe 60), taking samples of a gradient loaded with buffer only and centrifuged with the other gradients.

Chromatographic separation of adenine nucleotides.

To measure hydrolysis of ATP by the ghosts during uptake experiments, samples of the incubation media were analyzed chromatographically for their content of adenine

nucleotides. At various times during the incubation samples of 5 or 10μl, about 120,000 or 250,000 (^{3}H) d.p.m., were withdrawn from the media and applied on strips (3x22cm) of DEAE-cellulose paper (Whatman DE 81). The chromatograms were run in 0.5-0.6M formic acid, 10mM EDTA, adjusted to pH 3.1 with concentrated ammonia solution (Apps & Nairn, 1977), for after 2-3 hours at room temperature, until the solvent front had moved about 14cm. The strips were dryed and cut into pieces of 1cm. To count the associated radioactivity, the pieces were immersed in scintillation fluid and counted. The counting efficiency for tritium was only about 15%.

The respective Rf values for the three adenine nucleotides depended on the concentration of ammonium formate in the chromatography buffer. Controls of radioactive ATP, ADP or AMP were run separately and the following respective Rf values were obtained: in 0.50M ammonium formate: 0.09, 0.33, 0.70; in 0.55M ammonium formate: 0.20, 0.50, 0.70; in 0.60M ammonium formate: 0.47, 0.71, 0.82; for ATP, ADP and AMP respectively.

The same method was used to analyze the composition of adenine nucleotides within the ghosts after incubation. Ghosts were separated from the medium and lysed as described above. Because NaHepes interfered with the chromatography at high concentrations the lysis in these experiments was done in water only. The lysate was lyophilized overnight and resuspended in a minimal volume of water. Because of the very low counting efficiency it was necessary to chromatograph a large sample. Therefore paper strips of a

width of up to 5cm were used and up to 100µl resuspended lysate were applied. After chromatography the cut filter bits were folded to be placed into vials for counting. Samples of incubated ghosts were also put directly on chromatograms in an attempt to analyze their content more directly, but no radioactivity could be traced after development of the chromatograms.

Hydrolysis of PEP to pyruvate during incubation in a medium could also be assessed with this chromatography system. In 0.55M ammonium formate the Rf values were 0.45 and 0.70 for PEP and pyruvate respectively.

Enzymatic assays of adenine nucleotides.

To assess the amount of endogenous adenine nucleotides present in a preparation of ghosts, coupled enzymatic assays were used adapted from Bergmeyer (1963). The assay of ATP is based on the phosphorylation of 3 — phosphoglycerate to 1,3-diphosphoglycerate by the enzyme phosphoglycerate kinase. 1,3-Diphosphoglycerate can then be reduced to glyceraldehyde phosphate and phosphate by glyceraldehydephosphate dehydrogenase, in presence of NADH, which is oxidized to NAD^+. The decrease of NADH is recorded spectrophotometrically at 340nm.

A solution of 1ml 10mM NaHepes, pH 7.2, 0.03% Triton X-100 and 1mM $MgSO_4$ was used in both reference and assay cuvette. To the assay cuvette 50µl 100mM glyceraldehyde-3-phosphate was added and a base line of zero absorption at 340nm recorded. Then 50µl 4mM NADH, pH 7.0, was

added and a second line recorded; the difference reflects the absorption of NADH. After the addition of 40µl of a ghost preparation (3-6mg/ml) a further additional absorbance is observed. The reaction is started by the addition of 5µl of a combined enzyme solution containing phosphoglycerate kinase and glyceraldehydephosphate dehydrogenase. In presence of ATP in the ghost preparation a decrease in absorption ought to be observed. The assay is standardized by adding a known amount of ATP. In the above case the decrease of absorption by 0.05 units represented 10nmol ATP. Theoretically it should be 0.054 as calculated from the molar extinction coefficient of NADH (6.22 at 340nm).

The assay for ADP is based on the dephosphorylation of phosphoenolpyruvate to pyruvate by pyruvate kinase which is coupled to the phosphorylation of ADP to ATP. The resulting pyruvate is then oxidized to lactate by lactate dehydrogenase and this reaction is coupled to the oxidation of NADH to NAD+. The amount of NADH oxidized is therefore proportional to the amount of ADP present in the medium.

For the assay a solution of 1ml 10mM NaHepes, pH 7.2, 0.03% Triton and 1mM $MgSO_4$ was used in both reference and assay cuvettes. To the assay cuvette 150µl of 20mM PEP, pH 7.0, and 2µl pyruvate kinase was added and an absorbance base line recorded. 50µl 4mM NADH, pH 7.0, were added which results in an increase of absorption at 340nm. 40µl ghosts at 3-6mg protein/ml were added which increased the absorbance further but then a slight decrease as a result of some endogenous enzymic activity in the ghost preparation

was observed. When this decrease was linear with time, the assay reaction is started by the addition of 2µl lactate dehydrogenase solution and the rapid oxidation of NADH monitored until only the slight linear decrease of absorption was observed again. The distance between these two straight lines of 'background' oxidation represents the amount of ADP present in the ghost preparation. The assay was standardized by adding known amounts of ADP. A decrease of 0.05 absorption units represented 20nmol ADP.

AMP was assayed after the estimation of ADP in the same cuvettes. To the assay cuvette 10µl 200mM ATP, pH 7.0, and 5µl myokinase were added. This catalyzed the conversion of one AMP and one ATP molecule into two ADP molecules which were then detected quantitatively as described above, therefore only half of the amount of ADP formed originates from AMP. The addition of ATP could also be omitted because enough ATP had been formed from the assay of ADP done beforehand.

Estimation of protein.

Protein contents of preparations were estimated by the coomassie blue dye-

binding assay (Bradford, 1976; Read & Northcote, 1981). For the reagent 12mg coomassie brilliant blue G are dissolved in 5ml ethanol, and 10ml concentrated phosphoric acid added dropwise and stirred at room temperature for 60 min. after which the volume is adjusted to 100ml with water. The reagent is filtered through glass wool and a glass fibre

filter to remove unreacted blue dye, and it is kept refrigerated in a dark bottle. One ml of this reagent gives a linear response between 1 and 8μg protein.

For the assays 1ml reagent was added to the unknown protein (2-5μg) in 0.1ml 150mM NaCl. The samples were mixed immediately and the absorption at 595nm was measured after 30 min. but before 60 min. incubation at room temperature, against a blank sample containing 0.1ml NaCl and 1ml reagent. Samples were normally prepared in triplicate. For a standard curve, the absorption of 1 to 10μg bovine serum albumin (BSA) in 0.1ml 150mM NaCl was measured. Samples were prepared from a stock solution of 1% BSA, kept at -20 °C, which was itself standardized by measuring its absorption at 280nm (the extinction coefficient for a 1% solution is 6.6). Samples were allowed to stand at room temperature for 15-20 min. only before measurements were taken since BSA as a soluble protein was expected to react more quickly with the dye than the membrane bound proteins from chromaffin granule ghosts.

Other assays.

The assay for the mitochondrial marker enzyme cytochrome c oxidase was performed as described by Mason et al. (1973). Dopamine β-hydroxylase activity was measured with a method adapted from Friedman & Kaufman (1965) and catecholamine content of chromaffin granule ghost preparations were estimated according to Euler & Lishajko (1961). Both these methods have been recently described in detail by Grouselle & Phillips (1982).

Electrophoresis.

Slab gels. To separate proteins according to their molecular weight, polyacrylamide gel electrophoresis in presence of SDS was performed as described by Laemmli (1970). Slab gels 13x13cm, 1-1.5mm thick, were poured with an exponential gradient of either 6-15% or 10-15% w/v acrylamide. A stacking gel of 4.5% w/v acrylamide with a variable number of wells was used. Gels were run overnight at room temperature at a constant voltage between 45 and 60V. They were fixed in 10% acetic acid, 20% methanol in water at 40°C for 10-20 min, stained in 0.25% w/v coomassie blue R in 7.5% acetic acid, 50% methanol in water at 40° C for 5-15 min, and destained in 7% acetic acid, 10% methanol in water for several hours. The destaining solution was changed once and excessive dye was trapped by adding a piece of colourless polyurethane foam. Gels were dryed under vacuum onto a piece of Whatmann 3MM filter paper on a slab gel dryer (Hoeffer Scientific Instruments) which was heated for 20 min. Details of the composition of gel solutions and buffers are given in Table 3.

Isoelectric focussing. Two-dimensional maps of chromaffin granule proteins were obtained by isoelectric focussing in the first dimension in rod gels, and SDS polyacrylamide gel electrophoresis in the second dimension (O'Farrell, 1975, Apps et al., 1980). The pH-range of the focussing gel was varied to give an optimal resolution of

Table 3:

Solutions and buffers for SDS acrylamide gel electrophoresis.

Gels (for % acrylamide:)	6%	10%	15%	stack gel
H_2O (in ml)	11.3	7.3	1.15	5.1
30% (w/v) acrylamide (BDH) + 0.8% NN'-methylene bisacrylamide	6.0	10.0	7.5	1.8
1.5M TrisCl pH 8.8, + 8mM EDTA, 0.4% SDS	7.5	7.5	3.75	
0.5M TrisCl pH 6.8, + 8mM EDTA, 0.4% SDS				3.0
3% (w/v) polyacrylamide (Aldrich), 0.1% NaN_3	5g	5g	2.5g	2g
add (to polymerize): (in µl)				
10% (w/v) ammoniumpersulphate	15	15	7.5	20
TEMED	120	120	80	250
Total volume (in ml)	30	30	15	12

Sample buffer (5x concentrated; dilute with sample accordingly)

0.5M TrisCl pH 6.8	40ml
0.2M EDTA pH 7	4ml
SDS (for 5% final conc.)	25g
Glycerol	40g
Mercaptoethanol (if required)	4ml
add H_2O to give 100ml	
0.5% Bromophenolblue	0.5ml

Electrode tank buffer

Tris base	30g
Glycine	144g
SDS	5g
Disodium EDTA	3.72g
add H_2O to give 1000ml (gives pH 8.8)	

Solutions and buffers for the two-dimensional gel electrophoresis.

Cathode tank solution	1% (v/v)	ethanolamine
Anode tank solution	0.5% (v/v)	phosphoric acid
lectrofocussing gels (for 8 ml)	4.76 g	urea (ultrapure)
	0.84 ml	45% (w/v) acrylamide, 0.6 (w/v) bisacrylamide
	1.60 ml	10% (w/v) Nonidet P40
	2.32 ml	H_2O
	0.40 ml	ampholines
	to start polymerisation, add 40 ul 10% (w/v) ammonium-persulphate	
Sample buffer	2.28 g	urea (ultrapure)
	0.80 ml	10% (w/v) Nonidet P40
	0.20 ml	ampholines
	0.20 ml	2-mercaptoethanol
	1.20 ml	H_2O
	4 µl	5mg/ml bromophenol blue
Overlay solution	1.0 ml	5M urea
	10 µl	ampholines
$1^o \rightarrow 2^o$ dimension transfer buffer	50 ml	10% SDS
	3 ml	2-mercaptoethanol
	10 ml	0.5M Tris-Cl pH 6.8
	10 ml	glycerol
	10 µl	bromophenol blue 1mg/ml
	add	H_2O to give 100ml
	to seal gels: 1% agarose in this buffer	

Solutions and buffers for the antibody-blot.

Transfer buffer	28.8g	glycine	gives pH 8.6
	6.0g	Tris	
	400 ml	methanol	
	1600 ml	H_2O	
Horse serum		inactivate by incubating at 56°C for 30 min., filter, store in aliquots at -20°C	
Tris-Salt buffer 2x conc.	4.8g	Tris, pH 7.2 with HCl	
	1.8g	NaCl	
	in 500 ml	H_2O	dilute before use
Horse serum buffer	2.5g	BSA	
	50 mg	NaN_3	
	5 ml	inactivated horse serum	
	in 100 ml	Tris-Salt buffer (1x conc!)	
BSA buffer	3 g	BSA	
	50 mg	NaN_3	
	in 100 ml	Tris-Salt buffer (1x conc!)	

Sterilize solutions through membrane filter for storage at 4°C.

the proteins. To separate membrane proteins, a mixture of ampholines from two sources was used. Their concentration in the gel was 1.8% v/v ampholines of pH-range 3.5-10 (Bio-Rad) and 0.2% v/v of pH-range 9-11 (LKB). For separation of lysate proteins, 2% v/v ampholines pH-range 4-6 (Bio-Rad) were used. The gels were focussed at room temperature overnight at 400V. At the beginning the voltage was increased stepwise in order not to exceed a current of 0.5mA per tube. The focussing gels were either immediately further used or stored frozen in 2-D sample buffer at -20°C before re-use. Electrophoresis in the second dimension was performed on SDS polyacrylamide gels as described above. The pH-gradient of the focussing gels was assessed by cutting gels into segments of 1cm, eluting the ampholines into water and measuring the pH.

Sample preparation. Samples for one dimensional electrophoresis were prepared in 2 or 5% w/v final SDS concentration. 5% SDS dissolved the samples better and less distortion by the lipids of the chromaffin granule membrane was observed at the bottom of the gels. In some experiments samples were delipidated in 1:1, v/v, acetone/ethanol before dissolving in sample buffer for the electrophoresis. For the isoelectric focussing samples were dissolved in 2% w/v Nonidet P40, 8M urea and 5% v/v β-mercaptoethanol, but not all proteins seemed to be solubilized equally well and only with membranes prepared on the same day, which had not been frozen previously, were successful results obtained. It was therefore also not possible to delipidate the samples as above because a lot of proteins would then not enter the focussing gel.

Electrophoretic transfer of proteins and detection with antibodies.

Proteins separated on gels in one or two dimensions were transferred electrophoretically onto nitrocellulose sheets (Towbin et al., 1979). Slab gels which had not been fixed previously, were put on nitrocellulose sheets (Sartorius) soaked in transfer buffer. The gel and the sheet was put between two pieces of soaked Whatman 3MM filter paper, two scouring pads and two supporting metal grids serving as electrodes. The assembly was secured with two elastic bands. An electrophoretic destaining tank was used for the transfer. With the anode facing the nitrocellulose paper, 50V (6V/cm distance between the electrodes), 1.6A were applied for 1 hour. After the transfer protein remaining on the gels could be shown on fixed and stained gels.

For the reaction of the transferred protein with antibodies, the nitrocellulose sheet was first washed for 60 min. at 37° C in BSA-buffer to saturate the filter with protein (30mg/ml BSA, 0.5mg/ml NaN3, 20mM TrisCl, pH 7.2, 0.9% NaCl, sterilized by filtering, and kept at 4°C), and then incubated for 90 min. at 37° C with the required antiserum. Serum was diluted 1:50 into horse serum buffer which was used as a carrier (25mg/ml BSA, 0.5mg/ml NaN_3, 5% v/v inactivated horse serum, 20mM TrisCl, pH 7.2, 0.9% NaCl). The nitrocellulose sheet was then washed 5 times within 30 min. in a salt buffer (20mM TrisCl, pH 7.2, 0.9%

NaCl) to wash off unbound antibody. Remaining bound antibodies were radiolabelled by incubating with (^{125}I)-labelled protein A (0.5-1.0x10^6 d.p.m./ml salt buffer) at 37° C for 60 min. Excess label was washed off with 5 changes of salt buffer within 30 min. at 37° C. The labelled nitocellulose sheet was dried in air and autoradiographed for 1 to 3 days. Antisera and radiolabelled protein A were generous gifts from Dr.D.K.Apps.

Autoradiography.

For autoradiography, dried slab gels and nitrocellulose filters were exposed to X-ray films (Curix, Agfa). In order to detect the low energy radiation of nuclides such as tritium and ^{14}C, the gels were treated with a fluorescent agent (Chamberlain, 1979). The fixed but unstained gels were washed several times with water to remove all traces of acid and then soaked in 1M sodium salicylate at room temperature for 40 min. Gels were dried as normal and exposed to X-ray films at -70° C for several weeks. To ensure good contact between the film and the dried gel, it was sandwiched between two glass plates. Coomassie blue stain was shown to interfere with the fluorography and gels were therefore only stained once autoradiographs had been obtained. Gels were resoaked in water and stained as described above. Unfortunately, soaking in salicylate, drying and resoaking etc. always had some effect on the gel size and it was therefore difficult to identify blackenings on films with stained proteins on dried gels merely by superimposing films and gels.

Gel-electrophoresis at low pH.

Some work was done to adapt a low pH gel system (Jones et al., 1981) to be used similarly as the Tris-glycine system described above. Lithium dodecyl sulphate (LiDS) was used instead of SDS because it is, in contrast to SDS, soluble at low pH. Acrylamide, bisacrylamide and polyacrylamide were used as before. The buffer in the gel was 1M citric acid, 125mM phosphoric acid and Tris, at pH 4.0 for the separating gel and at pH 3.0 for the stacking gel. For the electrophoresis buffer glutamic acid and Tris, pH 4.0 with 1% LiDS was used. Although the published concentration for glutamic acid in this buffer is 0.51M, the largest concentration which could be obtained at pH 4.0 was about 60mM. Coomassie blue stained bands of proteins separated in such a gel were not as sharp as on SDS gels and this was thought to be partly due to the rather low ionic strength of the used running buffer. There seems to be no other suitable zwitterion with a high enough solubility buffering around pH 4, and it was therefore not possible to improve the obtained results.

Organic preparation of radiolabelled ATP analogues.

Several structural analogues of ATP which were known from kinetics experiments to inhibit substrate transport into chromaffin granule ghosts were synthesized in a radioactive form to be used as affinity labels. They were

first made as unlabelled compounds in micro to millimolar amounts to investigate the steps of the synthesis, followed by the labelled compound first of low and then of very high specific radioactivity.

Dialdehyde ATP. The synthesis of open ATP (oATP), the 2',3'-dialdehyde derivative of ATP, was essentially performed as described by Easterbrook—Smith et al. (1975), and Kumar et al. (1979). It comprises a reaction in one step where the ribosyl group of the nucleotide is oxidised by periodate. 1ml of 200mM ATP, put at pH 7.0 with NaOH, was mixed with 1ml of freshly prepared 220mM $NaIO_4$ and allowed to stand in the dark at 4°C for 1 hour. To stop the oxidation, excessive periodate was neutralized by adding 12µl concentrated ethanediol (200µmol). The product was separated from the reaction mixture on a Sephadex G10 column (2x20cm) eluted with water. The eluate was monitored for its absorption at 259nm and the fractions with the highest readings were pooled, giving a total volume of 6ml. The samples were stored frozen at -20°C. Chemical purity was assessed chromatographically on DEAE paper with 0.8M ammonium hydrogen carbonate as developing buffer. Only one compound with a Rf value of 0.78 was observed under UV light (Rf for ATP was 0.69).

Radioactive dialdehyde ATP was prepared from (^3H)ATP (24Ci/mmol), 1mCi/ml in 50% ethanol. Because of a possible reaction with the ethanol an excess of periodate was used. 50µl (10µCi) (^3H)ATP (2.1nmol) were mixed with 100µl of 100mM $NaIO_4$ (10µmol), put at pH 7.0 with 4N NaOH, and

incubated in the dark on ice for 60 min. The reaction was stopped by the addition of 12µl 1M ethanediol and the mixture was separated on a Sephadex G10 column (4x100mm). Samples (1µl) of the eluated fractions were monitored for radioactivity and those containing the label were pooled. Purity was checked chromatographically as before and pieces of the chromatogram were immersed in scintillation fluid to count for radioactivity. Additionally, chromatography on polyethyleneimine sheets with 2M LiCl saturated with H_3BO_4 at room temperature as developping buffer was performed. The respective Rf values were 0.1 for dialdehyde ATP and 0.6 for ATP. With neither of the two systems could unreacted radioactive ATP be detected.

Radioactive dialdehyde ADP and —AMP were synthesized from the respective radioactive adenine nucleotide in the same way. All three products were stored in aqueous solution at -20 °C and were stable for over a year. The nucleotides retained their phosphorylation state during oxidation and storage. This was assessed by DEAE paper-chromatography with the ammonium—formate buffer described in an earlier chapter. Respective reduced and oxidized adenine nucleotides had the same Rf values.

<u>FSBA.</u> 5'-p-fluorosulphonylbenzoyladenosine was synthesized from adenosine and p-fluorosulfonyl-benzoylchloride as described by Pal et al. (1975) and by Wyatt and Colman (1977). All steps were strictly carried out in the fume cupboard. 1.142g adenosine (Boehringer, Mannheim) (4.27mmol) were dissolved in 10ml

hexamethylphosphoric triamide (Aldrich) in a 50ml quick-fit test tube by warming in a waterbath to 50°C for 30 min. The clear solution was cooled to room temperature and 1.31g p-fluorosulfonylbenzoylchloride (Aldrich) (5.88mmol) were added and shaken in intervals for 30 min. A yellow oily solution was obtained. The test tube was stoppered and left overnight (18 hours). The reaction mixture was then extracted with 30ml petroleum ether (40°-60°)

by shaking for 10 min. in a separating funnel. The two phases were separated after 30 min. The product was precipitated from the lower yellow oily layer by adding slowly 40ml ethylacetate/diethylether (1:1, v/v). The resulting white crystals were filtered on a glass sinter and dryed in an air—stream. The purity of the product was analyzed by thin layer chromatography on silica gel foils (Merck, Darmstadt) and compared with commercial FSBA (Sigma). The developing buffer was methanol/chloroform (15:85, v/v) and the running time 75 min. at room temperature. The separated substances were visualized under UV-light. Both FSBA preparations showed a major spot at an Rf value of 0.55 and a very minor one at 0.19. This is in agreement with a reported Rf for FSBA of 0.56 (Pal et al., 1975). The Rf of adenosine, run as a control, was 0.30, whereas ATP did not move from the origin. The UV — spectrum in methanol was similar to that shown by Pal et al., (1975). It had two peaks, one at 260nm (adenosine) and one at 232nm, with a shoulder at 240nm.

Radioactive FSBA was synthesized in much the same way but on a smaller scale. 1mCi (2-^{3}H)adenosine (Amersham)

(21Ci/mmol) in 1ml aqueous solution was used to which 100µg unlabelled adenosine was added as a carrier; giving it a specific activity of 2.4 Ci/mmol. This mixture was freeze-dryed and dissolved in 950µl hexamethylphosphoric triamide and reacted with 122µg p-fluorosulfonylbenzoylchloride. The product was extracted twice with a total of 3ml petroleum ether, precipitated with 4ml ethylacetate/diethylether and filtered on a small glass-fibre filter. 73% of the radioactivity was recovered. Chromatographic analysis was performed as above and traces of products identified under UV-light were scraped off the foil and suspended in scintillation fluid. This was adapted to the dark for 2 days, because the silica gel contained a fluorescent UV-indicator, before counting the radioactivity. 70% of the radioactivity applied to the chromatogram was associated with the main product (Rf 0.55), most of the rest migrated like adenosine to Rf 0.16. The presence of adenosine was not considered to interfere in labelling experiments and no further purification was performed therefore. The dryed product was dissolved in dimethylformamide (DMF) or methanol for labelling of chromaffin granule membranes.

AzidoATP. The labelling experiments descibed in the results section have been performed with commercial 8-azido-(γ-^{32}P-)ATP (NEN), but some preliminary work was done to synthesize 8-azidoATP. A synthesis in three steps has been published by Wagenvoord et al. (1977). The starting material is AMP which is brominated to 8-bromoAMP. The

bromo group is then exchanged with an azido group yielding 8-azidoAMP which in turn is phosphorylated to 8-azidoATP. A similar synthesis of 8-azidoAMP performed on a very small scale has been published by Boos et al. (1978). This method seems especially suitable to synthesize the compound radioactively starting with radioactive AMP. A more direct method is described by Schaefer et.al. (1978), in which 8-bromoATP is synthesized according to Ikehara & Useugi (1969) and the bromo group is then exchanged for an azido group with N_3H. Apart from azidoATP this method also yields azidoADP and azidoAMP. Although this method seems to be very direct, it uses N_3H which is highly explosive, and preference was therefore given to the method described by Boos et al. (1978). It was tried, however, to short—cut the synthesis by brominating ATP rather than AMP and then exchange the bromo group by an azido-group directly on the ATP molecule.

For the bromination the method of Useugi & Ikehara (1968) was followed. 100µmol disodiumATP were dissolved in 4ml 1M NaAcetate, pH 4.0, and 940µl freshly prepared bromine-water (15µl Br_2 added to 1.5ml water with vigorous stirring) were added dropwise. It is crucial for the bromination to keep the pH strictly between 3.9 and 4.0 (K.S.Boos, personal communication). The mixture was allowed to react overnight in the dark at room temperature. The resulting pale yellow colour was discharged by adding 10mg sodium — metabisulphite ($Na_2S_2O_5$). The reaction mixture was separated on a DEAE — cellulose column (2.2x40cm) equilibrated with 60mM triethylammonium-carbonate buffer, pH

7.4. The buffer was prepared from a solution of triethylamine in water and the pH was adjusted with solid CO_2 (dry ice). The column was eluted with a linear gradient of 60 to 600mM triethylammonium-carbonate, pH 7.4, and the eluate monitored spectrophotometrically at 280nm. One broad peak was obtained and absorption spectra of the fractions were measured. They had absorption maxima at 263, 265 and 259nm, at the beginning, middle and end of the peak, respectively. They correspond to a side-product, azidoATP and unreacted ATP at the beginning , middle and end of the peak respectively. The appearance of some side-product with an absorption maximum of 263nm was expected (Boos et.al. (1978), but the amount obtained here was rather large, and it was difficult to obtain a significant amount of pure material with an absorption maximum at 265nm.

For the second reaction step tetraethylammonium-azide was prepared as described by Boos et.al. (1978). Tetraethylammonium-hydroxyde and periodic acid dissolved in p-dioxane are mixed with an aqueous solution of potassiumazide. The potassium precipitates as the periodate salt and is removed from the solution. Resulting tetraethylammonium-azide is extracted from the solution by evaporation and purified by recrystalizing it several times from chloroform and then from hot acetone. In two preliminary experiments tetraethylammonium-azide was reacted with the rather impure bromoATP obtained from the above preparatrion. The mixture of products obtained after the exchange reaction was separated on the same DEAE column as above. Four products with absorption maxima between 276 and

280nm were obtained. The reported maximum for azidoATP is 282nm, and for azidoADP and azidoAMP 281nm (Wagenvoord et al., 1977). Tested in chromaffin granule transport assays, one of the products showed an inhibitory effect on serotonin uptake which was dependent on irradiation with UV-light (see results section), and might therefore have indeed contained some azidoadenine nucleotide. However, it was considered that the above synthesis steps needed a lot more refinement and that in the end it might not actually be suitable to produce a compound of a high specific radioactivity which is necessary for affinity-labelling experiments. Apart from maintaining the right pH during the bromination, which is preferentially done with a pH-stat, it seemed necessary to maintain all organic substances strictly water-free and to use only dry or redistilled solvents. A better approach for the synthesis of the radiolabelled compound might be to synthesize unlabelled azidoATP or azidoAMP and then introduce the radioactivity either with an exchange- or a phosphorylation reaction with (^{32}P)phosphate. This has furthermore the advantage that the radioactivity of ^{32}P is of a higher energy which makes it easier to pick it up on autoradiographs of radiolabelled proteins separated on gels.

RESULTS PART 1: STUDIES ON TRANSPORT ACTIVITIES OF RESEALED & DISCUSSION CHROMAFFIN GRANULE VESICLES (GHOSTS).

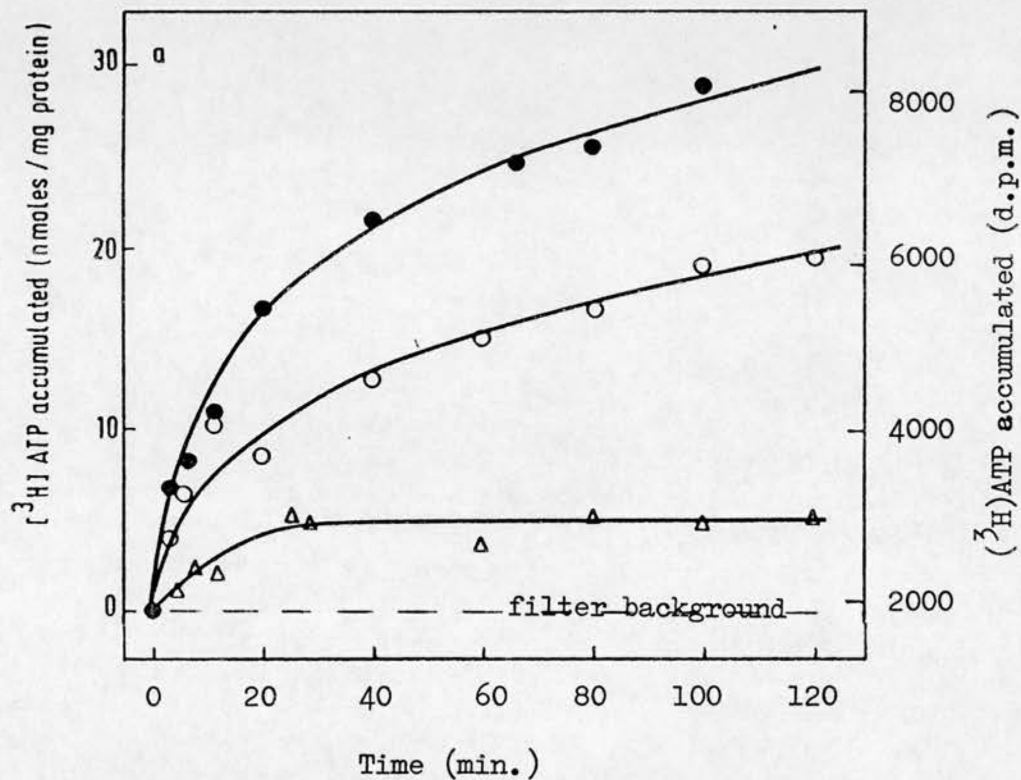
I. Characterisation of an adenine nucleotide transport.

Introduction.

Bovine chromaffin granules, the catecholamine storage vesicles isolated from the adrenal medulla, were lysed in a hypotonic buffer and resealed by restoring the osmotic pressure of the medium as described in the materials and methods section. These resealed membrane vesicles are called ghosts henceforth. In this chapter, experiments are discussed where ghosts are incubated in a buffered medium with radioactive adenine nucleotides; samples are withdrawn at variable intervals during the incubation to give time courses of adenine nucleotide uptake into ghosts. The incubation is stopped by diluting the withdrawn sample into a quenching medium; the diluted sample is then filtered on nitrocellulose filters which retain the ghosts. The amount of adenine nucleotide associated with the ghosts is measured by counting the filters for radioactivity. In initial interpretations of the experiments, all the radioactivity which is associated with the ghosts is referred to as uptake, and no distinction is made between adenine nucleotides bound to the ghost membrane or transported through the membrane.

ATP is taken up into chromaffin granule ghosts.

When chromaffin granule ghosts are incubated in a medium containing (^{3}H)ATP, a time dependent uptake of radioactivity into the ghosts is observed. Fig.1 shows the uptake of ATP into ghosts incubated at 37° C and 0° C. To start the incubation ghosts were added to a medium to give a final protein concentration of 0.5mg/ml. Additionally to the ghosts the incubation medium contained 5mM (^{3}H)ATP (2mCi/mmol), 1mM $MgSO_4$, 10mM KCl, 30mM NaHepes (pH 7.2) and 0.3M sucrose. Samples of 100µl were withdrawn at the time-points indicated in Fig.1 and diluted into a quench solution made up of 4ml 10mM NaPyrophosphate and 10mM NaHepes (pH 7.2) in 0.3M sucrose. The quenched ghost suspensions were filtered immediately on presoaked cellulose-nitrate filters and the radioactivity associated with the filter-trapped ghosts was counted. For further details of the filter-assay see the Materials and Methods section. To measure unspecific binding of radioactivity to the filters, equal amounts of incubation mixture but without added ghosts were filtered and counted. In this way a constant background of 1800 to 2000 (^{3}H) d.p.m. was measured which is also shown in Fig.1 but was subtracted to calculate the uptake in nmol ATP/mg protein in the experiments unless otherwise stated. The radioactivity of 10µl unfiltered incubation medium was counted as a standard. From this the amount of ATP represented by the radioactivity was calculated.



<u>Fig.1:</u> Uptake of (^3H)ATP by chromaffin granule ghosts.
Ghosts were incubated in the standard medium either at 37°C (•, o) or at 0°C (△). One incubation medium was supplemented with 10mM PEP and pyruvate kinase (2 units/ml) (o).

As seen in Fig.1, ATP is taken up at 37° C in a time-dependent process . During the time span monitored the ATP uptake curve does not reach a plateau. The total amount of ATP taken up within 100 min. at 37° C is 30nmol ATP/mg ghost protein, which is equivalent to a concentration of 7.5mM ATP inside the ghosts at a concentration in the medium of 5mM. For this calculation an internal ghost volume of 4μl/mg protein is assumed as reported by Phillips & Allison (1978). This result suggests that ATP finds its way into the chromaffin granule vesicles but is not accumulated to any great extent therein. Much of this thesis deals with the clarification and interpretation of these two initial observations.

To show that ATP is taken up by vesicles formed from chromaffin granules, ghosts incubated as above were analysed on a sucrose density gradient. After 2 hours' incubation at 37°C, 800μl of the incubation medium was run down a Sephadex G25 (medium) column (1.0x10cm) equilibrated with Hepes-buffered 0.3M sucrose to separate the loaded ghosts from remaining free radioactive ATP in the incubation medium. The ghosts were collected in 1ml elution buffer, applied to a linear 0.4-1.5M sucrose gradient (volume of gradient: 11.6ml), and centrifuged to equilibrium in a Beckman SW41 rotor at 41,000 r.p.m. (200,000xg) for 3 hours.

The gradient was fractionated in portions of 240μl (10 drops) from the bottom and samples were counted for (^{3}H)radioactivity, i.e. (^{3}H)ATP uptake. 50μl of every

fraction was assayed for Dopamine-beta-hydroxylase (DBH), a chromaffin granule membrane marker. The DBH assay method was adapted from Kaufmann & Friedman (1965) and is based on the hydroxylation of (^{14}C)tyramine by the DBH. The radioactive octopamine produced is then oxidized to p-hydroxy-benzaldehyde which, in turn, is extracted into toluene and counted for (^{14}C)radioactivity.

Fig.2 shows the comigration of ATP with the chromaffin granule membrane marker DBH (ATP-concentration and DBH-activity are given in arbitrary units). The second ATP peak at lower sucrose densities is probably due to free ATP which was either contaminating the eluate after the sephadex column step, or had leaked out of the ghosts when they were subjected to high sucrose densities on the gradient. Although the Sephadex G25 column retained most free ATP, a total separation into free ATP and loaded ghosts could not be obtained with the sample- and column-sizes used in the experiment. A longer column would have given a better separation, but the eluted ghosts are then in a bigger volume for which there is no room on the gradient. Additionally, the ghosts might shrink somewhat in the higher sucrose densities further down in the gradient and therefore release some of the internal ATP. In later experiments, samples fractonated from the gradients were filtered and only filter-retained radioactivity was counted which lead to a disappearance of this (^{3}H)ATP peak at lower sucrose densities. Sucrose densities were calculated from refractive index of fractions from a second gradient run in parallel.

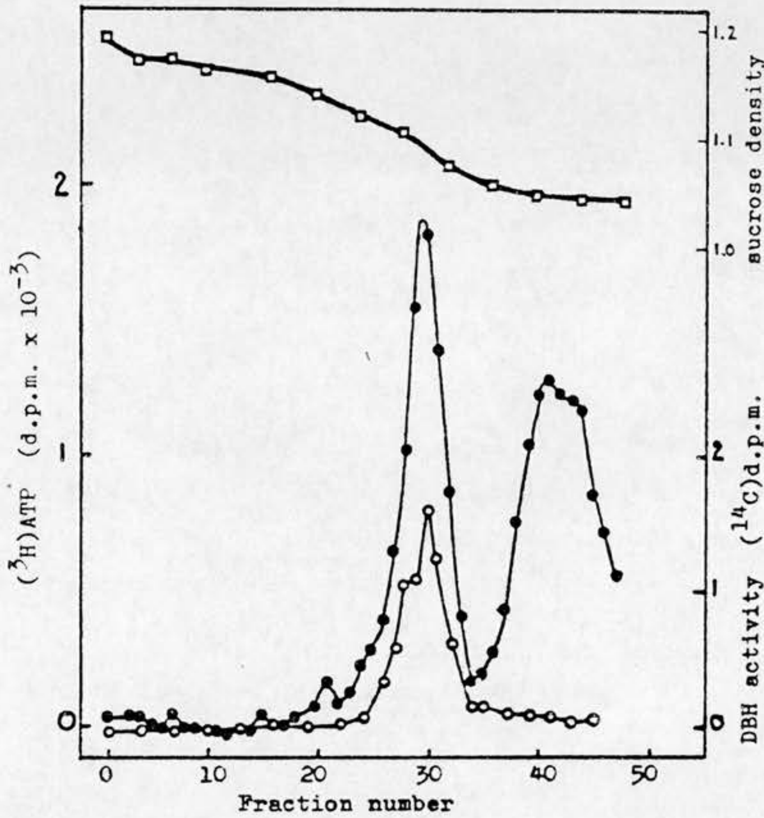


Fig.2: Separation of ghosts incubated with (^{3}H)ATP at 37°C for 2 hours on a sucrose density gradient. Portions of the fractionated gradient were counted for (^{3}H)ATP (•) or assayed for the ghost marker protein DBH (o). Sucrose concentrations were determined from a gradient run in parallel (□).

Hydrolysis of ATP during incubation.

In the uptake experiment above ATP is present in the incubation mixture at a concentration of 5mM. Due to the ATPase activity of the ghosts some of this ATP will become hydrolysed during the incubation at 37°C. To measure the extent of this hydrolysis, samples of 5µl were withdrawn from the incubation medium at various time points and analysed for their content of ATP, ADP and AMP. The mixtures of adenine nucleotides were separated by chromatography on DEAE-paper and analysed for radioactivity. In Fig.3 the time course of the hydrolysis of ATP into ADP and AMP is shown for such an experiment. Adenine nucleotide levels are given as percent of total radioactivity recovered from the chromatrograms.

The rate at which ATP is hydrolyzed is fast compared with the 2 hours during which ATP uptake was monitored: In less than 30 min. after the start of the incubation only half of the initial amount of ATP was left. This means that the ATP uptake is a process which uses a substrate of quickly changing concentration. A further complication is the appearance of ADP and AMP in significant amounts which might interfere with the uptake of ATP. Since the uptake of ATP as seen in Fig.1 is a slow process, which cannot easily be observed over the very short time period during which the concentration of ATP could assumed to be constant, effects of ADP and AMP on the uptake mechanism had to be taken into consideration.

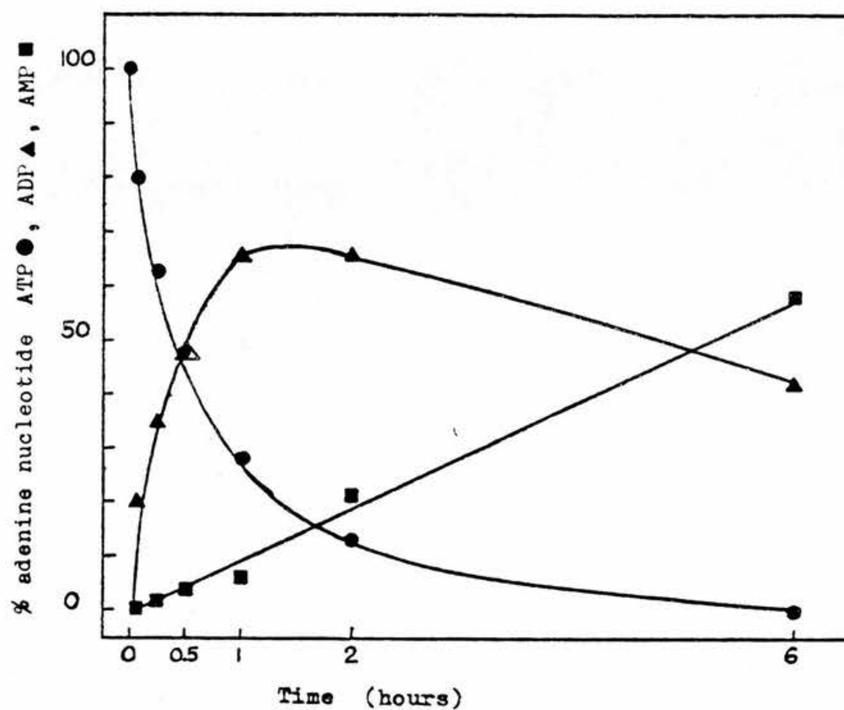


Fig.3: Hydrolysis of ATP in the incubation medium. Samples of the incubation medium at 37°C were analysed on DEAE paper-chromatography for their respective content of ATP (●), ADP (▲) and AMP (■).

ADP and AMP are also incorporated by the chromaffin granule ghosts.

Since ADP appears very quickly and in considerable amounts in the incubation medium during an uptake experiment, it is useful to know whether ADP itself is taken up itself. In an experiment similar to that described above, all ATP was replaced by ADP (Fig.4). Because of the absence of ATP, the ATPase was without a substate, therefore $MgSO_4$, an obligatory cofactor for the ATPase, was omitted as well.

In a second experiment 5mM EDTA was additionally added to the medium. EDTA, by complexing any trace amounts of Mg^{2+} should completely inhibit the ATPase. Analyses as in Fig.3 of adenine nucleotide levels in ghost suspensions incubated at 37°C with ATP, ADP or AMP in presence of EDTA showed that after 2 hours the nucleotides were still in the same phosphorylation state, ATP: 96%, ADP: 92% and AMP: 98%, and not hydrolysed as seen in Fig.3. In a third experiment the adenylate kinase inhibitor di(adenosine-5')pentaphosphate (AP5A) was present to prevent the formation of ATP from ADP and therefore to ensure that the measured radioactivity was ADP and not ATP.

All three experiments gave a similar uptake pattern which showed that ADP is taken up into the ghosts. The observed uptake must obviously be independent of the ATPase and was therefore interpreted as an unenergized uptake of ADP into ghosts. In Fig.5 batches of ghosts from a same

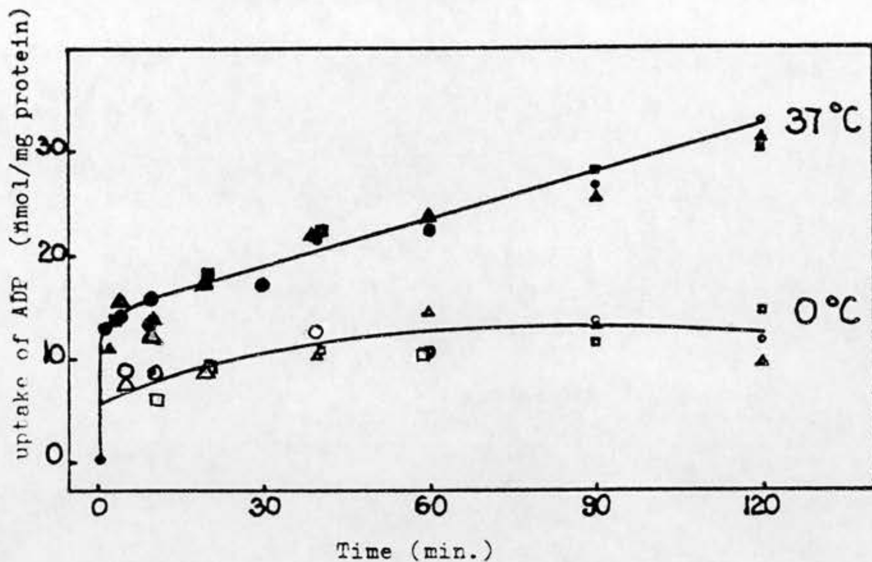


Fig.4: Uptake of (^3H)ADP into ghosts at 37°C (filled symbols) and 0°C (open symbols). No $MgSO_4$ was present and the following additions were made: 2.5mM EDTA (■,□); 100μM AP5A (▲,△); control (●,o).

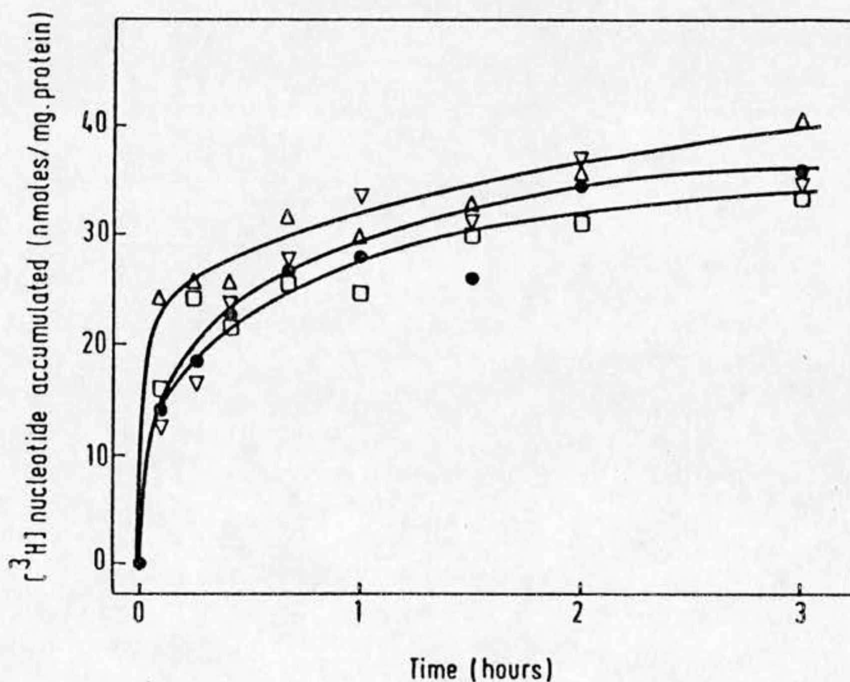


Fig.5: Uptake of (^3H) nucleotides by ghosts. Ghosts were incubated in standard uptake media containing 5mM (^3H)ATP, 5mM EDTA and no $MgSO_4$ (●); 5mM (^3H)ATP and 2.5 mM $MgSO_4$ (▽); 5mM (^3H)ADP and 5mM EDTA (△) or 5mM (^3H)AMP and 5mM EDTA (□).

preparation were incubated with ATP, ADP or AMP, and a similar uptake was observed for all three adenine nucleotides. The extent of uptake of adenine nucleotides normally varied somewhat between the different preparations of ghosts. Therefore in any one experiment only ghost batches from the same preparations were used. Fig.5 also shows how difficult it is to obtain defined curves from a series of measured points which scatter considerably. Therefore, in order not to over- interprete experiments at that stage, it was concluded that ATP, ADP and AMP are taken up into chromaffin granule ghosts in a largely similar pattern and that the shape of the uptake curves varies considerably between the ghost preparations.

The observation that the uptake does not distinguish between the different adenine nucleotides and that no difference in uptake of ATP is observed in presence of $MgSO_4$ or EDTA suggests that the adenine nucleotides are transported through the membrane in an unenergized process (i.e. one that is not dependent on ATP hydrolysis). This implies that these nucleotides merely penetrate the membrane until a concentration equilibrium across the membrane is reached. This agrees with the observation that in none of the presented experiments is the concentration of accumulated adenine nucleotide greater than twice the outside concentrations. This is in sharp contrast with the transport of serotonin across the membrane, where a gradient of up to 20,000 is obtained with ghosts prepared in the same way (Phillips & Apps, 1981), and with experiments presented in the next section.

Introduction of an ATP-regenerating system.

Since the main aim of this study was to find a mechanism by which ATP is accumulated in chromaffin vesicles, to give the high concentrations of internal ATP which are measured in intact granules (Winkler & Westhead, 1980), the interest was to characterise the uptake of ATP, rather than that of ADP or AMP. Therefore an ATP regenerating system was introduced, to keep the ATP concentration constant at its initial level. Furthermore, an uptake mechanism which is dependent on the ATPase requires a constant amount of ATP in order to avoid artefacts caused by a variable degree of energization.

The incubation medium as described in Fig.1 was supplemented with 10mM phosphoenolpyruvate (PEP) and 2 to 4U/ml pyruvate kinase (PK). In a coupled reaction pyruvate kinase transfers the high energy phosphate group from PEP to ADP giving ATP and pyruvate as products. The effect of the ATP regenerating system is seen in Fig.6: Adenine nucleotide levels were measured after 2 hours of incubation at 37°C. Normally MgATP is hydrolyzed to ADP and AMP as discussed before, but this can be prevented if $MgSO_4$ is replaced by EDTA. If PEP and PK are added to the medium containing $MgSO_4$, the ATP concentration remains constant over 2 hours. When only PEP but no PK was added, the amount of ATP hydrolyzed was smaller than in the absence of PEP. This effect was dependent on Mg^{2+} and is probably due to contaminating pyruvate kinase activity.

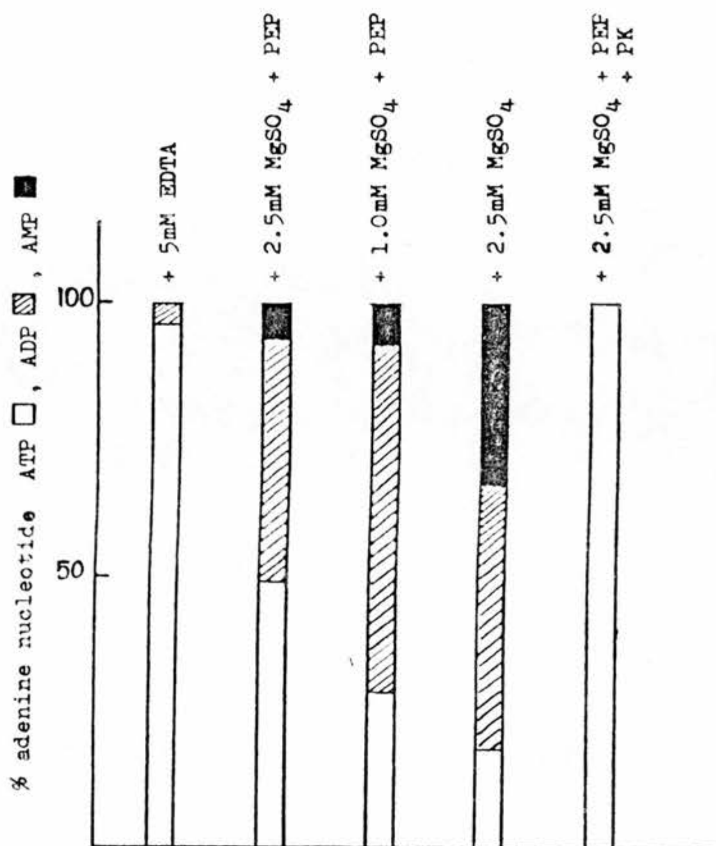


Fig.6: Adenine nucleotide composition of various media after 2 hours at 37°C. Ghosts were incubated with (^{3}H)ATP at 37°C in media as described for Fig.1, $MgSO_4$, EDTA, PEP and pyruvate kinase were only added where indicated. After 2 hours incubation the adenine nucleotide composition in the media was analysed on DEAE paper-chromatography. ATP (□), ADP (▨) and AMP (■) contents are given in % of total recovery of radioactivity.

The ATP regenerating system (10mM PEP, 2U/ml PK) was added to the incubation medium and the uptake of ATP was measured as described and shown in Fig.1. ATP remained triphosphorylated for the whole time period monitored. Somewhat surprisingly, the ATP uptake was inhibited in extent and initial rate of uptake when measured at 37° C. One explanation was that when ATP is hydrolyzed, products, ADP and AMP, are formed of which one might be taken up more efficiently than ATP. But as discussed above (Fig.5), it seems as if all the adenine nucleotides are taken up much to the same extent.

PEP, PK and glycerol (since pyruvate kinase was supplied as a solution in 50% glycerol), were tested independently for their inhibitory effect on the ATP uptake. Of the three substances only PEP proved to be inhibitory. Subsequent uptake experiments with radioactive PEP showed that PEP is itself taken up, and it seems likely that it therefore inhibits ATP uptake competitively.

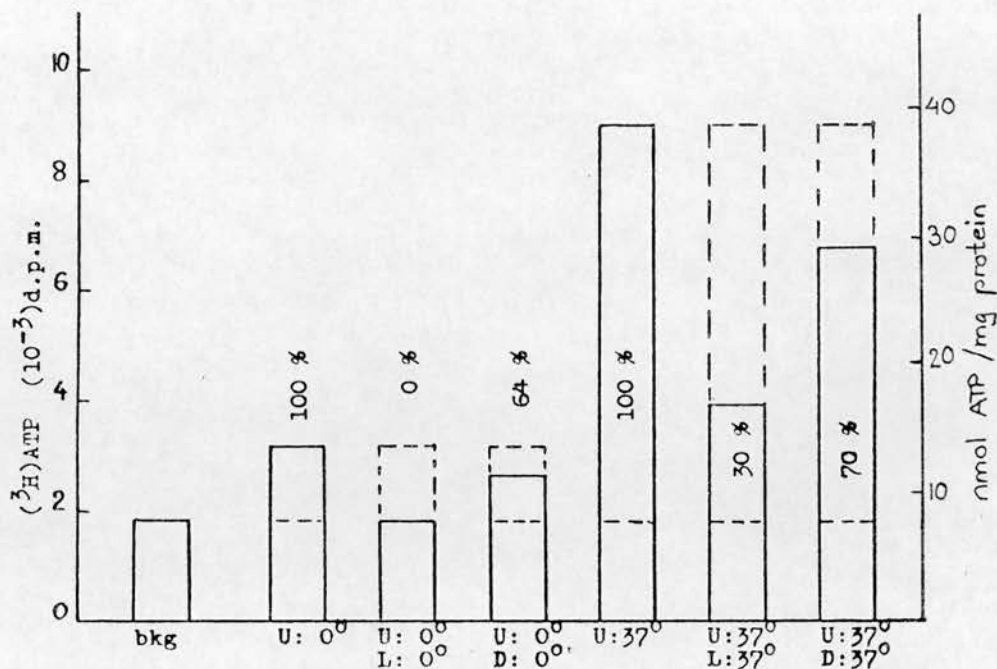
State of accumulated adenine nucleotides.

As outlined above, the concentration of adenine nucleotides inside the ghosts, after 2 hours incubation at 37° C, approximately equals the concentration in the surrounding medium. It could be argued that this observed uptake merely represents binding of ATP to the ghosts. One way of distinguishing between ATP bound to the membrane and ATP free within the ghost matrix, is to subject loaded ghosts to lysis in a hypotonic medium. In the absence of an

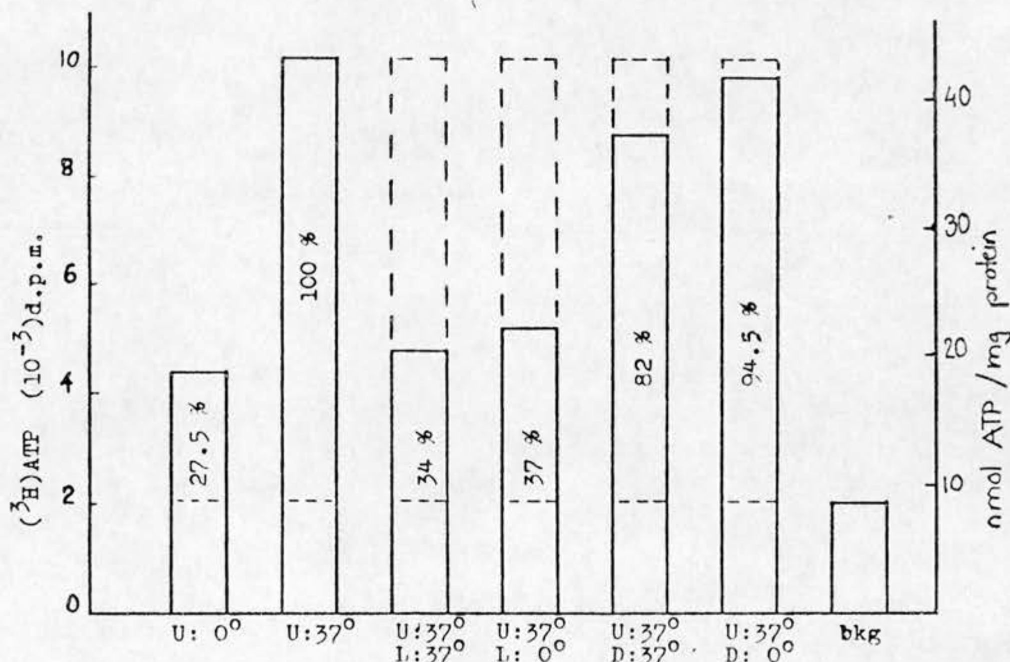
isoosmotic buffer water quickly penetrates through the membrane which makes the ghosts swell until they lyse and release the components taken up into the vesicle.

Ghosts were loaded with (^{3}H)ATP for 45 or 60 min. at 37° or 0°C. Samples of 100µl were lysed in 10mM NaHepes, pH 7.2, which contained no sucrose as osmotic stabiliser, or were diluted 20-fold into isotonic buffer, 10mM NaHepes, pH 7.2, in 0.3M sucrose. Additional samples were diluted into isotonic buffer and filtered directly to determine the total uptake of ATP. The lysed and diluted samples were further incubated at 37° and 0°C for 25 to 30 min. Before filtering, sucrose was added to the lysed samples to give the same final concentration as in the control experiments.

Fig.7 shows the results in d.p.m. of (^{3}H)ATP retained on the filters. 65 – 70% of the ATP taken up at 37°C is released with lysis of the ghosts. By contrast, 20-30% of the ATP is released from the ghosts when they are diluted and further incubated at 37°C in isotonic buffer, presumably by slow reversal of the uptake process. Further incubation of ghosts loaded at 37°C in isotonic buffer at 0° C retains 90% of the ATP. Radioactivity associated with the ghosts at 0°C is completely sensitive to lysis, suggesting that it represents accumulation into the ghost matrix and not binding to the membrane. In an experiment not shown, lysis in presence of 0.1% Triton X-100 removed all counts associated with ghosts under any conditions to background levels.



Experiment 2: 45 min. uptake (U); 25 min. further incubation: dilution (D) or lysis (L);



Experiment 1: 60 min. uptake (U); 30 min. further incubation: dilution (D) or lysis (L).

Fig.7: Dilution and lysis of ghosts incubated with (^3H)ATP. Ghosts were incubated and further treated as described in the text. The two experiments shown were performed independently with ghosts from two different preparations. Percentages of ATP taken up and retained are calculated from (^3H) d.p.m. after subtraction of the amount of label unspecifically bound to the filters (= bkg).

A time-course experiment showed that the proportion of ATP which is sensitive to lysis varies from the over-all uptake with time (Fig.8). ATP taken up in the first 2 to 5 min. is rather insensitive to lysis, suggesting that a binding process is occuring. Between 5 min and 3 hours (this upper time limit varied in different experiments), when the uptake reached a plateau level, most of the adenine nucleotide was sensitive to lysis. The amount which was insensitive to lysis increased linearly from 2 min. to 3 hours and continued thereafter at the same rate at the expense of lysis-sensitive adenine nucleotide. No regenerating system was present in this experiment so that the nucleotides accumulated varied in composition through this experiment.

The dilution experiments in Fig.7 suggest that ATP once loaded into ghosts is not irreversibly trapped there, but is able to leak out slowly again, which means that an uptake curve as shown in Fig.8 describes a sum of two events, uptake of ATP into the ghosts and leakage of accumulated ATP from the ghost matrix back into the medium. The plateau of net ATP uptake observed after 3 hours would then represent a dynamic process of uptake and leakage in which the rate of influx equals the rate of efflux. This is more clearly seen in Fig.9. An incubation medium which contains 5mM AMP, 2.5mM $MgSO_4$, 10mM KCl, 10mM NaHepes, pH 7.2, in 0.3M sucrose and ghosts at a protein concentration of 0.5mg/ml is split into three portions. A trace amount of (^{3}H)AMP is added at the start of the incubation at 37°C to one portion; the two other batches receive the same amount of radioactive tracer

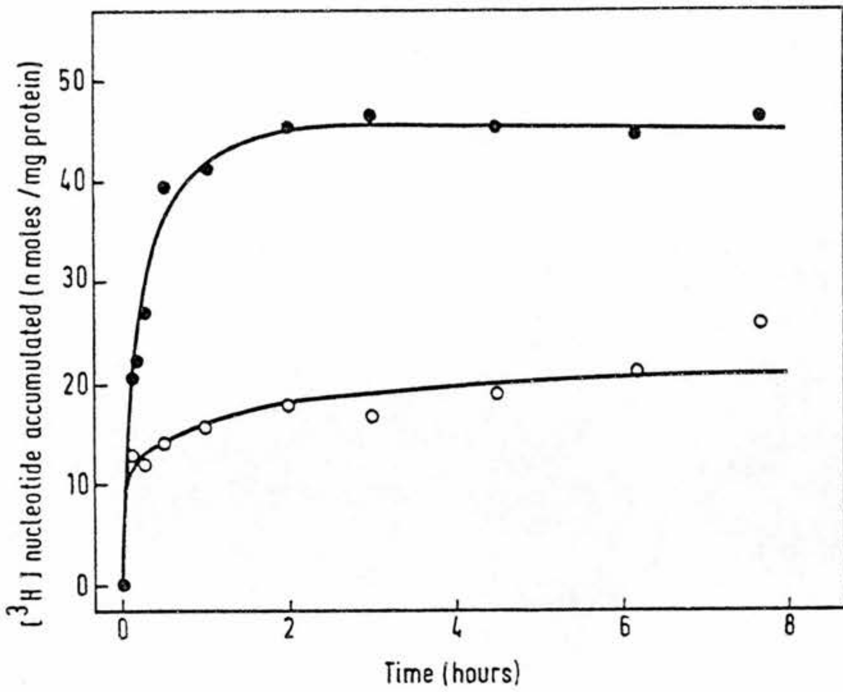


Fig.8: Release of accumulated (^{3}H)ATP by hypotonic lysis. Ghosts were incubated in a standard medium and samples were either diluted into isotonic buffered sucrose before filtration (●) or were first subjected to lysis in buffer lacking sucrose (o).

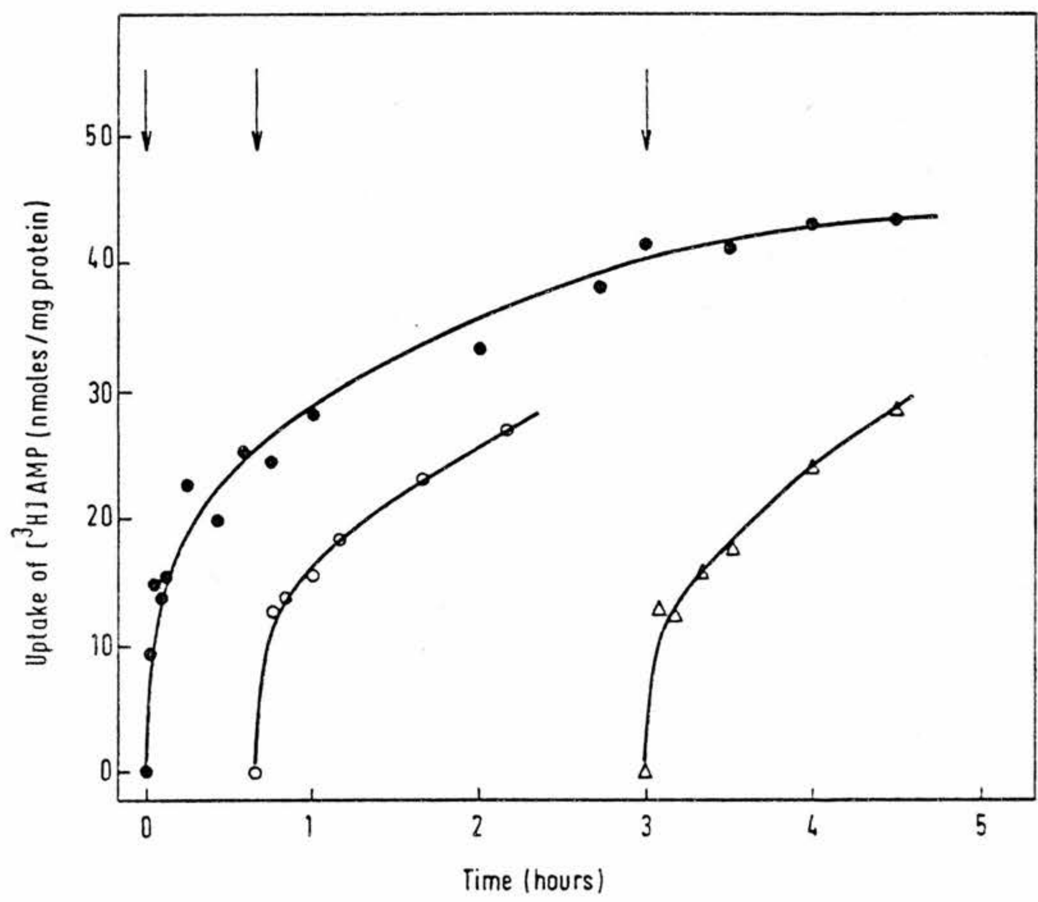


Fig.9: Exchange of (^{3}H)AMP across the ghost membrane. Ghosts were incubated with AMP and uptake of (^{3}H)AMP was monitored after the addition of the radioactivity to parallel incubations at 0 min. (●), 40 min. (o) or 180 min. (△) after the start of incubation.

40 min. or 3 hours after the start of the incubation. Although the rate of AMP uptake has slowed down markedly after 3 hours, newly added radioactivity equilibrates quickly across the membrane at a rate which is at least six times bigger than the rate of total AMP uptake at that time. The shape of the curve of this quick equilibration resembles more the shape of the AMP uptake during the first hour. Since there is a rapid uptake there must also be a rapid efflux, so that the curve at 3 hours represents an equilibrium across the membrane. This may explain why the adenine nucleotides do not appear to be concentrated inside the ghosts.

AMP was chosen for this experiment because it is not further hydrolysed by the ghosts and therefore all radioactivity measured represented the monophosphate. In an experiment to analyse the nature of the radioactivity actually taken up by the ghosts, it was shown that it all represented AMP (see below).

Analysis of adenine nucleotides loaded into the ghosts.

The original observation, that ATP is taken up by the ghosts and at the same time is hydrolyzed to ADP and AMP, led to the observation that all three adenine nucleotides can be taken up by ghosts. The phosphorylation state of the adenine nucleotides was assessed in the incubation medium as described previously (Fig.3). It remained unclear in what phosphorylation state the adenine nucleotides are stored within the ghosts and whether the composition of the

incubation medium reflected the composition of the internalised nucleotides. To clarify this, experiments were performed in which the adenine nucleotides in the lysate from loaded ghosts were analysed.

After incubation with ATP, ADP or AMP, batches of ghosts were separated from the medium at 4°C on small Sephadex G50 columns (0.4x8cm) as described before. Samples of the eluate containing the ghosts (identified by their turbidity) were measured for their content of free and filter-retained radioactivity. 50% to 70% of the radioacivity could normally be trapped on filters. 600-800µl of eluate containing the ghosts (ca. 250µg) were lysed in 25ml water for 10 min. on ice. The lysed ghosts were centrifuged; the lysate was freeze dried, resuspended in water, and about 100µl were analysed by chromatography on DEAE paper. The pellet was counted for radioactivity to measure the amount of label retained by the membranes after lysis in water (normally about 15%).

It was assumed that the composition of adenine nucleotides resolved by chromatography reflected the situation within the ghosts at the time of lysis. The counting efficiency for radioactivity on the DEAE paper was only about 10% and therefore the main problem was to apply enough radioactivity on the chromatograms to obtain significant results.

Fig.10a shows the analysis of a lysate from ghosts incubated with ATP, at twice the normal specific radioactivity, for 95 min. in presence of PEP and pyruvate

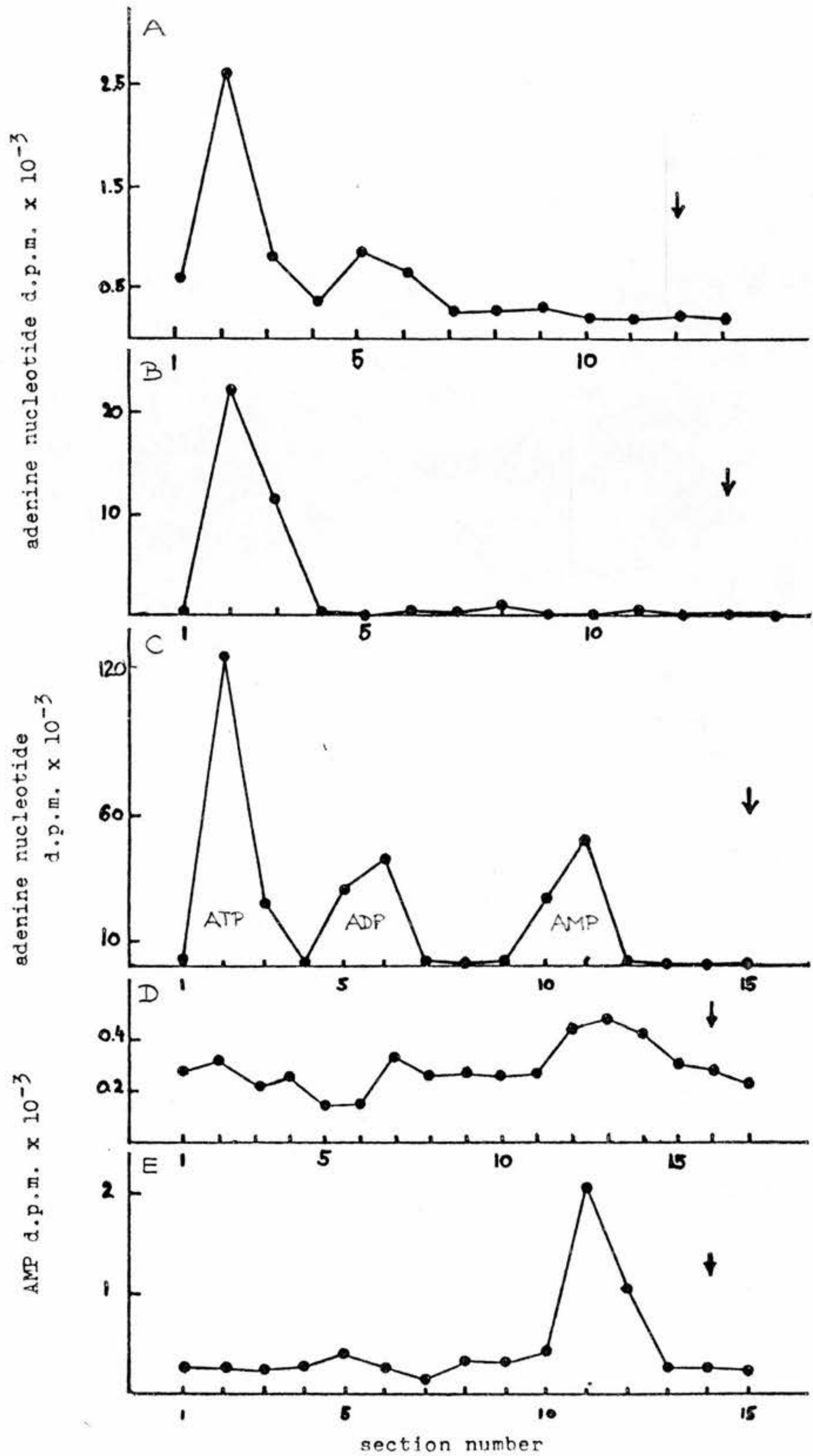


Fig.10 : Nature of adenine nucleotides inside the ghosts:

a. analysis of a ghost lysate after incubation with (^{3}H)ATP in presence of PEP and pyruvate kinase at 37°C for 95 min.

b. analysis of the external medium in a.

c. control chromatography (mixture of (^{3}H)ATP, (^{3}H)ADP and (^{3}H)AMP

d. analysis of a ghost lysate after incubation with (^{3}H)AMP in presence of EDTA at 37°C after 110 min.

e. analysis of the external medium in d.

kinase. 67% of the label in the lysate is ATP which indicates that the ghosts take up at least some of the radioactivity as ATP. The analysis of the medium shows that in presence of PEP and pyruvate kinase no ADP is found at 95 min. The finding of ADP in the lysate might be due to hydrolysis during the preparation of the sample. In Fig.10d the lysate of ghosts from the experiment described in Fig.9 is analysed. Although the amount of label is rather small, it seems to indicate that all label within the ghosts at 90 min. is AMP, as is the case for the label in the medium (Fig.10e).

In a somewhat different experiment, ghosts were loaded for 60 min. in an ordinary medium with (^{3}H)ATP but no PEP and pyruvate kinase. At the end of the incubation 500µl of the medium was diluted into 15ml of an ice-cold buffer containing 30mM Hepes, pH 7.2, 0.3M sucrose, 5mM ATP, 10µM 4-chloro-7-nitro — benzofurazan (Nbf-Cl), 2mM EDTA; the latter compounds inhibit the ATPase of the membrane, and the dilution prevents further uptake of labelled ATP. The diluted ghosts were centrifuged, 30 min. at 35,000xg at 4° C, and then lysed by resuspension in 100µl 10mM Hepes, pH 7.2, containing 10µM Nbf-Cl and 2mM EDTA, but no sucrose. After standing on ice for 30 min., while mixing occasionally on a vortex mixer, the lysed ghosts were centrifuged as above and the 100µl lysate was analysed chromatographically as above.

The adenine nucleotides of the lysate consisted of 60% ATP and 40% ADP, whereas no AMP was detected. The

composition of the medium after 60 min. incubation however, was 44% ATP, 51% ADP and 5% AMP. Although this result is somewhat ambiguous, it shows that some ATP is trapped inside the ghosts which is not hydrolyzed to the same extent as the ATP in the medium within 60 min. The experiment in Fig.9 suggests that, if left long enough, the composition of adenine nucleotides inside the ghosts will come to equal the composition in the medium. This equilibrium might not be achieved after 60 min. in the above experiment.

Generally, it can be concluded that ghosts take up adenine nucleotides in their existing form, and that the putative carrier in the membrane seems to catalyse an exchange process in addition to a net uptake. Only marginal concentration gradients are achieved (maximally 2-fold); this cannot be attributed to 'leaky' ghosts because they accumulate high concentrations of both serotonin and protons (Phillips & Apps, 1980).

II. Comparison of the adenine nucleotide uptake mechanism with the transport of serotonin into chromaffin granule ghosts.

Introduction.

Chromaffin granule ghosts have been extensively used as a model system for the mechanism of catecholamine uptake into intact chromaffin granules. The proton pumping activity of the ATPase has been linked with the energisation of the catecholamine uptake via a proton electrochemical gradient. According to a current model one catecholamine molecule is exchanged for two protons when transported across the ghost membrane (Phillips and Apps, 1980). Obviously it is of considerable interest to compare such a well-defined transport mechanism with the ATP uptake discussed so far. Furthermore, the uptake of catecholamines into ghosts, when monitored concomittantly with the uptake of ATP, can serve as a control for the intactness of the ghosts and for the state of the proton and potential gradients.

For many studies of catecholamine accumulation into chromaffin granule ghosts another biogenic amine, serotonin (5-hydroxytryptamine), which is endogenously present in the brain and in serotonin vesicles in blood platelets, has been used instead of adrenaline or noradrenaline. It has greater chemical stability, and although it is not a natural substrate in the adrenal medulla, it has a high affinity for

the uptake mechanism of chromaffin granules (Phillips, 1974b).

Uptake of ATP and serotonin into chromaffin granule ghosts.

To the standard mixture, as introduced in Fig.1: 5mM (^{3}H)ATP (10μCi/ml), 1 or 2.5mM KCl, 10mM NaHepes, pH 7.2, in 0.3M sucrose with 0.5mg/ml ghost protein, 25μM (^{14}C)serotonin (10-50nCi/ml) was added in dual-isotope experiments to monitor ATP and serotonin uptake at the same time. This incubation mixture was used for all experiments, unless otherwise stated. In some experiments PEP and pyruvate kinase were added to ensure a constant ATP concentration (although one must bear in mind that PEP inhibits the uptake of ATP slightly, as discussed above).

A comparison of serotonin and ATP uptake is seen in Fig.11. The two time courses are clearly different in shape and extent of uptake. Serotonin is taken up quickly, in a process with a high initial rate; the concentration gradient reaches a plateau after one hour and begins then to leak out as the pH gradient decays, since there is no ATP regenerating system present. The amount of serotonin accumulated at 60 min. is 16.5nmol serotonin/mg protein, which represents a concentration of 4.1mM within the ghosts, equivalent to a 180 fold concentration gradient. The ATP uptake is comparatively slow apart from the rapid event within the first few minutes, and continues steadily over 2 hours, after which 40.5nmol adenine nucleotide/mg protein are accumulated. This equals a concentration of 10.1mM

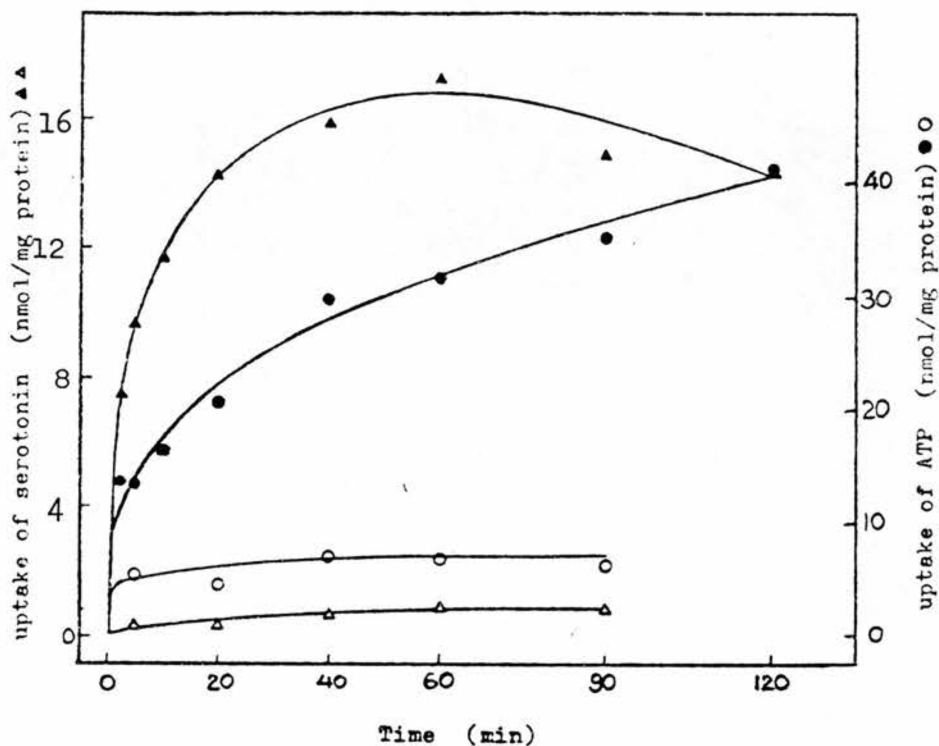


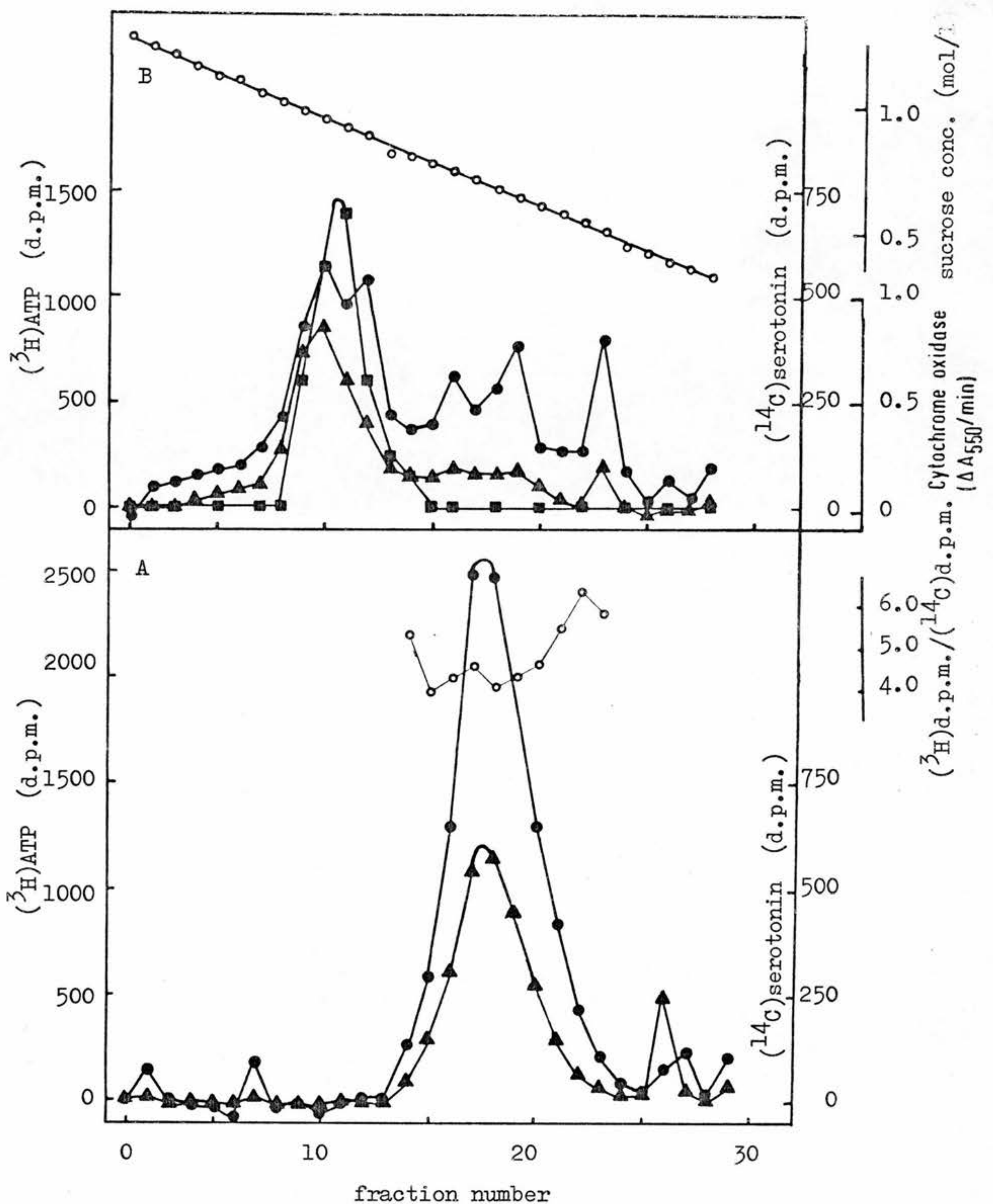
Fig.11: Dual isotope experiment. Uptake of (^{3}H)ATP (5mM) (•,o) and (^{14}C)serotonin (25µM) (▲,△) into ghosts in a standard incubation medium at 37°C (closed symbols) and 0°C (open symbols).

inside the ghosts, which represents only a two fold concentration of adenine nuicleotide across the ghost membrane.

Adenine nucleotides and serotonin are taken up into the same vesicles.

In Fig.2 it was shown that ATP loaded into ghosts comigrates with the ghost membrane marker dopamine β-hydroxylase on sucrose gradients. Dual label experiments allow one to show directly that ATP is taken up by the chromaffin granule membrane vesicles. Ghosts (1mg/ml) were incubated for 2 hours at 37° C in a standard incubation medium which contained PEP and pyruvate kinase. 500µl of the medium was then applied to a Sephadex G50 column (0.7x12cm) to separate the ghosts from free medium components. The eluted ghosts in 800µl 10mM Hepes pH 7.2, 0.3M sucrose were analysed on a sucrose density gradient as described before. The gradient was fractionated by pumping off portions of 400µl (15 drops). 250µl of each portion were diluted into 4ml 10mM NaHepes (pH 7.2) in 0.3M sucrose and filtered as for uptake assays. All these steps were performed at 4° C. In a parallel experiment a sample of mitochondria from adrenal medullary cells (1mg/ml) was labelled and fractionated in the same way.

In Fig.12a the two comigrating peaks of ATP and serotonin are shown. The ratios of the two radioactivities, corrected for their respective backgrounds, are calculated for each fraction of the peaks. These ratios vary only



<u>Fig.12a:</u> Analysis of ghosts incubated with (^{3}H)ATP and (^{14}C)serotonin on a sucrose density gradient. Samples of gradient fractions (250µl) were filtered and assayed for (^{3}H)ATP (●) and (^{14}C)serotonin (▲), the ratios of the two radioactivities are calculated for the peak area (o).
<u>Fig.12b:</u> Similar analysis of mitochondria incubated with (^{3}H)ATP (●) and (^{14}C)serotonin (▲). Samples of 60µl were assayed for cytochrome oxidase activity (■). Sucrose concentrations were measured with a refractometer (O).

little within the peak, an indication that both radioactivities are distributed in the same way, and that therefore ATP is taken up into the same vesicles as serotonin. Because the fractions from the gradient were filtered before they were counted, only radioactivity associated with the vesicles was measured and no second peak of soluble radioactivity, as seenin Fig.2, was observed.

The distribution of the radioactivity associated with the mitochondria on a sucrose gradient is shown in Fig.12b. 60ul of the fractions from this gradient were assayed for cytochrome oxidase activity, a marker of the mitochondrial inner membrane. The distribution of this activity showed that mitochondria migrate to a density equal to 0.95M sucrose, which is about two thirds down the gradient. Their position is clearly different from that of chromaffin granule ghosts at sucrose densities equal to 0.72M, about one third down the gradient. ATP as well as serotonin is associated with the mitochondria. The mitochondria were isolated from the preparation of chromaffin granule ghosts after the lysis step and are therefore probably mostly present as submitochondrial particles. This might explain why serotonin is present at densities typical for mitochondria, since serotonin is likely to accumulate inside the submitochondrial particles in exchange for the proton gradient established by the mitochondrial ATPase.

Portions of the gradient on which the ghosts were separated were analyzed for cytochrome oxidase as well but no activity was found. Similarly, there is also no

serotonin or ATP radioactivity found at densities to which mitochondria are expected to migrate. It is concluded therefore, that the uptake of ATP observed so far, represents uptake into the chromaffin granule ghosts and does not stem from contaminating mitochondria.

If ADP and AMP are also taken up by ghosts, as shown in Fig.5, it is of interest to know whether these nucleotides comigrate with serotonin as well. Ghosts were incubated in a dual labelling medium in which (^{3}H)ADP or (^{3}H)AMP was present instead of (^{3}H)ATP, and $MgSO_4$ was replaced by 2mM EDTA. Under these conditions however, no serotonin is taken up by the ghosts. Therefore, after an incubation for 42 min. at 37°C, a pulse of unlabelled 5mM MgATP was given to energize the ghosts for 10 min. and to enable them to take up enough serotonin, to be seen in sucrose gradient fractions, but no ATP. The inset in Fig.13 shows the kinetics of this uptake for ADP. The loaded ghosts were then analysed on sucrose gradients as descibed above and comigration of ADP, Fig.13a, and of AMP, Fig.13b, with serotonin was shown. This indicates that these two nucleotides are also taken up by the same vesicles as take up serotonin.

The effects of $MgSO_4$, Hepes and KCl on ATP uptake.

The observation that all three adenine nucleotides are equally well taken up into chromaffin granules, and that EDTA had no inhibitory effect on this uptake, questioned the necessity of Mg^{++} in the incubation medium. So far, $MgSO_4$

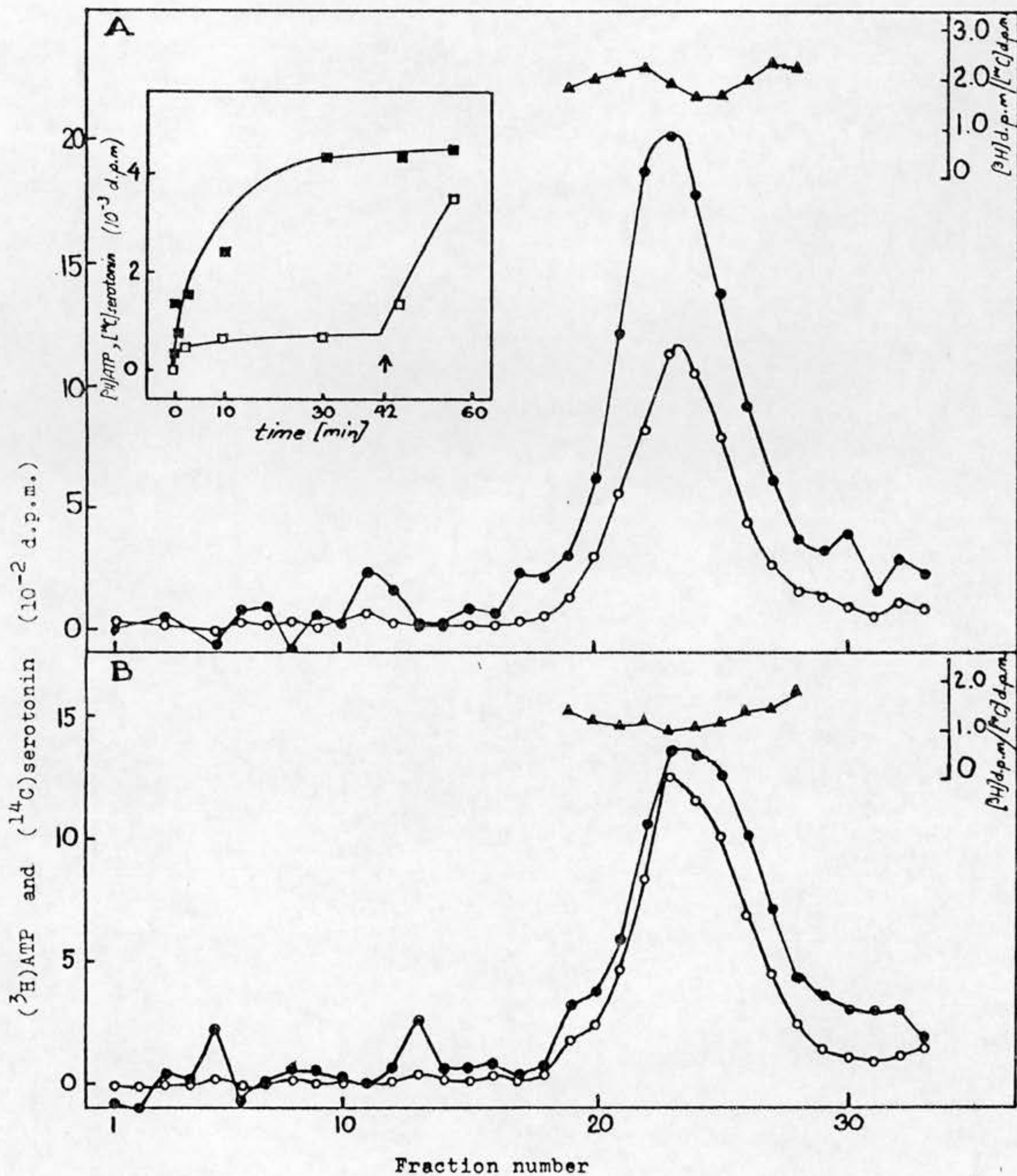
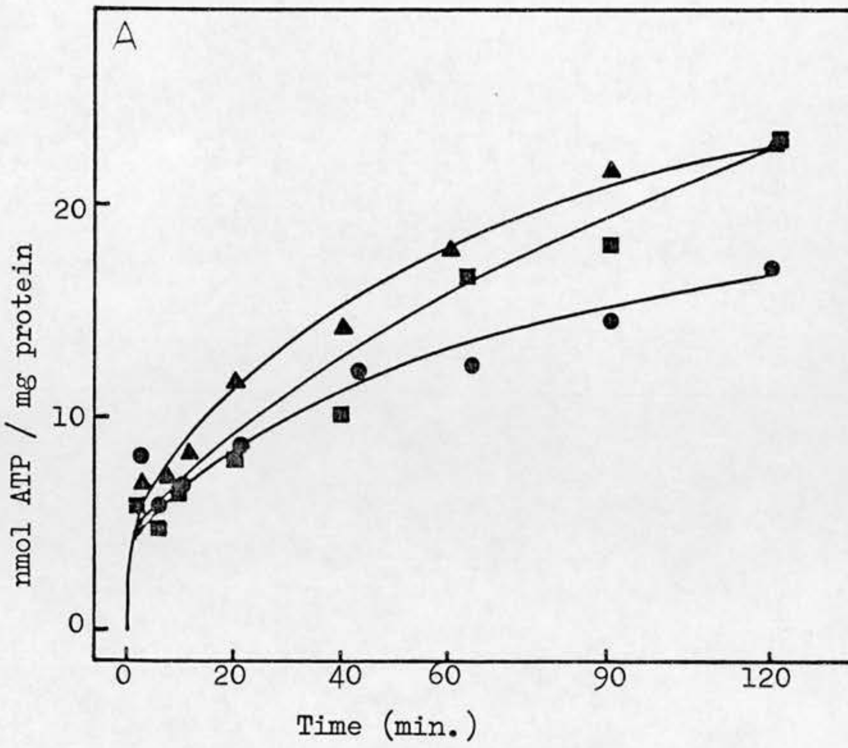


Fig.13: Analysis of ATP-pulsed ghosts incubated with (^3H)ADP (Fig.A) or (^3H)AMP (Fig.B) and (^{14}C)serotonin on sucrose density gradients. Samples of the gradient fractions were filtered and assayed for (^3H)ADP or (^3H)AMP (●) and (^{14}C)serotonin (o). The inset shows the ADP uptake (■) into ghosts to be analyzed and the effect of the pulse with ATP (arrow) on the uptake of serotonin (□). The ratios of ^3H to ^{14}C radioactivity is calculated for the fractions of the peaks (▲).

was routinely added because the ATPase uses ATP in form of a Mg-complex and cannot, therefore, energize the ghosts in absence of Mg^{++}. If the uptake of adenine nucleotides has no absolute requirement for Mg^{++} a correlation of ATP uptake with the amounts of Mg^{++} present in the medium could still indicate in which ionic form ATP is transported into the ghosts.

ATP uptake in presence of different $MgSO_4$ concentrations was measured in a standard double labelling experiment, with the only alteration that $MgSO_4$ as present at either 0.25mM, 2.5mM or 10mM and ATP at 5mM. Fig.14a shows qualitatively that more ATP was taken up at lower $MgSO_4$ concentrations, which might be an indication that free ATP is the substrate for the ATP transport. The result obtained for serotonin uptake (Fig.14b) was in agreement with the view that MgATP is required to energize the serotonin uptake, and that therefore the reduced uptake was possibly due to insufficient MgATP present as ATPase substrate.

Additionally it could also be argued that at higher $MgSO_4$ concentrations the sulphate anion competes with ATP for uptake. Phillips and Allison (1978) showed that ghosts take up sulphate to some extent as counterion for translocated protons, but, as for ATP, no significant concentration gradients could be established. In swelling experiments the passive ion permeability of ghosts has been investigated and it was shown that anions like acetate, sulphate or chloride were rather impermeant compared with thiocyanide or iodide (Phillips, 1977). Fig.15 shows that



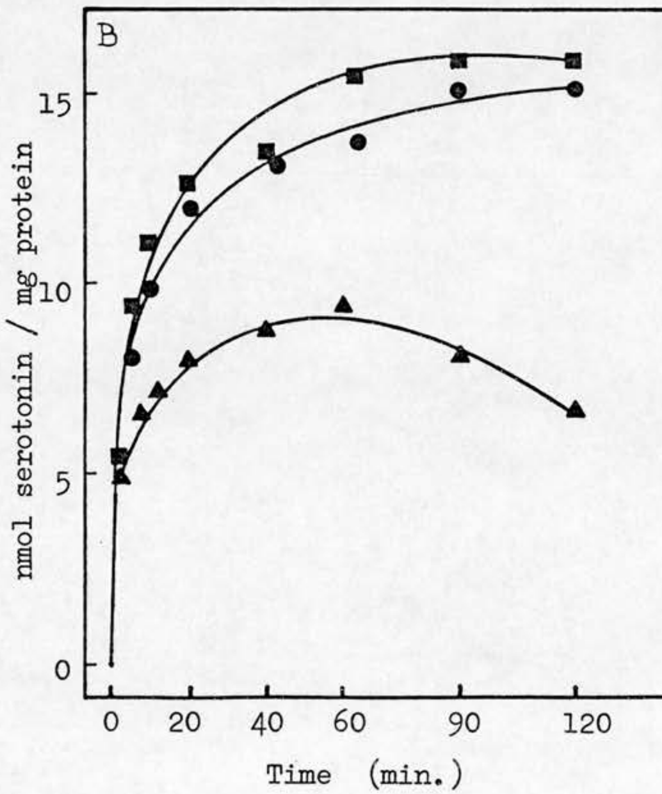
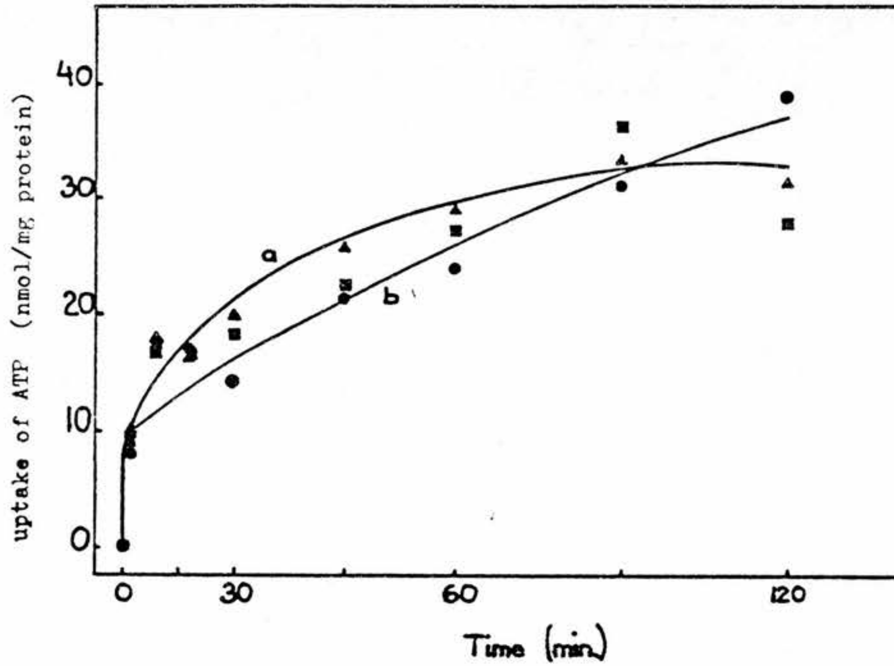


Fig.14: Effect of Mg^{++} concentrations on ATP (a) and serotonin (b) uptake. Ghosts were incubated at 37°C in a standard medium with 5mM ATP and 10mM $MgSO_4$ (●), 2.5mM $MgSO_4$ (■) or 0.25mM $MgSO_4$ (▲).

there was essentially no difference in ATP uptake when 2.5mM $MgSO_4$ was replaced by 2.5mM MgAcetate or 5mM EDTA. In this particular experiment individual time points scattered considerably and two possible interpretations of time courses are drawn, but it was not possible to assign distinct curves to the presence of one of the three anions. In other experiments $MgSO_4$ was replaced by $MgCl_2$ with no effect on ATP uptake. It is unlikely, therefore, that under standard conditions adenine nucleotide uptake is inhibited by either chloride or sulphate present in the medium.

High concentrations of NaHepes inhibited both, ATP and serotonin uptake. The extent of uptake at 30 min. was reduced by 30% in presence of 50mM NaHepes compared with 2mM NaHepes. It was therefore desirable to add only very little buffer salt, also because ATP and PEP are buffering substances themselves. However, since both ATP and PEP are hydrolyzed during the incubation their buffering capacity at neutral pH might change. Therefore 10mM NaHepes was considered to be the minimal concentration to give an adequate buffering capacity. In some experiments this was increased to to 30mM to give a higher capacity, knowing that this higher concentration inhibits the two uptake mechanisms somewhat. To the incubation media KCl at 10mM was routinely added because it enhanced the uptake of ATP as well as serotonin, but no further stimulation of the uptake was observed at higher concentrations.



<u>Fig.15:</u> Uptake of ATP into ghosts in a standard medium with either 2.5mM $MgSO_4$ (●), 2.5mM MgAcetate (▲), or 5mM EDTA (■). No significant differences in the ATP uptake is observed for the three experiments, curve a is rather suggested by the experiment with acetate, curve b in presence of $MgSO_4$.

ATP uptake as a function of the ATP concentration in the medium.

For further characterisation of the uptake of ATP into the ghosts, the dependence of uptake on the ATP concentration in the medium was investigated. If the transport is facilitated by a specific protein like a carrier or a pore it ought to be possible to saturate this protein, and such an uptake should therefore show kinetics similar to those of an enzyme.

Uptake of ATP was measured in media which contained ATP concentrations between 10mM and 0.5mM; $MgSO_4$ was present at a constant amount of 0.25mM. The ATP regenerating system was added, bearing in mind the inhibitory effect of PEP; but for a kinetic analysis it was considered to be necessary to work with a constant substrate concentration. Fig.16a shows the uptake of ATP at 37°C at the various concentrations. The shape of the curves suggests that the extent of ATP uptake is proportional to the amount of ATP present in the medium and that the ATP uptake is not saturable within the concentration range tested.

Fig.16b shows the serotonin uptake which was measured concomittantly. It was somewhat reduced at higher ATP concentrations, which is possibly due to the inhibitory effect of free ATP on the ATPase, which utilizes MgATP as a substrate. The ghosts seem therefore not to be fully energized at these ATP concentrations due to the partially inhibited ATPase, thus the lower uptake of serotonin; compare this also with Fig.14.

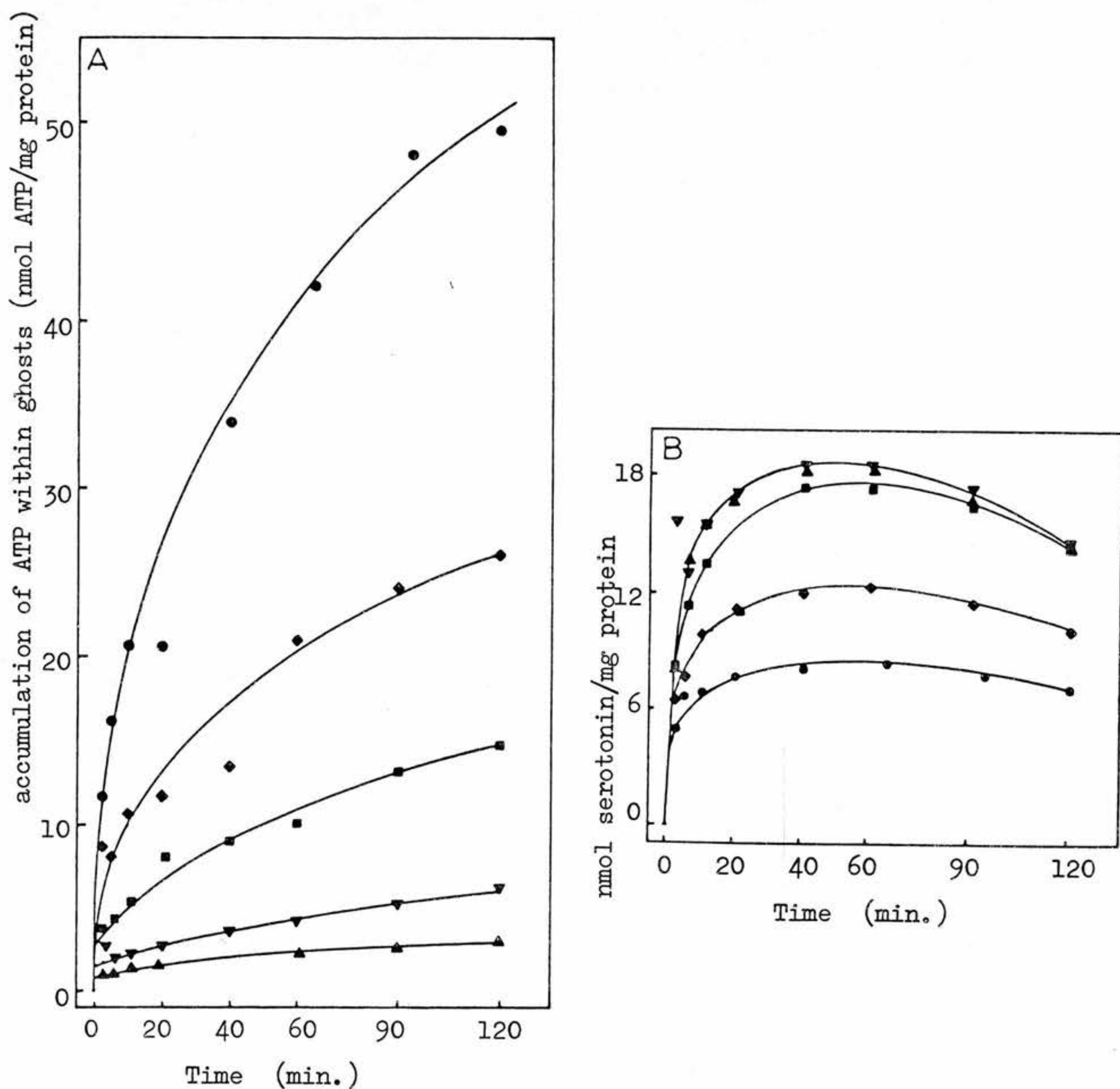


Fig.16: Effect of ATP concentration in the medium on the uptake of ATP (a) and serotonin (b) into ghosts at 37°C. $MgSO_4$ was 0.25mM for all experiments. ATP was 10mM (●), 5mM (◆), 2.5mM (■), 1mM (▼) and 0.5mM (▲).

To rule out unwanted effects caused by the presence of PEP or the low concentration of $MgSO_4$ present in the above experiment, a similar experiment with somewhat altered parameters was performed. ATP uptake was measured at three different ATP concentrations, and the ratio of ATP to $MgSO_4$ was kept constant at 5 to 1. PEP was omitted because of its inhibitory effect, bearing in mind that under these conditions the substrate is a mixture of changing amounts of the three adenine nucleotides. Since in the three incubation media only the increment of unlabelled ATP but not of radioactive ATP was changed, the specific radioactivity varies in the three experiments but at the same time it makes the uptake curves easier to compare. If the rate of uptake is proportional to the amount of ATP in the media, the three experiments ought to give the same uptake kinetics, when the uptake is expressed in terms of radioactivity rather than amounts of ATP taken up.

Fig.17a shows the curves for the three experiments: The result is somewhat ambiguous. The uptake of ATP at 1mM concentration seems to be a little faster than at 5mM and this one, in turn, a little faster than that at 20mM, which suggests some saturation. A hypothetical curve can be drawn taking the amounts of ATP loaded into ghosts at 30 min. (inset Fig.17a). With a guessed Vmax of 10nmol/min./mg, this speculative curve suggests a Km value of at least 30mM. Clearly experiments ought to be done with higher ATP concentrations which are at and above the proposed Km value to show that the uptake is saturable. However for kinetic

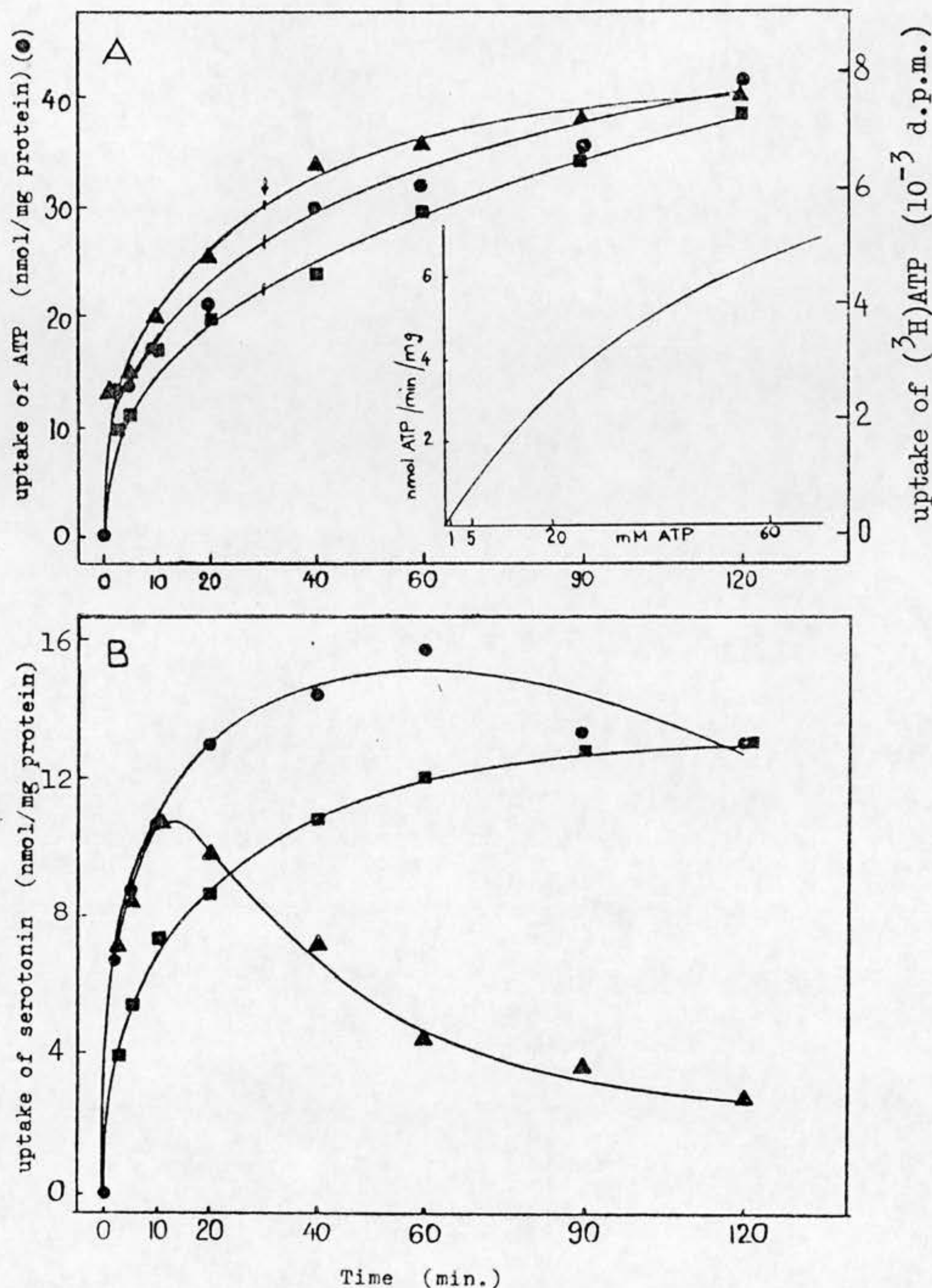


Fig.17: ATP and serotonin uptake into ghosts at different ATP concentrations. A: (^{3}H)ATP uptake is measured at variable specific radioactivity. The amount of ATP taken up is given as (^{3}H)d.p.m. and is calculated into nmol ATP per mg protein for the experiment with 5mM ATP only(●). These values can be multiplied by 4 for 20 mM ATP (■) and divided by 5 for 1mM ATP (▲) substrate concentration. The inset shows a hypothetical saturation curve.
Concomittant serotonin uptake is measured in B.

analysis initial rates ought to be determined, a task which is very difficult because of the large scattering of the measured points. But at these high ATP concntrations one would have to consider shrinking effects and other artefacts due to high salt concentrations. Additionally, as discussed for the lysis experiments (Fig.8), there are probably several events during the uptake of ATP and initial rates might measure indeed a different event from that seen at 30 min. and described in the inset.

Fig.17b shows concomitant serotonin uptake which coincides with the observations made so far: the optimal uptake is seen in presence of 5mM ATP and 1mM $MgSO_4$. At lower ATP concentrations serotonin leaks out of the ghosts, after an initial burst of uptake, when most ATP is hydrolyzed and no energy is available to maintain the concentration gradient of serotonin. At very high ATP concentrations, when in absolute terms the content of free ATP is higher, serotonin utake is inhibited in initial rate but continues over a longer time period because more ATP is available to energize the ghosts. The experiment also suggests that energization by the ATPase is not merely an all-or-none effect, but that the electrochemical gradient has to be maintained because it is dissipated constantly by serotonin beeing transported into and leaking out of the ghosts, and presumably by protons themselves leaking out.

In spite of the marked scattering of individual measurements in the time courses of ATP uptake, an attempt was made to determine initial rates of uptake by taking a

larger number of measurements over a short time period. Standard incubation media were used, in absence of PEP and pyruvate kinase, and the ATP to $MgSO_4$ ratio was held constant at 2 to 1. Time points were taken at 2 min. intervals over the first 20 min. and the uptake was assumed to be linear over this period. The initial rates of uptake were calculated from these time points using linear regression, and SD were calculated for the slopes. In Fig.18 the initial rates are plotted against the ATP concentrations in the media. As above, a straight line was obtained, indicating again that ATP uptake is a non-saturable process. However the straight line in Fig.18 seems not to pass through the origin and although the intercept with the y-axi is small and the significance of the observation questionable, considering the methods which lead to this curve, the behaviour of the ATP uptake at very low ATP concentrations was investigated. The uptake seemed to be proportional to the amount of substrate for ATP concentrations as low as 25µM. At even lower concentrations some saturable uptake or binding was observed. Estimations of the Km using the direct linear plot method (Eisenthal & Cornish-Bowden, 1974) suggested values of about 2µM for ATP and 40µM for ADP . The meaning of this observation was unclear and it was decided not to pursue it any further.

Sensitivity of ATP uptake to pH and PEP.

A comparison of various experiments showed that the extent to which PEP inhibited the ATP uptake varied considerably between the different experiments. Comparing

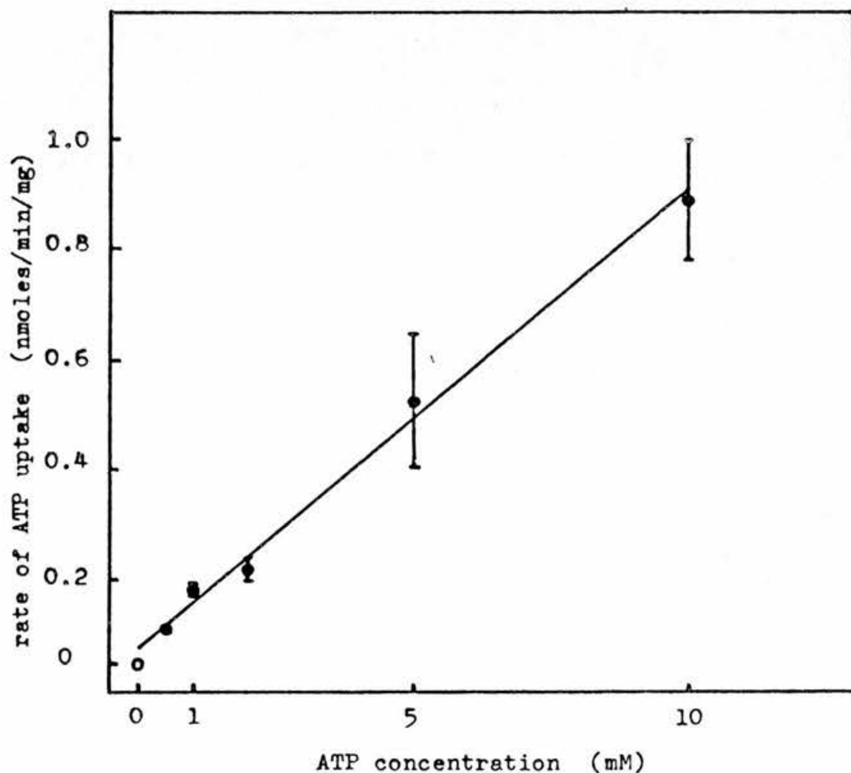


Fig.18: The rates of ATP uptake increase linearly with the substrate concentration. Initial rates of ATP uptake were ~~deter~~ determined as described in the text and are plotted against the the concentration of ATP in the incubation media.

all changing parameters in these experiments it was concluded that the extent to which PEP inhibited ATP uptake was pH-dependent. It was therefore also important to know how the ATP uptake itself varied when the pH of the incubation medium was altered.

Batches of incubation media to give final concentrations of 50mM Hepes (free acid), 10mM KCl, and 2.5mM $MgSO_4$ were prepared and the pH was adjusted with concentrated NaOH to give values of 6.4, 6.8, 7.2, 7.6 and 8.0. Ghosts were preincubated in these buffers at 37°C for 30 min. to allow for pH equilibration between the interior of the ghosts and the media. To start the uptake experiments, a mixture of (^{3}H)ATP, (^{14}C)serotonin, pyruvate kinase and where necessary PEP was added to give the same final concentrations as in a standard experiment. Since PEP and ATP have some buffering capacity, the pH values of the media were measured again between the first 10 and 20 min. of the incubation; these values remained stable for the time during which the uptake was monitored.

Fig.19 shows that the ATP uptake in presence and absence of PEP varies considerably at comparable pH values. The curves drawn from the measured time points, also intercepted the y—axis at very different levels. The curves suggested that the extent to which PEP inhibited ATP uptake varied with the pH. For a further analysis of the experiments rates of uptake at 1 min. were determined graphically and plotted against the pH values of the incubation media during the uptake experiments (Fig.20).

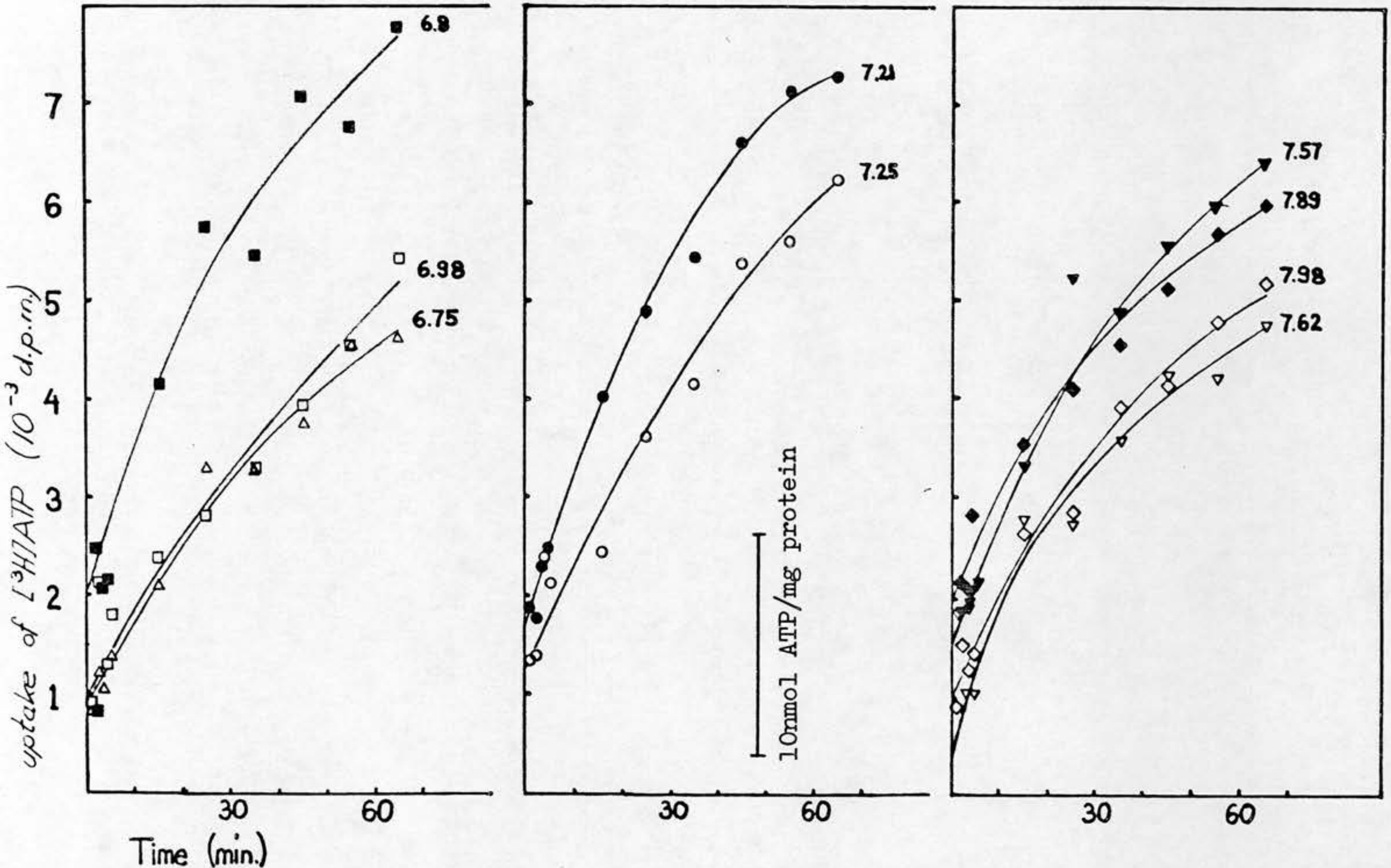


Fig.19: The effect of the pH in the incubation medium on the uptake of ATP into ghosts in absence (closed symbols) and presence (open symbols) of PEP. The pH of the media and a calibration for the conversion of d.p.m. into nmole ATP are given in the figure.

The uptake of ATP in absence of PEP is more marked at lower pH values than at higher ones. However if PEP is present in the medium this effect seems to be reversed, less ATP is taken up in more acidic media then in more alkaline ones. This effect of PEP seems to get lost at pH values higher than about 7.8. How could a change in pH cause such an effect? The only part of ATP which changes its its ionisation state at near neutral pH is one of the hydroxyl groups at the gamma phosphate. Its pK is in the region of 6.6 to 6.9 for free ATP (O'Sullivan and Perrin, 1964). However these pK values are strongly dependent on the ionic strength of the medium. In the above conditions there is half a mol $MgSO_4$ present per mol ATP, which forms complexes with the phosphate groups and therefore neutralizes negative charges thus lowering the pK value. Fig.21 shows titration curves of ATP and PEP measured in an ionic environment similar to that of the incubation medium; the curve for ATP indicates no marked ionistion at those pH values at which ATP uptake was measured. It is therefore concluded that the above pH effect is more likely to be a property of the transport mechanism than of the substrate.

Less clear is the effect of PEP on ATP uptake. If it is because of the transport mechanism that ATP uptake is stimulated at lower pH values then the effect of PEP could be a dissipation of this stimulation. But Fig.20 even suggests that PEP inhibits more strongly at low pH values than the stimulation observed in absence of PEP at the same values. This could be due to a change in charge of PEP or

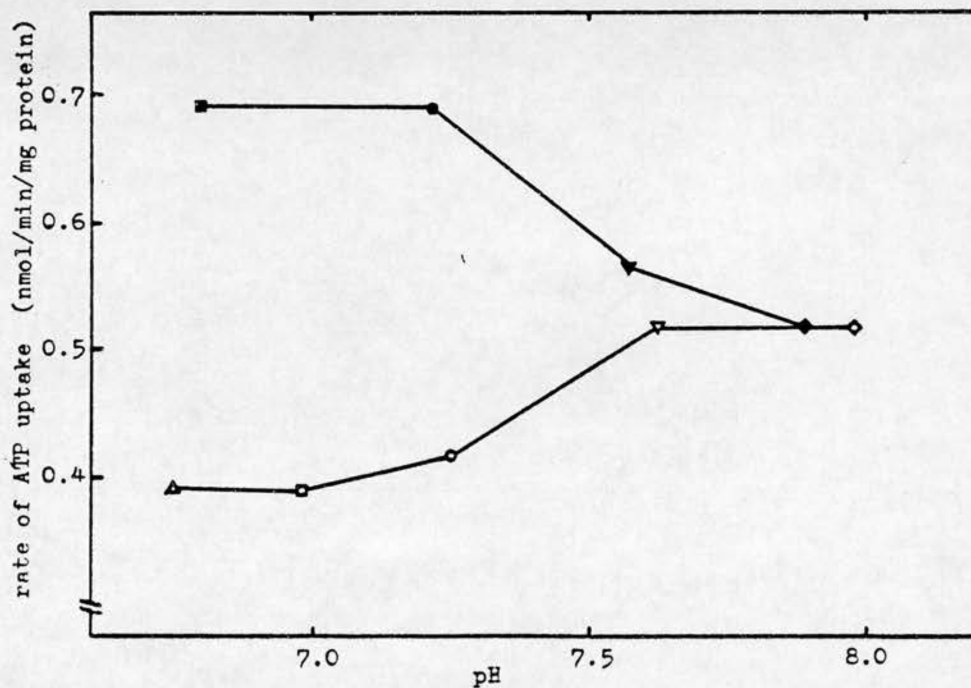
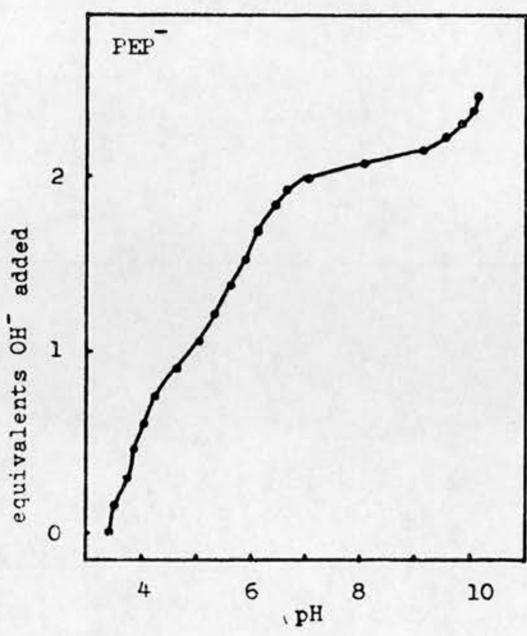


Fig.20: The effect of the pH in the incubation media on the rates of ATP uptake in presence (open symbols) and absence (closed symbols) of PEP.



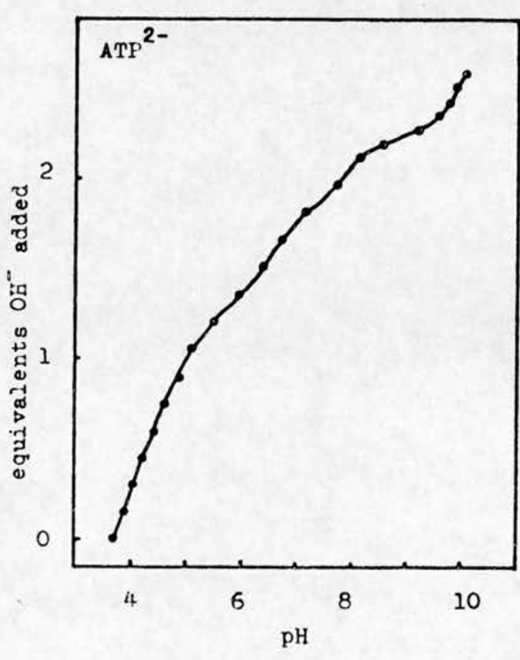


Fig.21: Titration curves of ATP and PEP, measured in an ionic environment similar to that of the incubation medium.

to a greater susceptibility to PEP of the transport system in its stimulated state.

Serotonin uptake in presence and absence of PEP at various pH values is shown in Fig.22. Initial rates are determined from the uptake of serotonin during the first 5 min. at which the uptake was approximately linear. The uptake decreases linearly with pH over the full pH range tested. The presence or absence of PEP did not alter the uptake. In contrast, Sherman and Henry (1981) have observed a decrease of the rate of uptake of noradrenaline into ghosts at lower pH values, whereas Knoth et al. (1981a) found that initial rates did not change with pH for uptake of dopamine into ghosts. Obviously there is some controversy of results. Sherman and Henry have interpreted their results to indicate the ionic nature of the transported substrate, but such interpretations have to be done with care because the pH can have an effect on both the substrate as well as the carrier mechanism.

Temperature sensitivity of the ATP uptake.

As has already been shown in Fig.1, the ATP uptake is dependent on temperature. In Fig.23 the uptake is measured at various temperatures between 0°C and 45°C in a standard incubation medium in presence of PEP and pyruvate kinase. A comparison of the series of uptake curves measured at various temperatures indicates that the uptake of ATP occurs in two phases which depend differently on temperature, an early phase between 0 and 5 min. and a later between 5 and

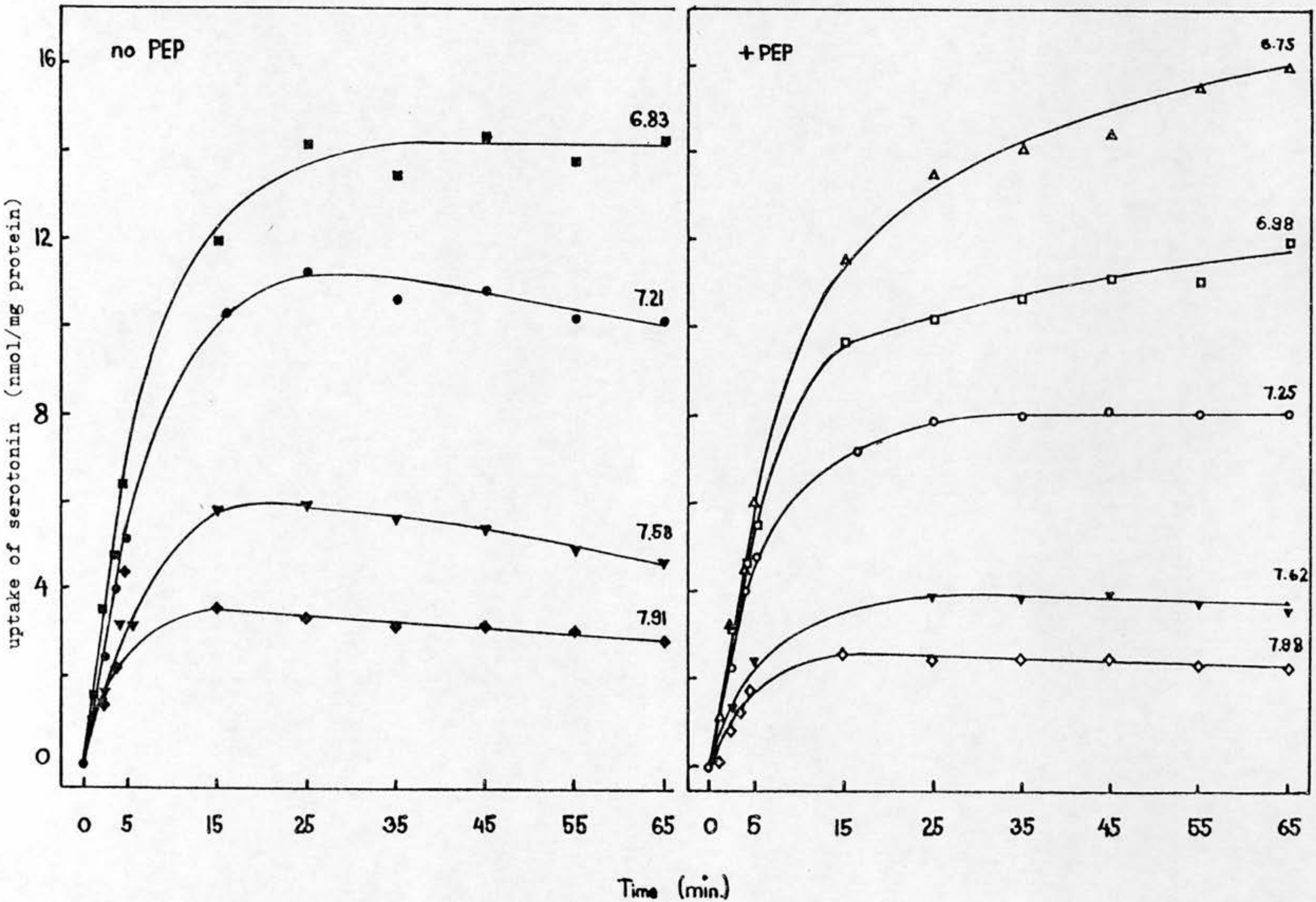
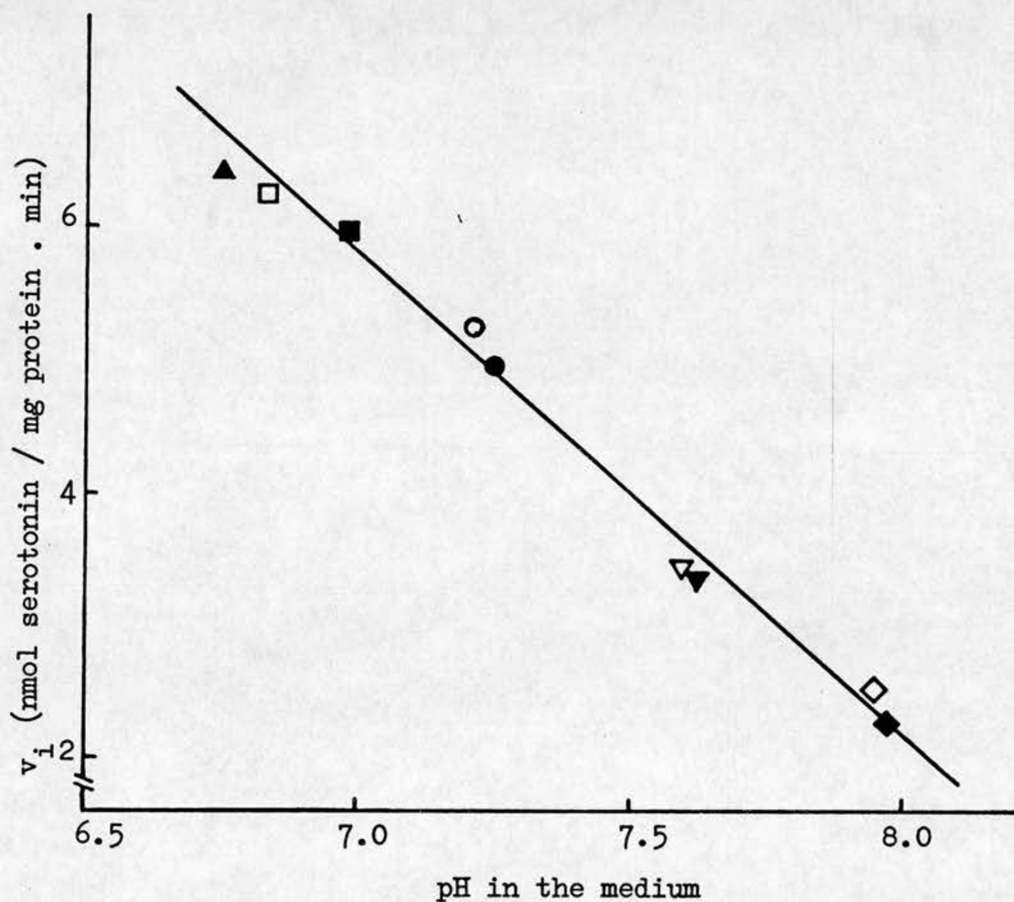


Fig.22a: The effect of the pH in the incubation medium on the uptake of serotonin into ghosts in absence (closed symbols) and presence (open symbols) of PEP. The pH of the media is indicated in the figure.

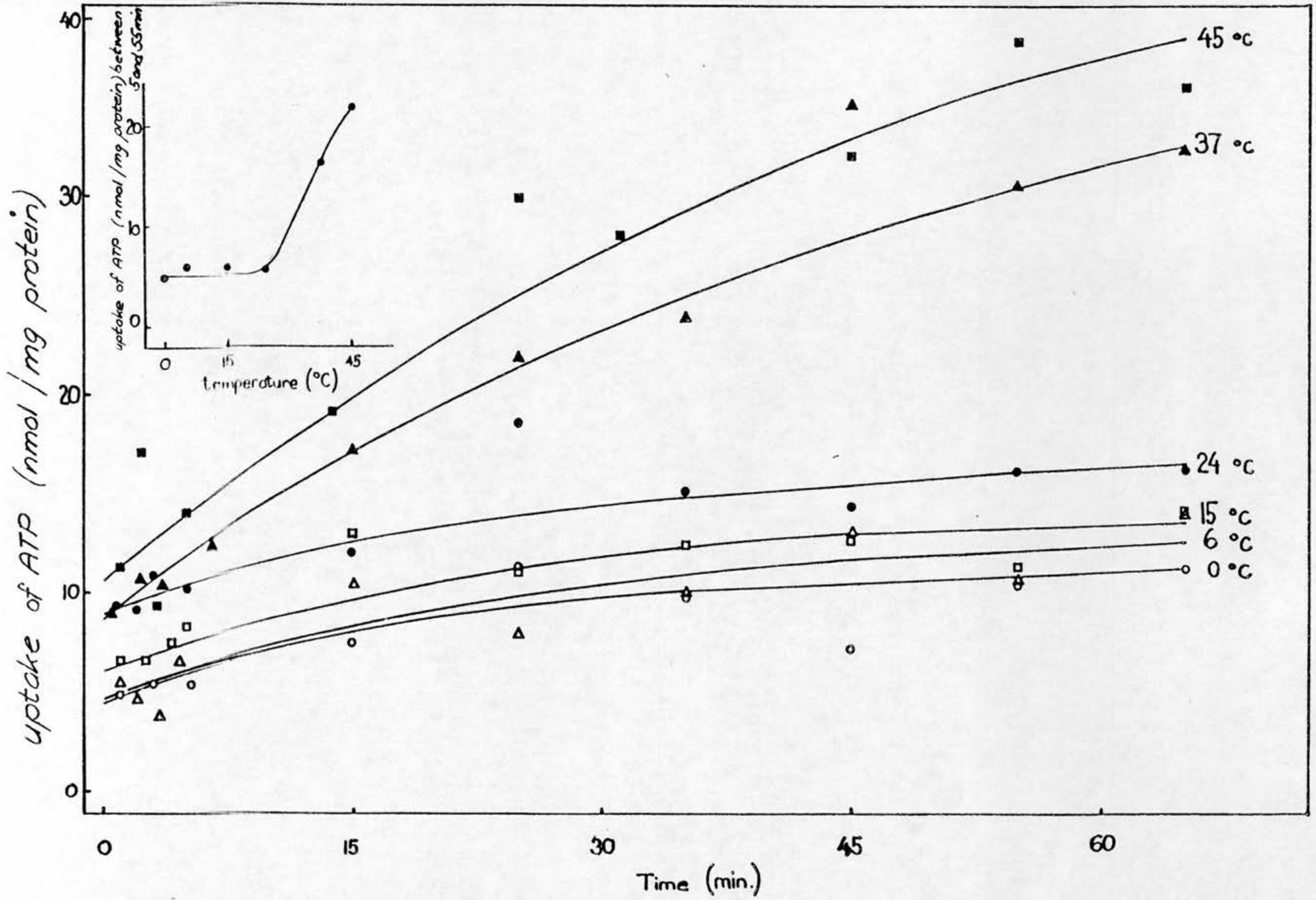


<u>Fig.22b:</u> The effect of the pH in the incubation media on the initial rates of serotonin uptake in presence (open symbols) and absence (closed symbols) of PEP.

55 min. This is best seen for the uptake measured at 24°C: the curve intercepts the y-axis at the same higher level as the 37 °C and 45°C curves but in contrast has a small slope similar to that of the experiments measured between 0 and 15 °C.

The uptake observed after the first 5 min. seems to become activated at 24°C and higher temperatures; this is more clearly seen in the inset in Fig.23, where the rates of uptake between 5 and 55 min. are plotted against temperature. Obviously it would be interesting to present these data in an Arrhenius plot, but for such an interpretation uptake ought to be monitored at more temperatures. It is however doubtful whether clear data could be obtained at all, since the scattering of the individual points in the uptake curves is large compared with the differences between the curves at various temperatures.

Fig.24 shows the temperature dependence of the serotonin uptake. Initial rates of uptake are determined from the linear part of the uptake which lasted normally for the first 5 min. The activation energy calculated from the Arrhenius plot shown in Fig.24a is 65 kJ/mol which indicates an enzymatic process.



<u>Fig.23:</u> Uptake of ATP into ghosts incubated at various temperatures between 0°C and 45°C. In the inset the ATP uptake measured between 5 and 55 min. is plotted against the temperature of the incubation medium.

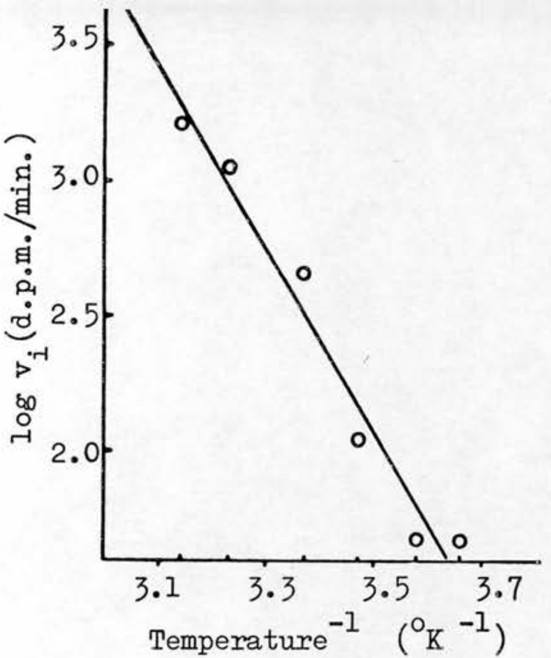
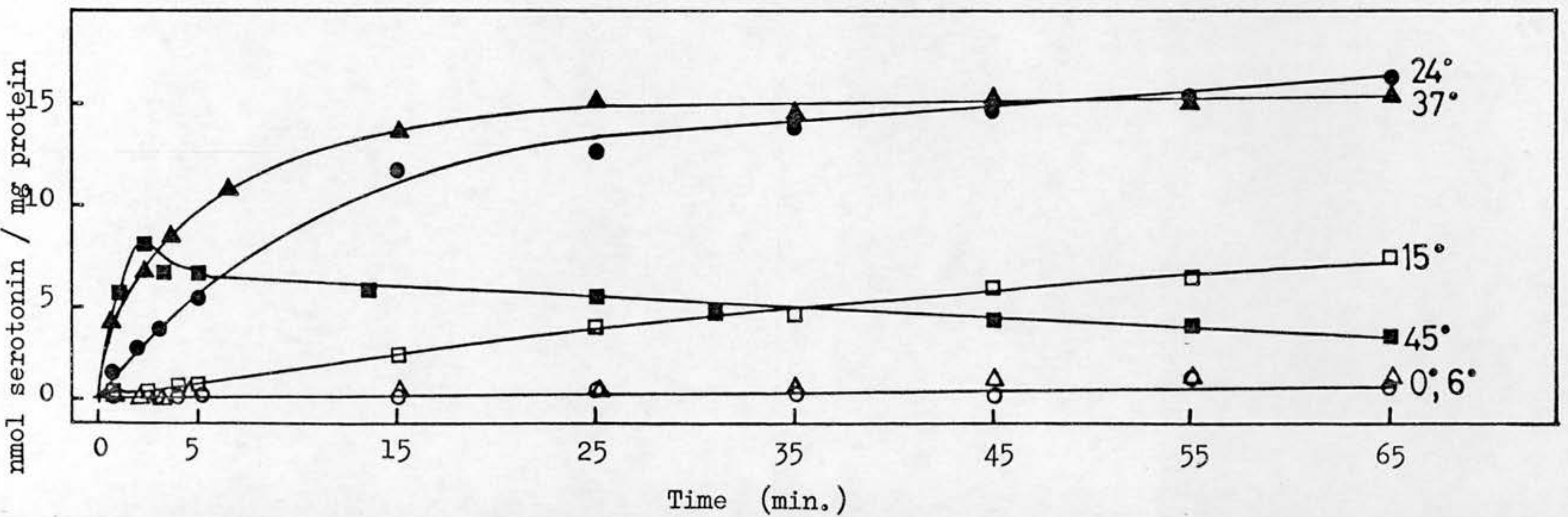


Fig.24: (below) Uptake of serotonin into ghosts incubated at various temperatures between 0°C and 45°C.

Fig.24a: (left) Arrhenius diagram of serotonin uptake into ghosts.



III. Inhibitor studies.

Introduction

In this section the uptake of adenine nucleotides into resealed chromaffin granule vesicles is characterized by its behaviour in presence of various potentially inhibitory agents. In most of the experiments serotonin uptake was measured concomittantly and served as a control for the way of action of the inhibitors. The substances which were chosen as potential inhibitors can essentially be divided into two groups according to their respective mode of action.

One group of substances might interfere with the energization of the ghosts by either inhibiting the ATPase or by interfering directly with the electrochemical potential of the energized ghosts. Most of these substances have been used previously in studies with mitochondria and their effects in that system are well established.

The other group of inhibitors are substances which might interact directly with a possible adenine nucleotide transport protein. Most of them were chosen because they inhibit a similar process in some other biological system. They are of very variable specificity and some of them modify proteins covalently and are therefore also part of the first class of compounds, if they bind to and inhibit

the chromafiin granule ATPase. For all experiments standard incubation media were used with 5mM ATP and 2.5mM $MgSO_4$, and PEP and pyruvate kinase were present when indicated. In some experiments it was necessary to preincubate the ghosts with the inhibitors; this and other alterations are described in the individual experiments.

ATP uptake and uncoupling agents.

In presence of MgATP the chromaffin granule ATPase pumps protons across the membrane which results in the establishment of a transmembrane proton gradient (ΔpH, acidic inside the ghosts), and an electrical potential ($\Delta\Psi$, positive inside the ghosts). Each of these two parts of the proton electrochemical potential ($\Delta\mu_{H^+}$) can be collapsed separately (Apps et al., 1980a).

When ammonium sulphate is present in the incubation medium, uncharged ammonia penetrates the membrane and is protonated inside the ghosts. This collapses the pH gradient but leaves the amount of positive charges in the matrix constant, i.e. $\Delta\Psi$ remains constant. In contrast, thiocyanate, a membrane-permeant anion, neutralizes positive charges inside the ghosts without reducing the amount of protons; $\Delta\Psi$ is abolished and the ATPase is stimulated, leading to an increase in ΔpH. Fig.25 shows the ATP uptake into ghosts in a medium supplemented with PEP and PK, in presence of 10mM KSCN or 5mM $(NH_4)_2SO_4$. The ATP uptake seems not to be affected by either of the two salts. Serotonin uptake in contrast is clearly inhibited in

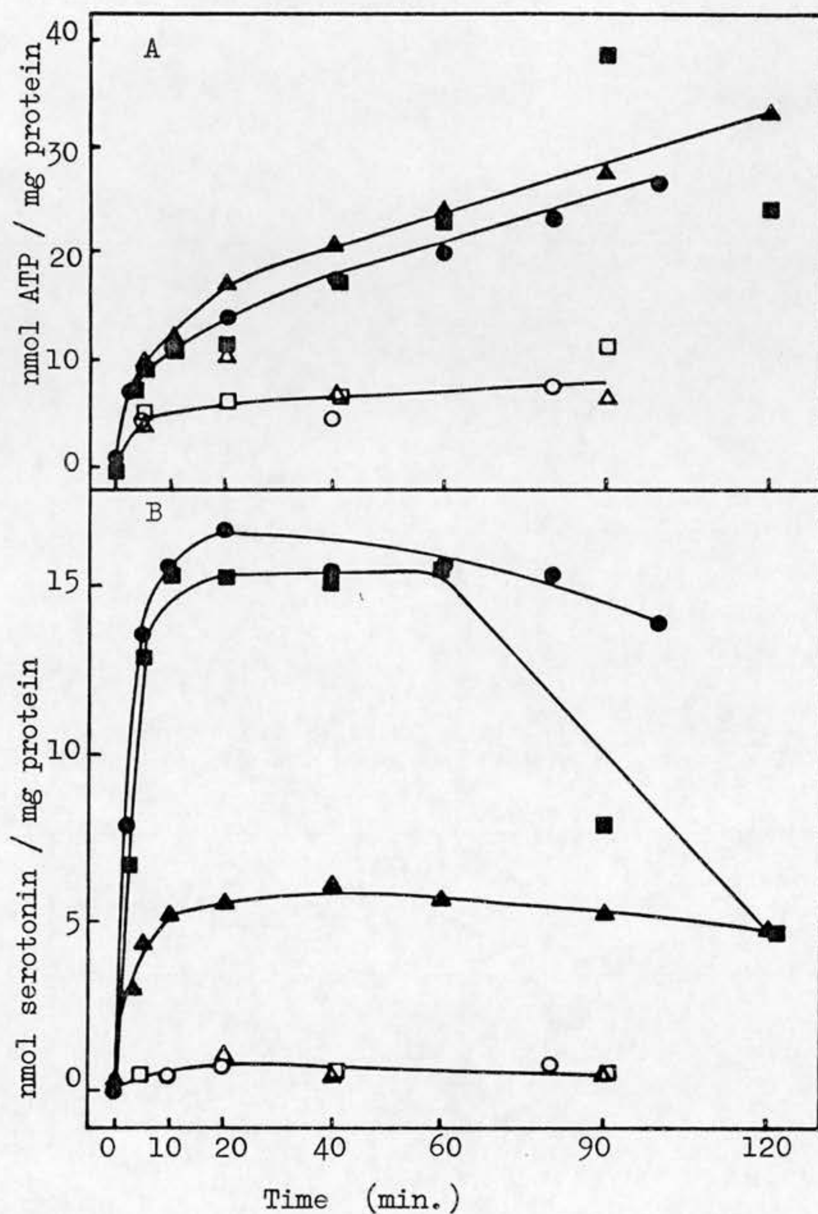


Fig.25: Uptake of ATP (a) and serotonin (b) into ghosts in presence of 5mM $(NH_4)_2SO_4$ (▲,△) or 10mM KSCN (■,□); control, no addition (●,○). Incubation at 37°C: solid symbols, 0°C: open symbols.

presence of ammonium sulphate, which indicates that the serotonin transport depends on the membrane proton gradient. More detailed studies (Apps et al.,1980 *a*; Johnson et al.,1979) have established that the accumulation of catecholamines into chromaffin granule ghosts is dependent on both the pH and the electric gradient but that some accumulation is also observed if only one of the two gradients is established, as indicated by Fig.25. When the concentration of the thiocyanate or ammonium ions was increased to 100mM the extent of ATP uptake was inhibited by about 40% in both cases and the uptake pattern resembled the uptake of lysis insensitive label as shown in Fig.8, i.e. the rapid initial binding was unchanged but no further uptake was observed after the first 20 min. The serotonin uptake was in both experiments completely reduced to background levels. This might be an artefact caused by the high salt concentrations, rather than an inhibition due to deenergization of the membrane.

The proton pumping activity of the ATPase can be shortcut by the protonophore FCCP, which has been extensively used as an uncoupling agent in mitochondria. In presence of 20µM FCCP the extent of ATP uptake is inhibited by about 25% for time points taken later than 30 min. No marked inhibition is seen during the first 15 min. of the uptake (Fig.26). The ghosts had not been preincubated with FCCP and this could account for the lagging effect of the uncoupler. Its action on the uptake of serotonin, however, was immediate; the uptake was almost completely inhibited right from the start of the incubation, and the delayed

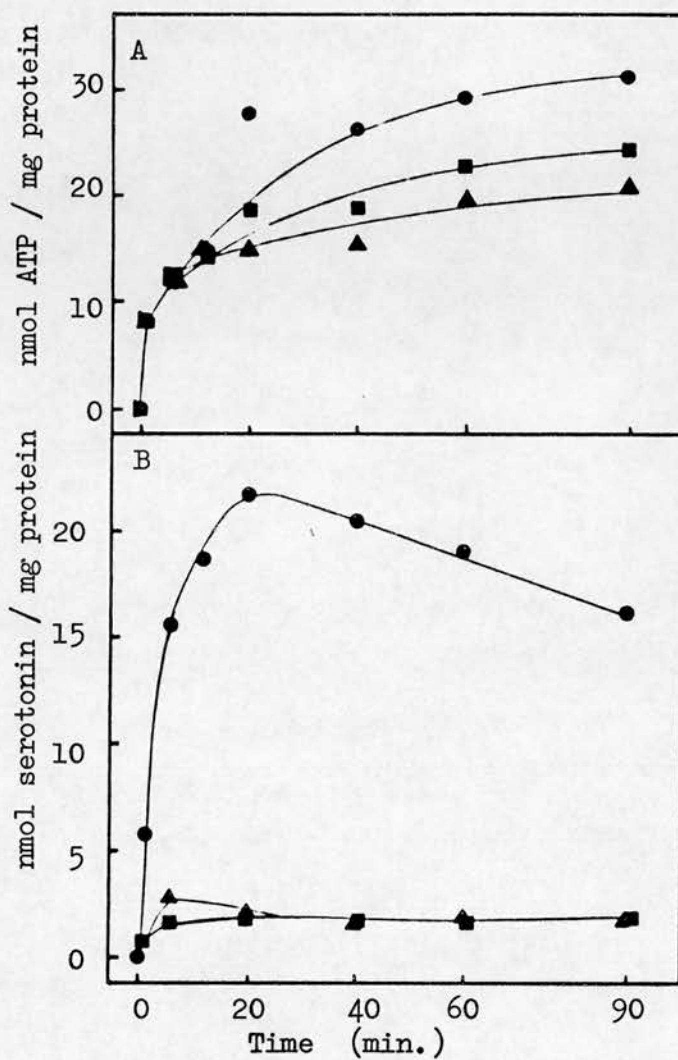


Fig.26: Inhibition of ATP (a) and serotonin (b) uptake into ghosts by 20µM FCCP (■) and 1mM NEM (▲); control (●).

effect on ATP uptake must therefore be for a different reason. A comparison with Fig.8 suggests that the residual uptake of ATP observed in presence of FCCP is similar in nature to the lysis-insensitive uptake of ATP. According to Fig.8 only the proportion of ATP taken up after about 15 min. is lysis-sensitive and the same amount of time has to elapse before a sensitivity to FCCP is observed. This is another indication that there are at least two phases of ATP uptake: a rapid binding and a slower uptake.

Of all the agents tested which dissipate a part or the whole of the electrochemical gradient or inhibit the energization of the ghosts by affecting the ATPase, FCCP was the only one which also inhibited the ATP uptake. In all other experiments where the energization of the ghosts was greatly diminished, as judged by their ability to accumulate serotonin, adenine nucleotide uptake was not inhibited, as discussed for ATP uptake in presence of EDTA, $(NH_4)_2SO_4$, KSCN and uptake of ADP and AMP itself. The partial inhibition of the uptake of ATP in presence of FCCP and the lack of inhibition by ammoniumsulphate which acts as an electrogenic protonophore (proton-sink within the matrix) suggests that at least a part of the ATP uptake might be indeed a response to the electrical part of the electrochemical membrane potential, as has been suggested for ATP uptake into intact chromaffin granules (Weber & Winkler, 1981). But if this is true, an inhibition by thiocyanate should be observed and this is clearly not the case. From these results it remains therefore unclear whether the proton pumping activity of the ATPase is

involved in the uptake of ATP into chromaffin granule ghosts.

<u>Inhibition of chromaffin granule transport mechanisms by protein modifying reagents.</u>

A similar inhibition of ATP uptake as with FCCP was observed in presence of 1mM N-ethylmaleimide (NEM, Fig.31) (Fig.26) and with 267μM dicyclohexylcarbodiimide (DCCD, Fig.31) (Fig.27). NEM is a thiol reagent which inhibits phosphate transport in mitochondria but it is obviously of broad specificity. DCCD inhibits the chromaffin granule ATPase by binding to the proton conducting part of the enzyme complex (Sutton & Apps, 1981). Although DCCD binds predominantly to this protein it is also a potentially unspecific agent which reacts with other proteins of the chromaffin granule membrane as well, as seen when (^{14}C)DCCD-labelled membrane proteins are separated on polyacrylamide gels (Apps et al., 1980a).

Another substance which was shown to inhibit ATP uptake into ghosts was 4 – chloro-7-nitrobenzofurazan (Nbf-Cl, Fig.31) (Fig.28), an agent which binds to the β-subunit of the mitochondrial ATPase (Ferguson et al., 1975). But again, Nbf-Cl is an inhibitor of broad specificity which inhibits in chromaffin granule ghosts the ATPase as well as the actual serotonin transport mechanism (Apps et al.,1980a). Radioactive Nbf-Cl was found to bind extensively to proteins in the chromaffin granule membrane and this will be discussed in the next section, on ATP

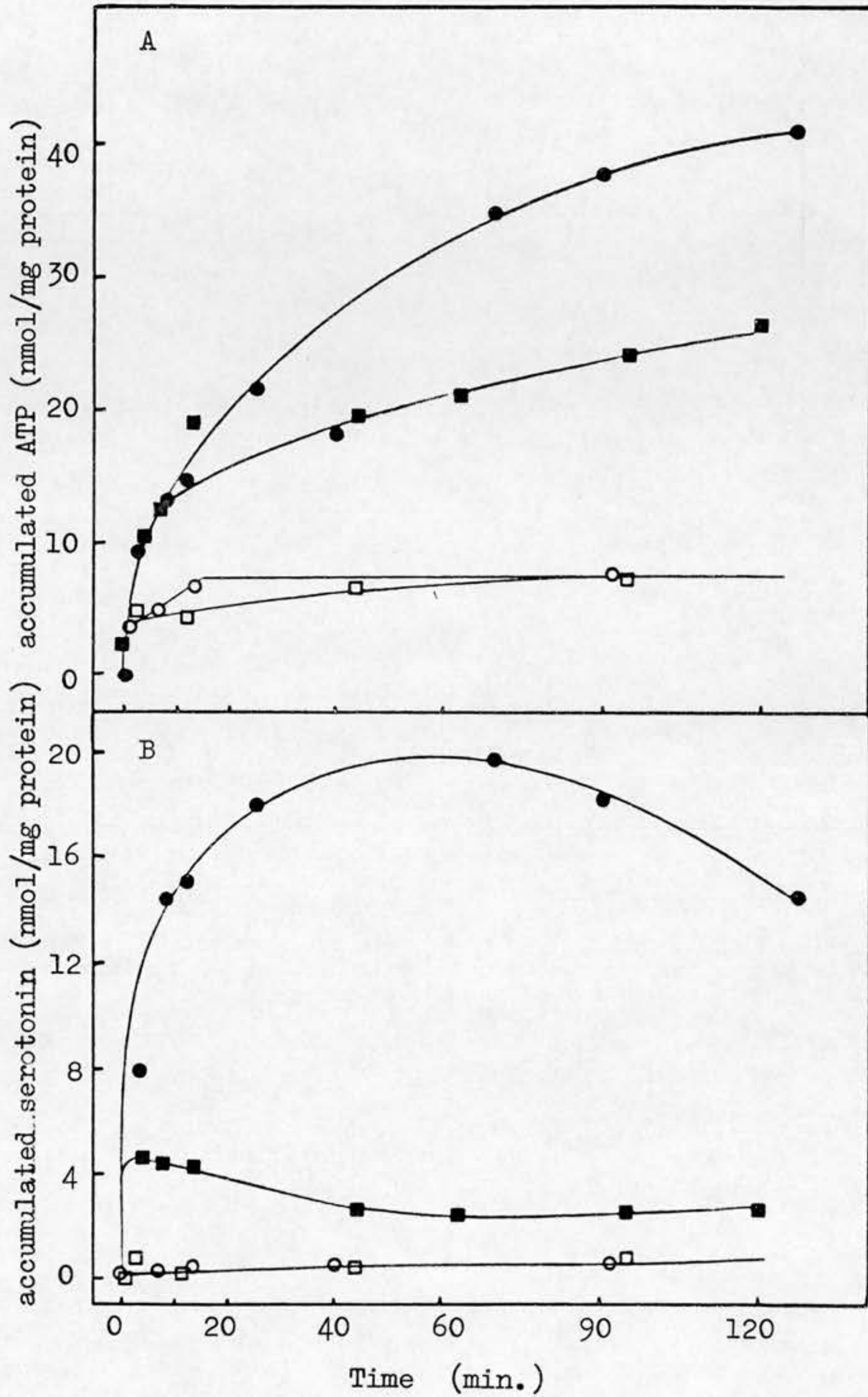
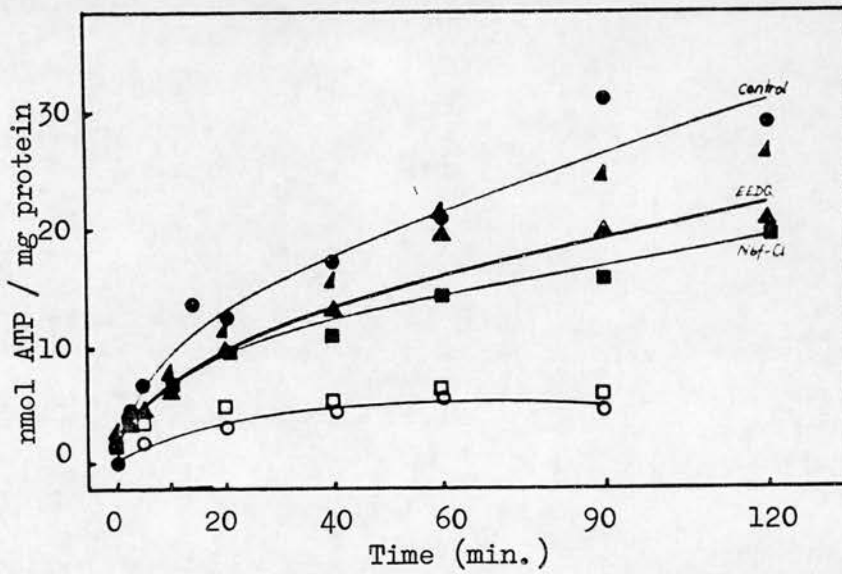


Fig.27: Inhibition of ATP (a) and serotonin (b) uptake by 267μM DCCD (■,□) at 37°C (solid symbols) and 0°C (open symbols); (●,○) control with no DCCD added.

uptake and uncoupling agents.

N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, Fig.31) (Fig.28), is a specific carboxyl reagent which irreversibly inhibits the mitochondrial ATPase, probably by binding like DCCD to the membrane sector of the enzyme complex, but also to the F_1-ATPase where it possibly interferes with the binding of MgATP to the nucleotide site (Pougeois et al., 1978). Ghosts were preincubated with EEDQ at 37 °C for 10 min. at 75 and 750μM to give 20 and 200μM inhibitor concentrations respectively during the incubation with ATP. The control experiment contained 0.5% methanol which was used as the solvent for EEDQ. At higher concentrations EEDQ inhibits initial rate and extent of ATP uptake in a similar way as Nbf-Cl (Fig.28). No inhibition of the uptake at 0°C was observed. The rapid binding of ATP as seen in other experiments (e.g. Figs.8 or 23) was absent, probably because of the preincubation with methanol.

The inhibitors tested so far are either substances which affect the electrochemical membrane potential of the ghosts or substances which modify proteins in a rather unspecific way. Because these agents do not exclusively bind to the ATPase their inhibition of the ATP uptake does not prove an involvment of the ATPase in the uptake mechanism. None of the inhibitors seems to inhibit the ATP uptake mechanism specifically; serotonin uptake was usually inhibited at least as much as ATP uptake by the substances tested above. Therefore further possible inhibitors were screened for activity with the aim of finding a substance which inhibits



<u>Fig.28:</u> ATP uptake into ghosts preincubated with 75μM (▲) or 750μM (▲) EEDQ, or 750μM Nbf-Cl (■,□). The uptake was measured at 37°C (solid symbols) and 0°C (open symbols). The the ghosts for the control (●,○) were preincubated in a similar amount of methanol as was present as solvent for the inhibitors.

ATP uptake but not the ATPase.

Uptake of ATP in presence of sulfonated aromatic compounds.

Sulfonated aromatic substances have been shown to inhibit the anion transporter, band 3 protein, of red blood cells (Cabantchik & Rothstein, 1974) and were therefore tested as possible inhibitors of the transport mechanism of ATP into chromaffin granule ghosts. Among the drugs active in erythrocytes are the stilbene derivatives SITS (4-acetamido — 4'-isothiocyano — 2,2'-stilbene disulfonic acid) and DIDS (4,4'-diisothiocyano — 2,2'-stilbene-disulfonic acid) (Fig.31). Their inhibitory effect is thought to be due to electrostatic interactions between the negatively charged core of the disulfonic stilbene and positively charged groups of the membrane proteins. After initial and reversible binding and inhibition, DIDS becomes covalently bound to an amino group in the protein which would make radioactive DIDS a possible probe for affinity labelling of anion transporting proteins.

Related to these substances are the polysulfonated compounds suramin and cibacron blue F3GA (Fig.31). Cibacron blue binds tightly to nucleotide-binding enzymes (Edwards & Woody, 1979) since the aromatic rings and the sulphonate groups resemble the heterocyclic bases and the phosphate groups respectively of nucleotides. However cibacron is not a very specific analogue of ATP because its steric conformation can apparently adjust to fit the active sites of many nucleotide requiring enzymes.

Fig.29 shows the inhibitory effect of SITS, suramin and cibacron on the uptake of ATP and serotonin into ghosts incubated in a standard medium in absence of PEP and pyruvate kinase at 37°C and 0°C. The inhibitors were added at the start of the incubation at a final concentration of 200μM. All three substances reduce ATP uptake at 37°C to some extent: SITS and suramin inhibit the rate of the uptake observed after the first five minutes, the initial rapid binding however is only slightly affected. The inhibition by cibacron is more marked, with an inhibition of the rate of the rapid binding and a complete inhibition of the subsequent uptake process. The radioactivity which was associated with the ghosts incubated in presence of cibacron showed very little sensitivity to hypoosmotic lysis, which supports the view that mostly lysis-sensitive ATP uptake is inhibited by cibacron. All the inhibitors also reduce the uptake at 0°C in a similar way as the uptake at 37°C, which indicates that the 0 ° C event is lysis sensitive uptake rather than binding.

Serotonin uptake was completely inhibited by all three agents which meant that the inhibitors acted also at sites different from the putative nucleotide carrier. Inhibition of ATP uptake only through binding of these substances to the ATPase was ruled out, because none of the specific ATPase inhibitors inhibited ATP uptake as completely as, for example cibacron.

The concentration of the inhibitors was reduced in an attempt to affect the transport of ATP, without effect on

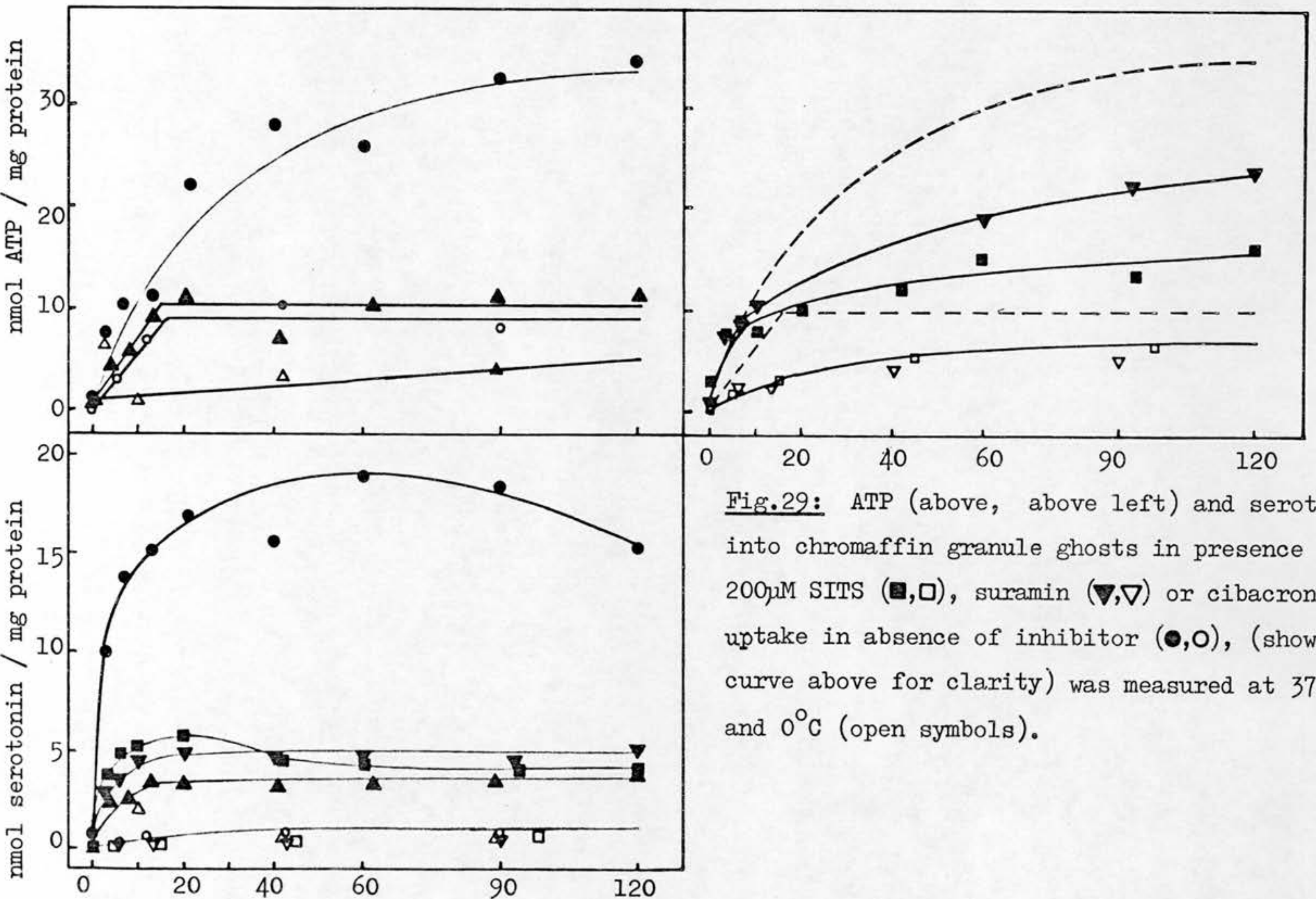
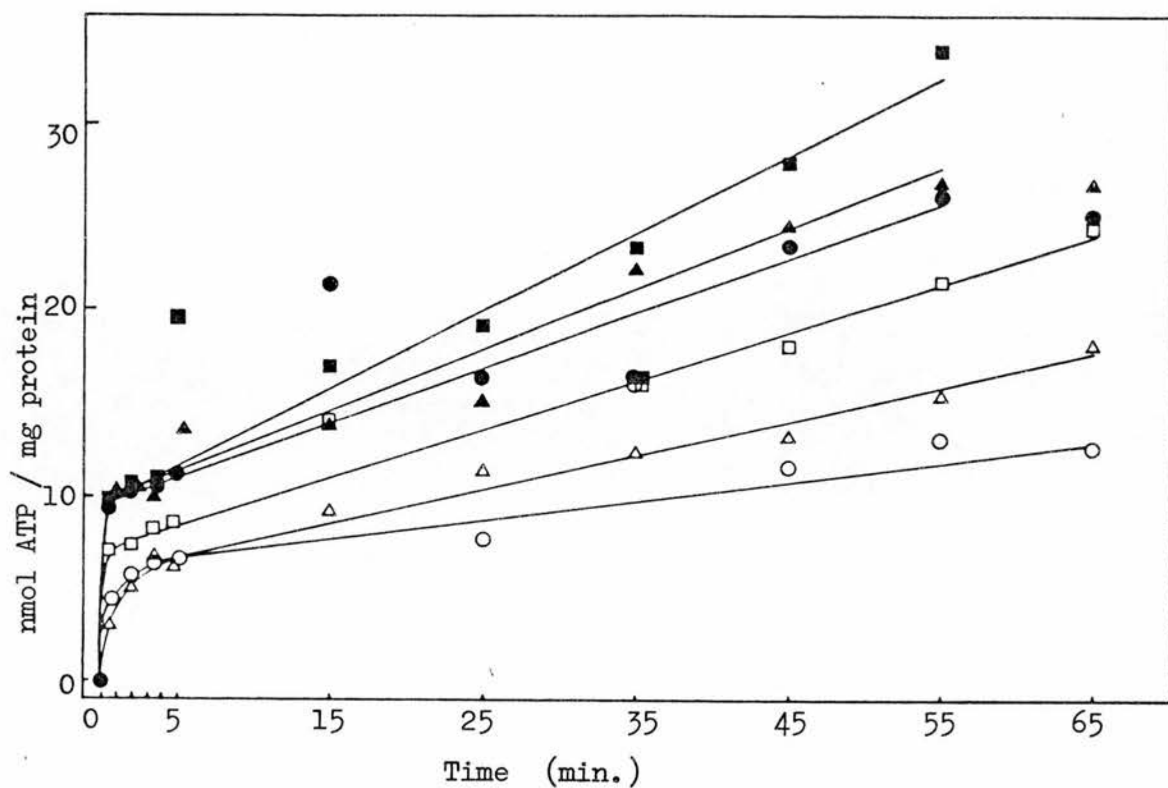


Fig.29: ATP (above, above left) and serotonin uptake into chromaffin granule ghosts in presence of either 200μM SITS (■,□), suramin (▼,▽) or cibacron blue (▲,△); and uptake in absence of inhibitor (●,○), (shown as dashed curve above for clarity) was measured at 37°C (solid symbols) and 0°C (open symbols).

the serotonin transport mechanism. Fig.30a,b shows ATP and serotonin uptake into ghosts, in presence of various cibacron concentrations between 1 and 400μM. The rates of uptake of ATP between 5 and 55 min. which eliminates effects of cibacron on the rapid binding, are plotted against cibacron concentration in Fig.30c. The inhibition of serotonin uptake is also shown. To obtain accurate values, initial rates of serotonin uptake were determined separately from time courses taken over 5 min. after a preincubation with cibacron for 5 min. to resemble the conditions at which ATP uptake was measured. One portion of the ATP uptake (ca. 30%) was sensitive to low cibacron concentrations which did not inhibit serotonin uptake. But both uptake mechanisms were sensitive to higher concentrations of cibacron with serotonin uptake being more sensitive than ATP uptake which was still present at a third of its original rate at very high cibacron concentrations. As observed in the previous experiment high cibacron concentrations did also inhibit the rapid binding of ATP to the ghosts. Of all the inhibitors tested cibacron was the only agent to have an effect on this rapid binding.

Inhibitory effect of atractyloside and carboxyatractyloside.

ATP/ADP exchange across the mitochondrial inner membrane is an adenine nucleotide transport process for which specific inhibitors are available. Atractyloside (ATR) and carboxyatractyloside (CAT), compounds isolated from the thistle Atractylis gummifera, bind to this carrier non covalently with a high affinity. The binding, which



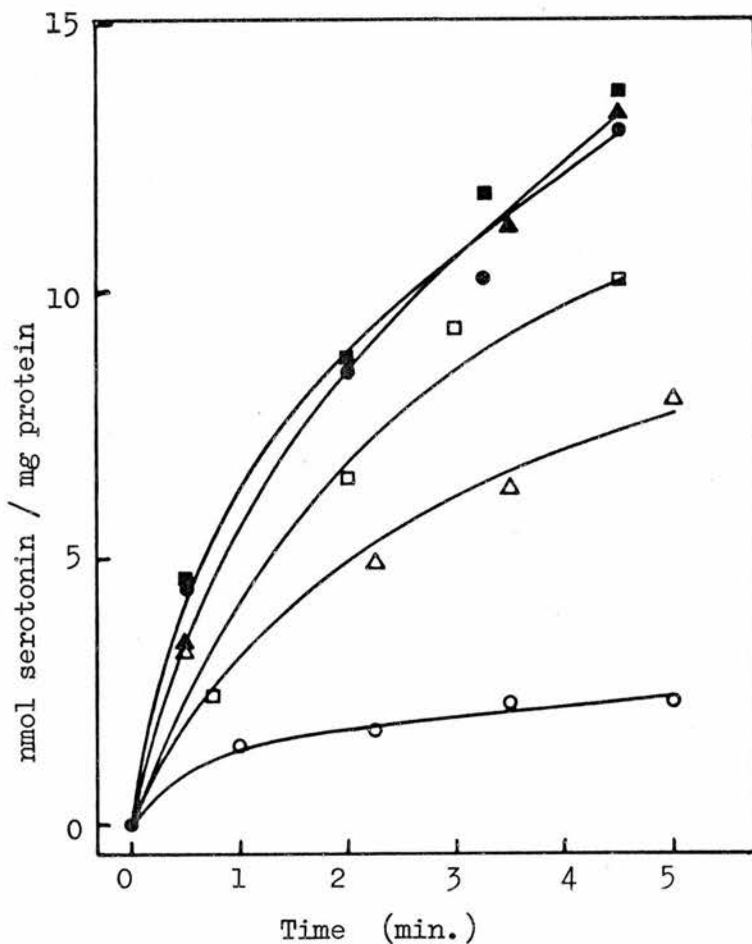


Fig.30a: (above) ATP uptake into ghosts at 37°C in presence of increasing amounts of cibacron blue.

Fig.30b: (left) serotonin uptake into ghosts at 37°C after preincubation with increasing amounts of cibacron blue.

Concentrations were: control (●), 1μM (▲), 5μM (■), 25μM (□), 100μM (△), 400μM (○).

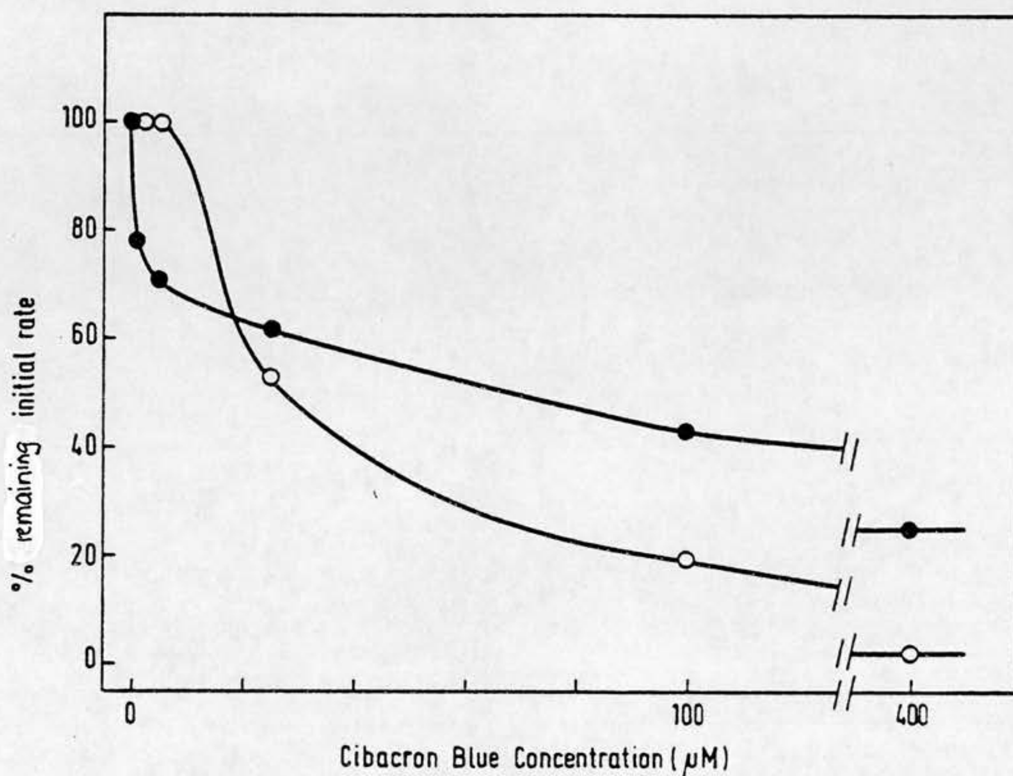


Fig.30c: Inhibition of ATP (●) and serotonin (○) transport into ghosts by cibacron blue. Effect of increasing inhibitor concentrations on uptake rates.

inhibits the exchange of nucleotides, is with respect to ADP competitive for ATR and non-competitive for CAT with respect to ADP (Vignais et al., 1973) and is strong enough that the mitochondrial carrier could be isolated as a CAT-protein complex (Klingenberg et al., 1978).

The effect of atractyloside and carboxyatractyloside on adeninenucleotide and serotonin transport into ghosts was measured under various conditions. Some inhibition was observed but it seemed that uptake of ATP was only inhibited by atractyloside when the incubation medium did not contain PEP and pyruvate kinase (Fig.32a,b). The potency of the inhibitor did not change when it was preincubated with the ghosts for 10 min. at 37° C. No marked increase in inhibition was observed at ATR concentrations as high as 1mM (data not shown).

In Fig.33 uptake of ATP and serotonin is shown at 250μM and 1mM carboxyatractylside, in presence of PEP and pyruvate kinase into ghosts which have been preincubated for 15 min. at 37° C with the inhibitor. Surprisingly the serotonin transport into ghosts was markedly inhibited by higher concentrations of carboxyatractyloside. The poor inhibition of ATP uptake by ATR and CAT when PEP was present in the medium was unclear and could possibly be due to PEP and ATR or CAT inhibiting a similar part of the energized ATP uptake so that their inhibitory effects are non-additive.

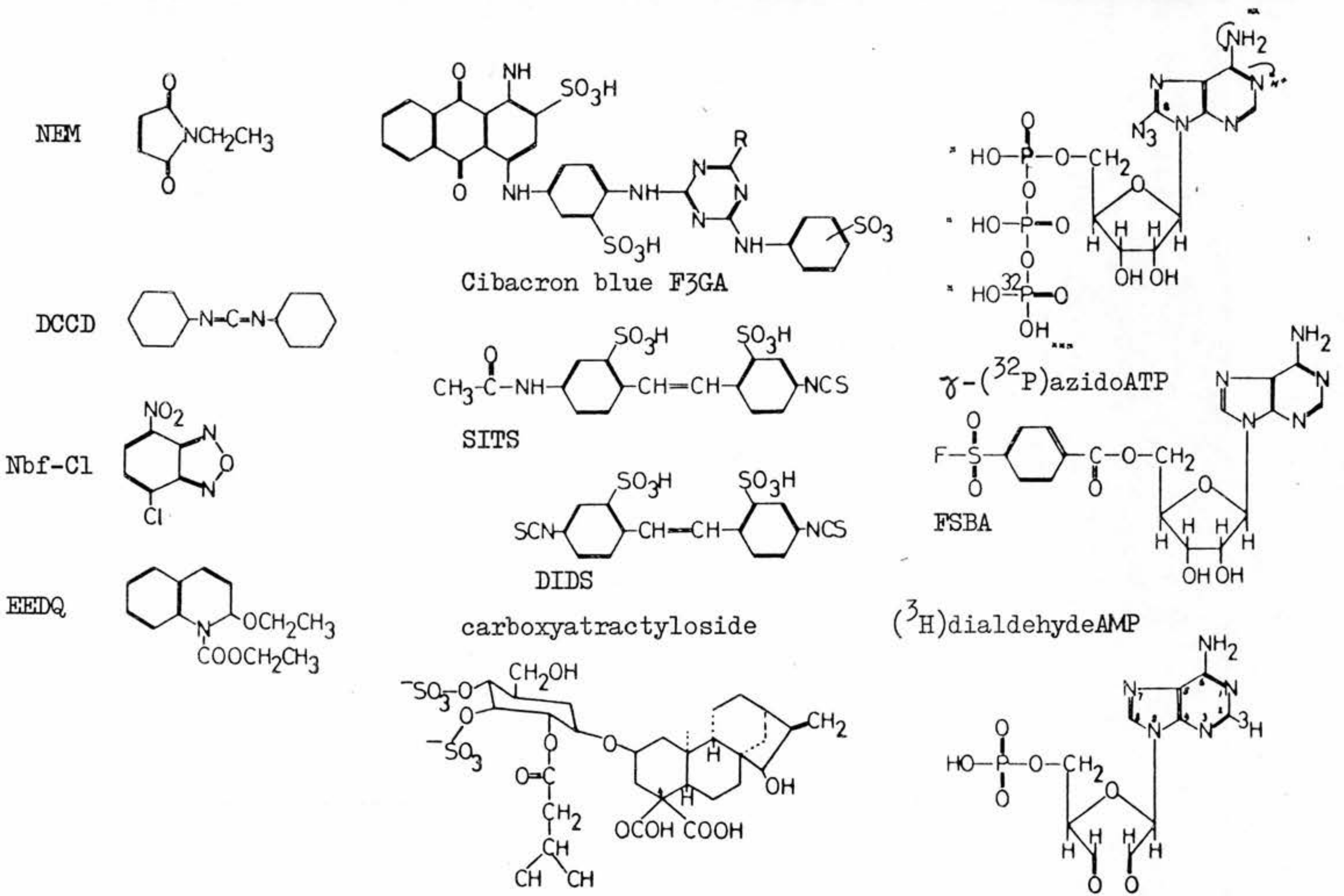


Fig.31: Chemical structures of some inhibitors.

Notes: cibacron: R= -Cl cibacron; R= -O-dextran blue dextran; R= -O-sepharose blue sepharose
carboxyatractyloside: atractyloside, without carboxy groups at position 4
azidoATP: ATP, without N_3 group at position 8, pK's for ATP: x pK 1-2; xx pK 3.7
xxx pK 6.7-6.9

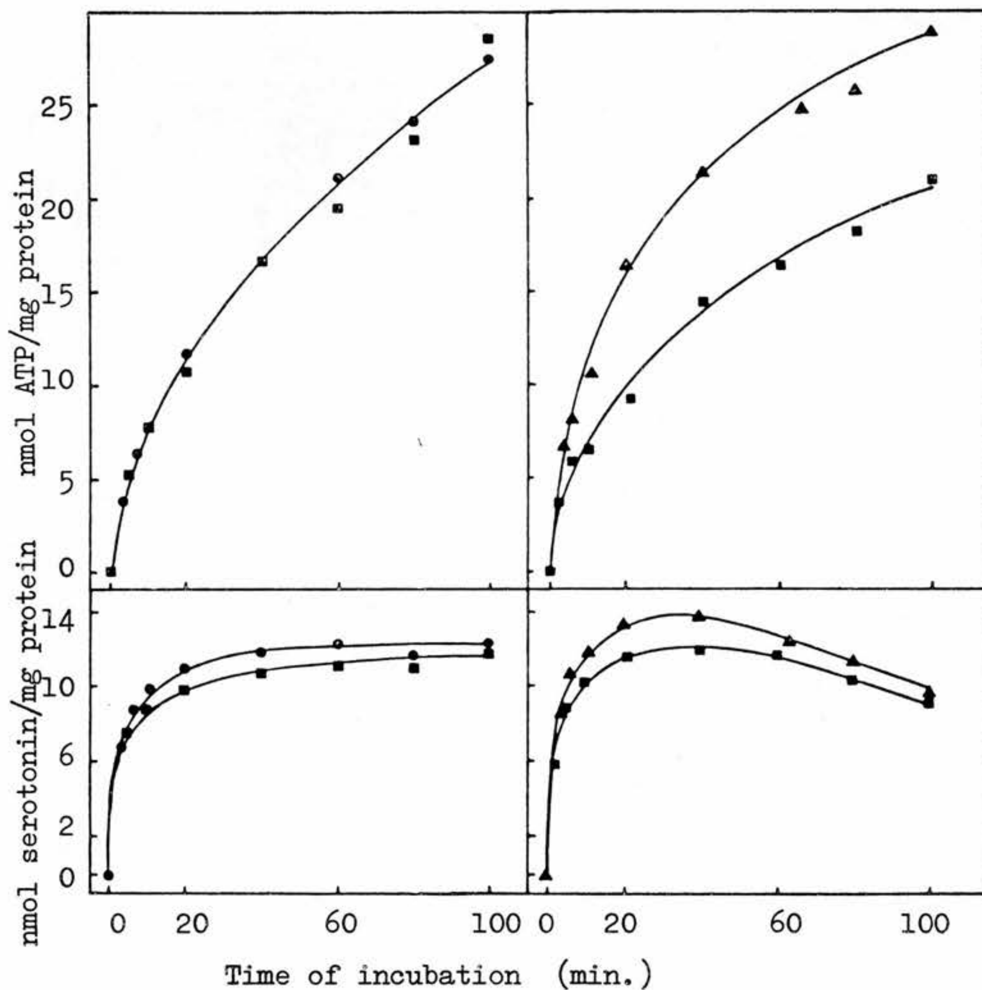
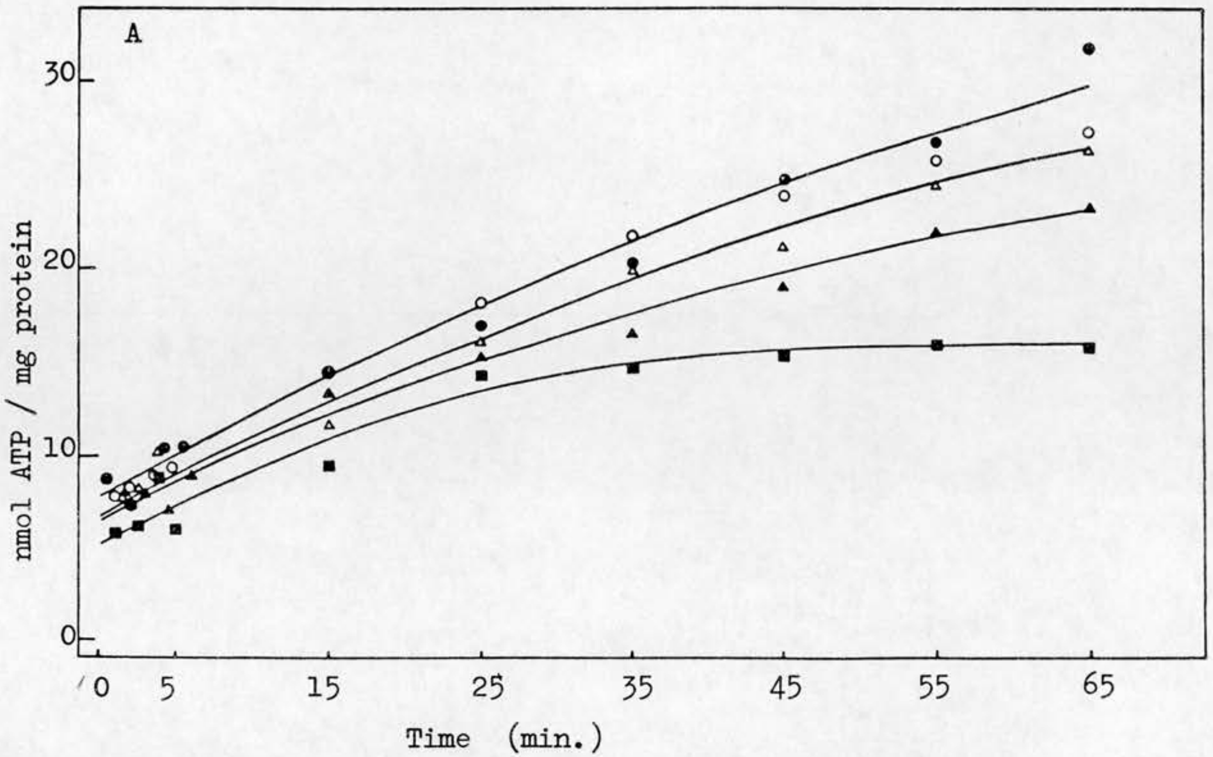


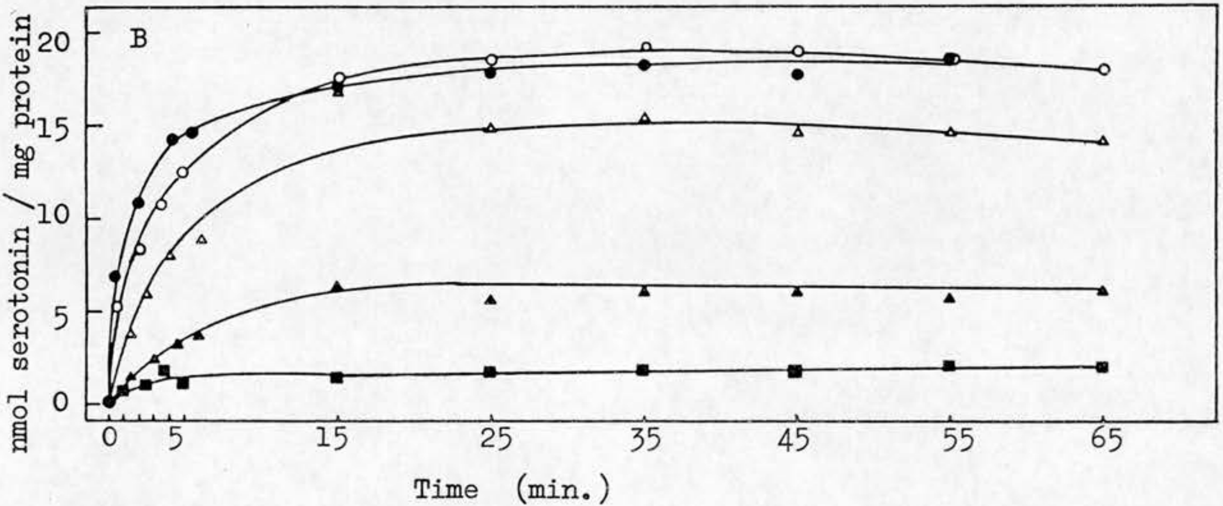
Fig.32: Transport inhibition by atractyloside. ATP (upper figures) and serotonin (lower figures) transport into ghosts is monitored in presence (●) and absence (▲) of PEP and pyruvate kinase in the medium. In both experiments the inhibition by 125µM atractyloside (■) was measured.

Analogues of adenine nucleotides and uptake processes into chromaffin granule ghosts.

A variety of analogues of adenine nucleotides were assesed for their inhibitory effect on transport of ATP into chromaffin granule ghosts. - Having established that all three adenine nucleotides are taken up by the ghosts two substances were checked which are analogues of adenosine rather than adenine nucleotides.

Nitrobenzylthioinosine (NBTI) at submicromolar concentrations inhibits nucleoside transport into erythrocytes (Jarvis & Young, 1980), but no inhibition of ATP or serotonin uptake was observed when used with the chromaffin granule ghosts (Fig.33). The adenosine analogue FSBA (5'-p-fluoro-sulfonyl-benzoyl adenosine) has been used to label the catalytic and regulatory sites of the porcine mitochondrial ATPase (Di Pietro et al., 1979). FSBA at 267µM added as a methanolic solution and measured against a control experiment in presence of methanol (0.5% final methanol concentration) inhibited only the serotonin uptake by about 15%. For some unknown reason the ATP uptake was slightly stimulated but this effect was not observed when ghosts were preincubated with FSBA (500µM FSBA and 1% methanol in preincubation) for 10 min. at 37° C, in which case the extent of serotonin uptake was inhibited by about 40%. Similar results were obtained with PEP and PK present in the medium or when FSBA was dissolved in DMSO. Although FSBA did not inhibit ATP uptake it was used in a radioactive form as an affinity label in an attempt to label proteins involved in serotonin transport. These labelling results



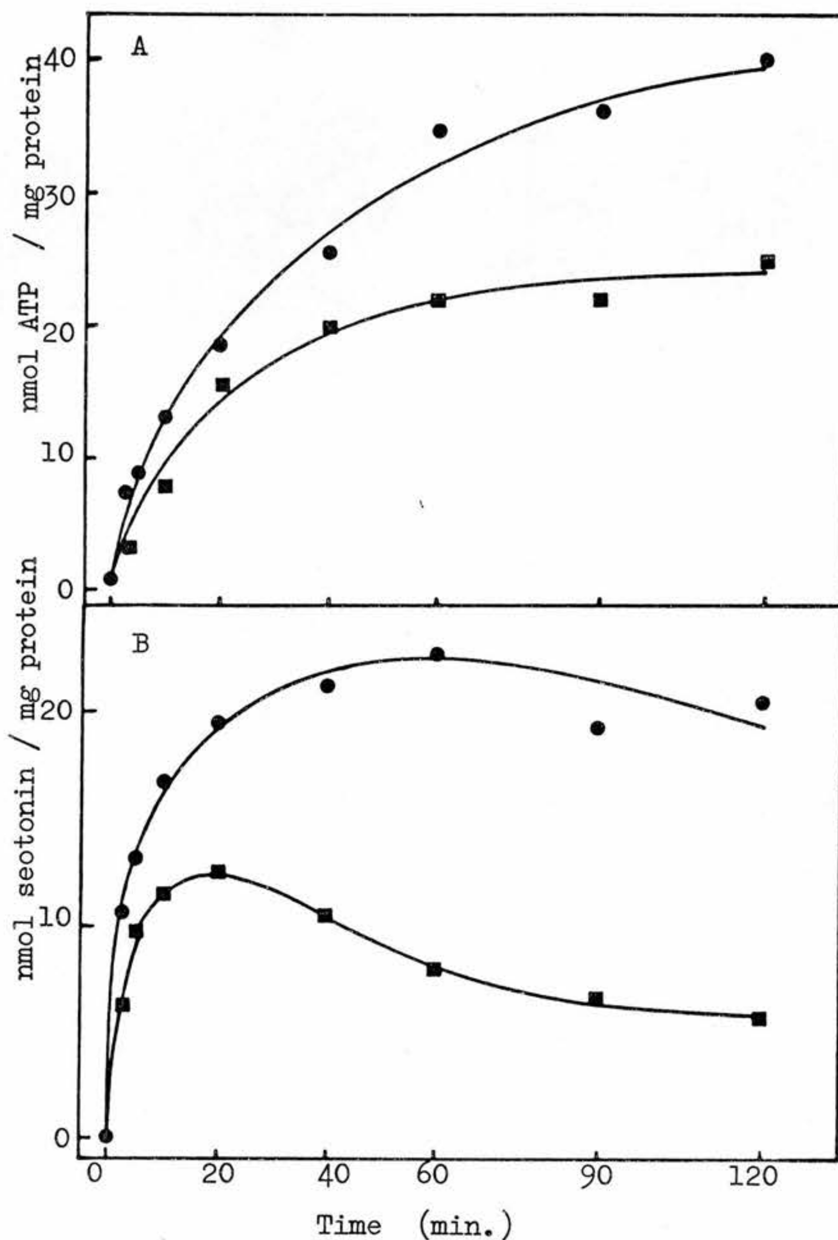


<u>Fig.33:</u> Uptake of ATP (a) and serotonin (b) into ghosts at 37°C in presence of either 250µM (△) and 1mM (▲) carboxyatractyloside, 400µM DIDS (■) or 100µM NBTI (○). Control, no addition, (●).

will be presented in the next section.

Two derivatives of ATP were used successfully as specific inhibitors of chromaffin granule adenine nucleotide transport. The 2',3'-dialdehyde derivative of ATP (Fig.31) has been used as an affinity label for an ATPase from Mycobacterium phlei (Kumar et al., 1979) and the ATP binding site of pyruvate carboxylase (Easterbrook-Smith, 1976). The dialdehyde system of the analogue reacts with amino groups and the label is therefore used as a lysyl group reagent. Dialdehyde ATP of various concentrations between 0.25 and 5mM was tested with ghosts preincubated at 37°C for 20 min. Fig.34 shows ATP uptake in presence of 1mM dialdehyde ATP (giving a final concentration of 140 μM); rapid binding and uptake of ATP were inhibited while serotonin uptake was less affected. Dialdehyde ATP was therefore the only inhibitor, apart from low concentrations of cibacron, which inhibited ATP uptake more strongly than serotonin uptake, an indication that this substance inhibits the ATP transport mechanism directly and not via the ATPase, the possible energy source.

A similarly potent inhibitor was 8-azido AMP (Fig.31). The azide group photolyses upon irradiation with UV light, forming nitrogen and a highly reactive nitrene group. Irradiation can be done with near-UV light (350 nm) at which little damage is done to the proteins. Azido ADP and ATP have been successfully used to label covalently the mitochondrial adenine nucleotide translocator protein (Schaefer at al., 1978), and the β-subunit of the



<u>Fig.34:</u> Effect of dialdehydeATP on the transport of ATP (a) and serotonin (b) into ghosts. Ghosts were preincubated for 20 min. at 37°C in presence (●) or absence (■) of 1mM dialdehydeATP before the uptake was measured at 37°C.

mitochondrial F_1-ATPase (Wagenvoord et al., 1977) respectively. In this study azido AMP was used as an inhibitor because of its commercial availability.

In initial experiments the effect of irradiation with UV-light on the ghosts was studied. The degree to which ghosts retained the ability to accumulate catecholamines was used as a parameter. Batches of 400µl ghosts (1.7mg protein/ml) in Hepes-sucrose buffer were exposed to UV-light in a small quarz cuvette (0.5x0.5x4.0cm). The light source was a Xenon-lamp from a fluorometer (Perkin-Elmer MPF-2A). The ice-cold samples were exposed at 4cm distance from the lamp for 40 s and then cooled on ice for 30 s, with this procedure samples did not warm up above 15°C. Two batches were treated in this way for a total of 4 and 12 min., respectively. The uptake of serotonin into these ghosts was measured in a standard medium which did not contain radioactive ATP. In Fig.36 the result is shown in comparison with a control sample of ghosts which were not exposed to UV-light. The exposure to UV obviously affects the integrity of the ghosts in a dose-dependent manner. To reduce the damaging effect of the irradiation, the exposure time was greatly reduced.

Samples of 1ml ghosts (0.9mg/ml) in Hepes-sucrose buffer were irradiated in four intervals of 15 s with 30 s cooling on ice in between. The samples contained 0.65mM $MgSO_4$ and either 0.65mM AMP or 0.65mM azidoAMP and had been preincubated with these compounds in the dark at 37°C for 10 min. before irradiation. One sample containing AMP was

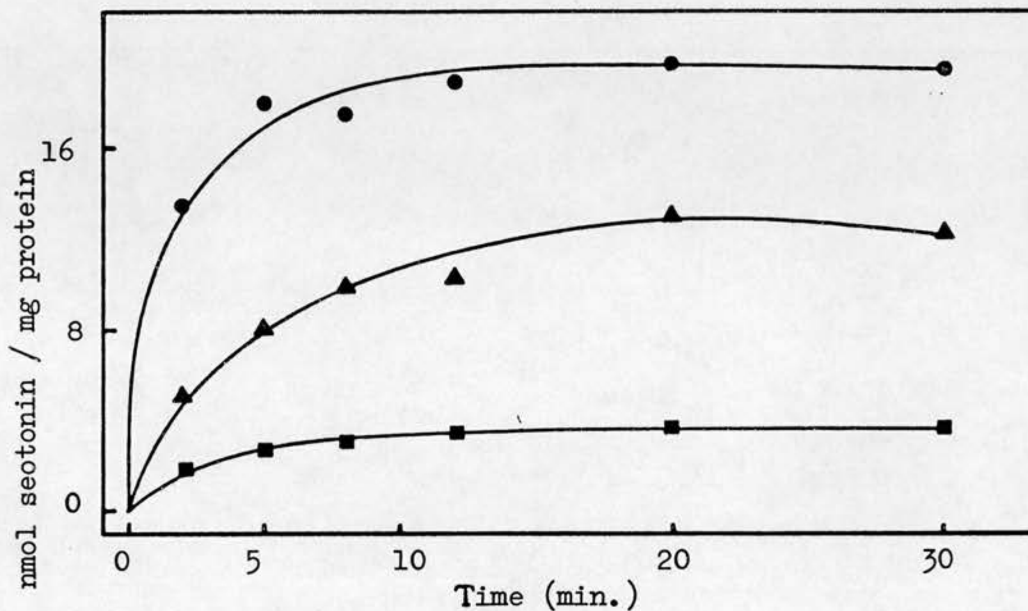


Fig.36: The effect of irradiation with UV light on the serotonin transport into ghosts. Batches of ghosts were exposed for 0 min. (●); 4 min. (▲); and 12 min. (■) to a strong UV light source, serotonin transport was measured as normal.

also preincubated but not exposed to UV and served as a control. The other control-experiment to assess the effect of azidoAMP without irradiation was not performed because the azido compound could also be activated by day-light and therefore experiments ought to have been done in a dark-room which was not feasable. The azidoAMP preparation contains 1mol/mol NH_4^+ and equivalent amounts of $(NH_4)_2SO_4$ were therefore added to the other samples before the transport assays.

Transport of radioactive ATP and serotonin into the three samples of ghosts prepared above was measured in a standard incubation medium and the time-courses are shown in Fig.37. The inhibitory effect the exposure to UV-light had on serotonin uptake (Fig.36) was lost due to a much shorter exposure time, but some inhibition of the uptake of ATP however was observed. AzidoATP inhibited both, ATP and serotonin uptake. The transport of ATP was not affected in initial rate but only in the extent of the uptake observed after the first 10 min. Only very little ATP was incorporated into ghosts treated with azidoATP between 20 and 80 min. This lag of the inhibitory effect was also observed for the action of NEM and FCCP (Fig.26), DCCD (Fig.27), and high concentrations of cibacron etc. (Fig.29). Obviously azidoAMP inhibits a similar part of the ATP transport as those agents.

Both, the dialdehyde - and the azido-analogue of ATP were used for further studies on labelling of chromaffin granule membranes. This is discussed in the next section.

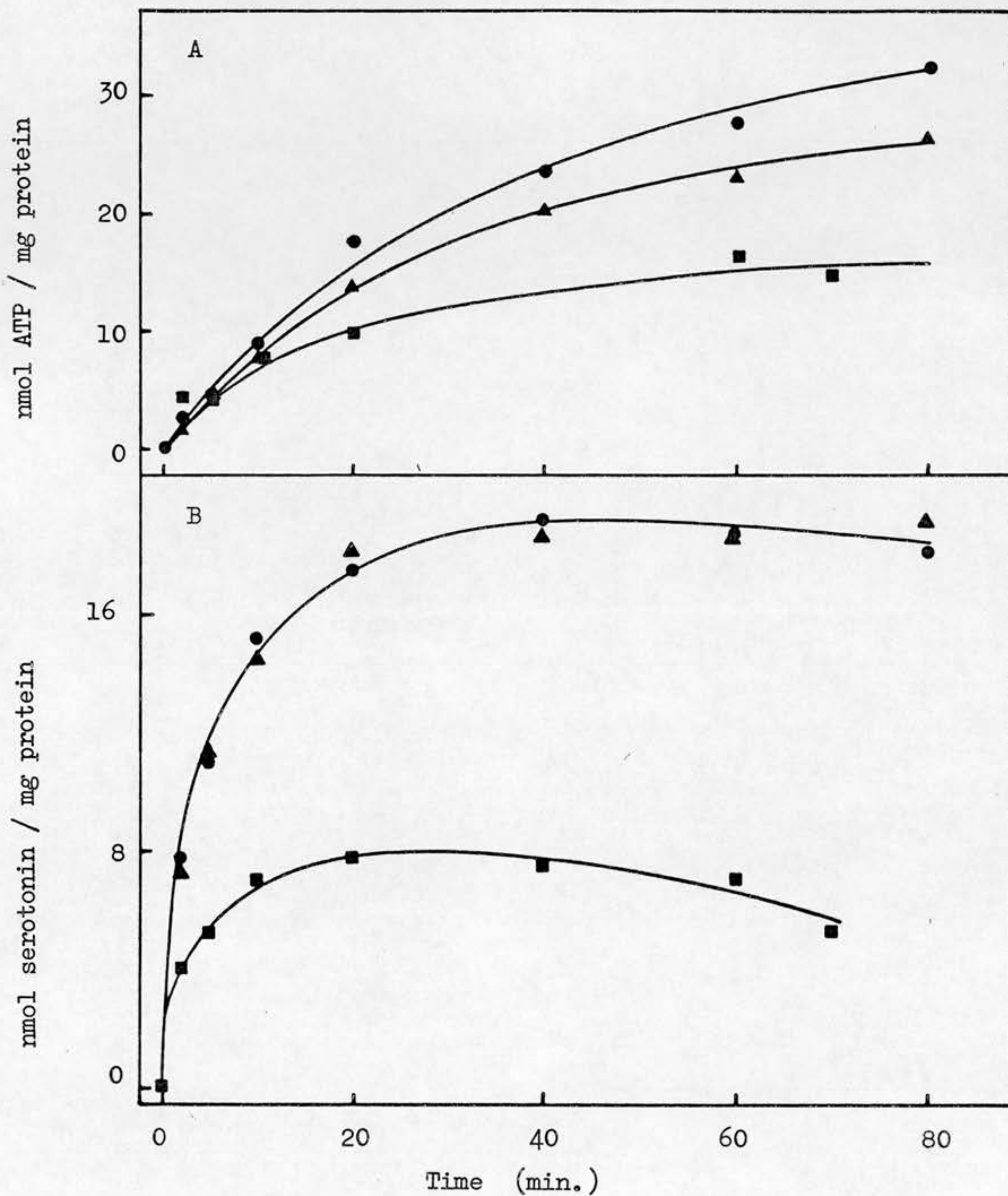


Fig.37: Uptake of ATP (Fig.a) and serotonin (Fig.b) into ghosts at 37°C which were either untreated (●), exposed to UV light (▲) or exposed to UV light in presence of azidoAMP (■).

IV. Phosphoenolpyruvate.

Transport of phosphoenolpyruvate into chromaffin granule ghosts.

As was shown in Fig.1 phosphoenolpyruvate (PEP) inhibited ATP uptake. The degree of inhibition varied somewhat between the experiments and it was shown that the inhibitory effect of PEP was dependent on the pH in the medium (Fig.19). Since PEP itself has only little buffering capacity at near neutral pH (Fig.21) an addition of 10mM PEP from a roughly neutralized 1M stock solution should not influence the pH of the medium too much, although Hepes was normally only present at 10mM. The variable extent of ATP uptake observed in presence of PEP in the various experiments might be indeed due to variations in the pH of the media, but some inhibition was always observed in presence of PEP. Since PEP is quite likely to be transported through the chromaffin granule membrane as well, ghosts were incubated with radioactive PEP to measure possible uptake. Fig.38 shows the uptake of (^{14}C)PEP into ghosts in presence of various MgATP concentrations. The time courses of PEP uptake were measured as described before for ATP and serotonin, with the incubation medium slightly altered: 10mM NaHepes, pH 7.2, 0.3M sucrose, 200μM PEP (0.5mCi/ml), and ATP and $MgSO_4$ were present in equimolar amounts at concentrations between 20μM and 2.5mM.

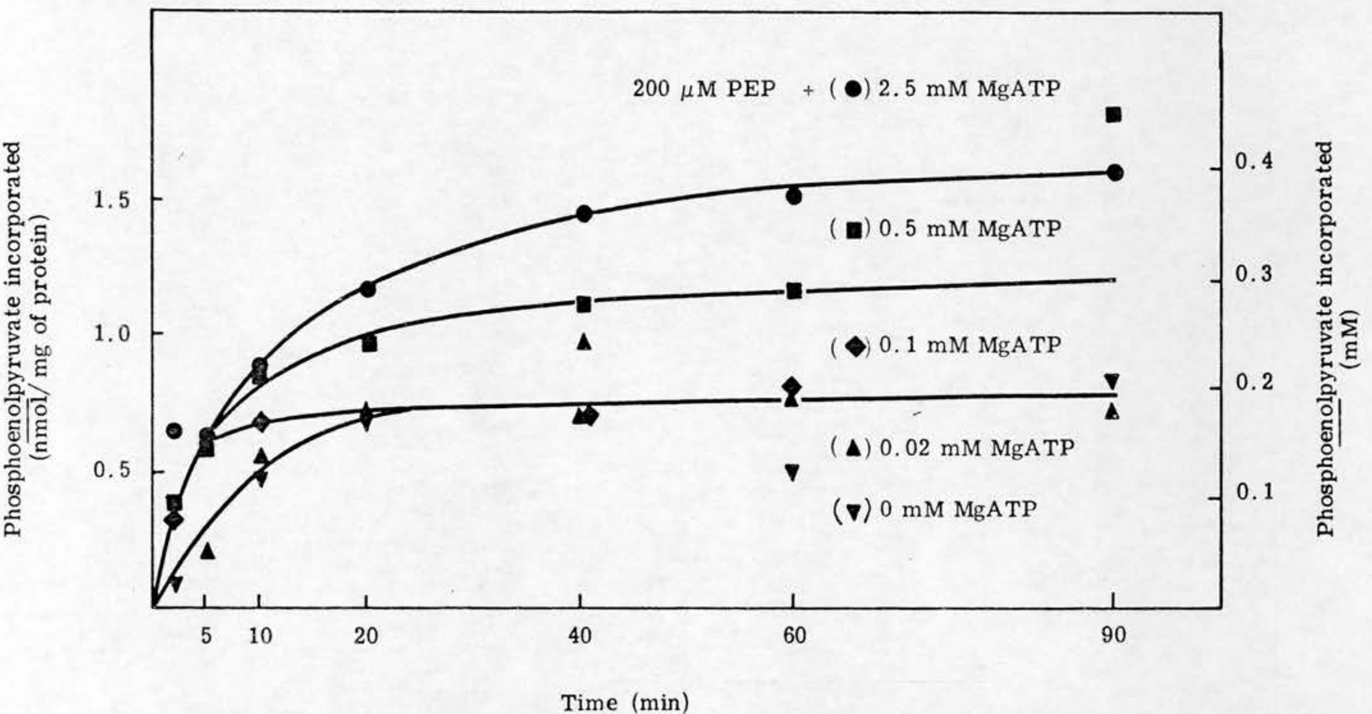


Fig.38: Uptake of (^{14}C) phosphoenolpyruvate into chromaffin granule ghosts at 37°C. Incubation media contained 200μM PEP and MgATP as indicated in the figure. The concentration of PEP obtained within the ghosts is also indicated.

PEP was transported into chromaffin granule ghosts in a temperature and energy dependent process. The uptake at 0° C was negligible regardless of the presence or absence of ATP. At 37° C in absence of MgATP or at very low MgATP concentrations (20µM) PEP is transported into ghosts at the same rate (56pmole PEP/min/mg), a plateau is reached after about 30 min. which accounted for a concentration equilibrium of PEP across the ghost membrane. At 100µM MgATP the initial rate of PEP uptake was increased to 154 pmole/min/mg but not the plateau level. Further additions of MgATP had no effect on the initial rates but the plateau values could be increased to give a maximal 2-fold concentration gradient of PEP inside the ghosts against the medium. This was achieved at 2.5mM MgATP and could not be increased by futher addition of MgATP. As shown in Fig.39 is the uptake of PEP not further stimulated when MgATP is five-fold increased to 12.5mM. This also sheds light on the question of competitive inhibition of PEP uptake by ATP: principally PEP seems to inhibit ATP uptake as discussed before but apparently PEP uptake is not inhibited by high ATP concentrations. This might suggest that PEP has the higher affinity for the putative ATP transport mechanism than ATP itself. An analogous observation has been made for the transport mechanism of catecholamines into chromaffin granule ghosts, which has a greater affinity for the non endogenous substrate serotonin than for adrenaline and noradrenaline, the substrates in vivo (Phillips, 1974b). When ghosts labelled with (^{3}H)ATP and (^{14}C)PEP were analyzed on a sucrose gradient in a similar experiment as described

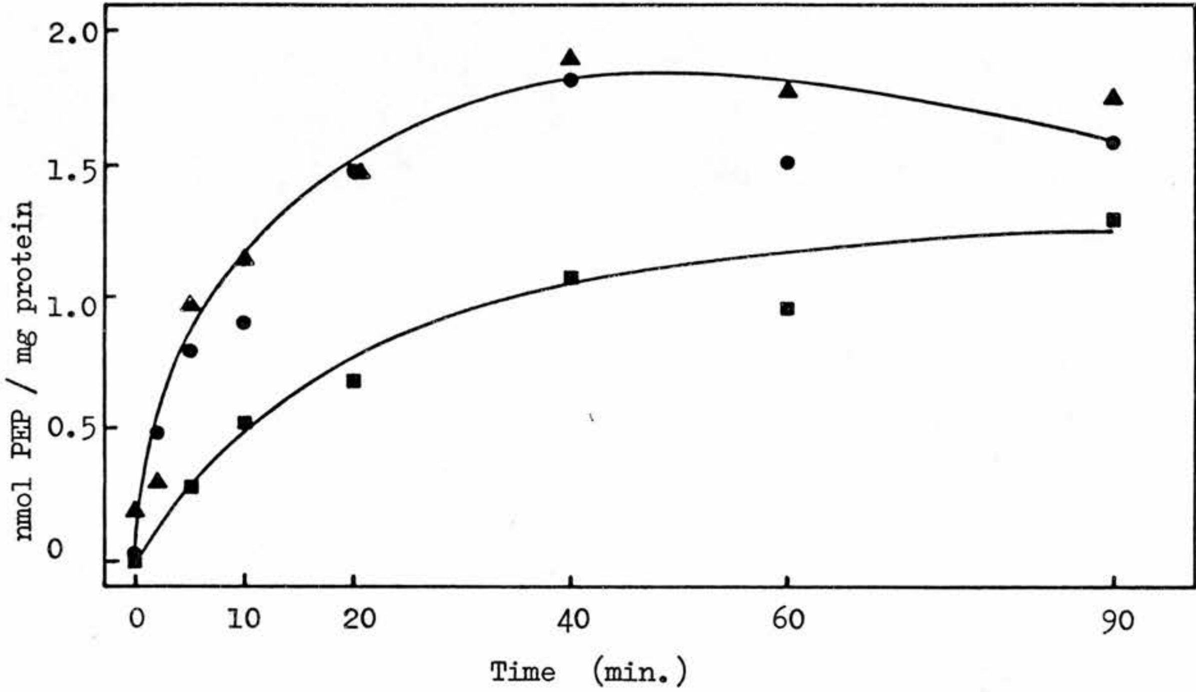


Fig.39: Uptake of PEP into ghosts at 37°C in presence of 2.5mM ATP and 2.5mM $MgSO_4$ (●). This medium was supplemented with another 10mM ATP and 10mM $MgSO_4$ (▲) or 200µM atractyloside (■). PEP in the medium was 200µM.

in Fig.12a comigration of the two radioactivities to similar densities was observed which showed that the two substances are incorporated into the same vesicles (data not shown).

Fig.39 also shows that atractyloside (ATR) at 200μM reduces the rate of PEP uptake to about a third of the control experiment. This means that PEP uptake is more susceptable to atractyloside than ATP uptake. However the fact that both transports are inhibited by ATR is a further indication that PEP is transported by a mechanism which facilitates at least part of the transport of ATP into the ghosts.

The PEP uptake resembles ATP uptake in that there is no large concentration gradient achieved and that a concentration equilibrium across the vesicle membrane can be obtained in absence of an energy source. The clear MgATP dependence of the additional uptake of PEP which results in a two-fold concentration gradient is in some contrast with the observations made for adenine nucleotide transport itself where no clear energy dependence could be shown but where up to two fold concentration gradients were in fact achieved. Scattering of measured time points was much less in time courses with (^{14}C)PEP and the rapid binding observed with (^{3}H)ATP appeared to be absent. On the whole it was easier to obtain clear results with PEP uptake and it was for that reason that its characterisation was pursued further. Especially the question about ghosts energization was better defined for PEP because the transported substrate was not at the same time the substrate for the ATPase as in

ATP uptake experiments. It ought to be kept in mind however, that although not monitored as radioactive uptake, one has to assume that any ATP added to the medium is transported into the ghosts concomittantly with PEP and that the question about competition remains therefore unsolved.

Characterisation of PEP transport.

To investigate whether the uptake of PEP is saturable, initial rates of PEP transport into ghosts were measured at concentrations between 50µM and 2mM. As for ATP, the initial rates of PEP uptake increased linearly with the increased substrate concentration, so there is no indication that the transport process can be saturated. This is shown in Fig.40, where PEP was present at a constant specific radioactivity of 1.67mCi/mmol. To measure unspecific binding to the filters, samples containing equivalent amounts of radioactivity but no protein, were filtered. This binding is shown as uptake at 0 min. and not subtracted as background like in most other experiments. The rates of uptake were determined from the linear part of the curves measured between 2 and 10 min. In Fig.41 the linear increase of the rates of the PEP uptake with increasing substrate concentration is shown. The data were obtained from two different preparations of ghosts. The fact that PEP uptake was not saturable, was taken as a further indication that it might cross the membrane in the same way as adenine nucleotides.

Since PEP uptake was partially energy dependent as shown

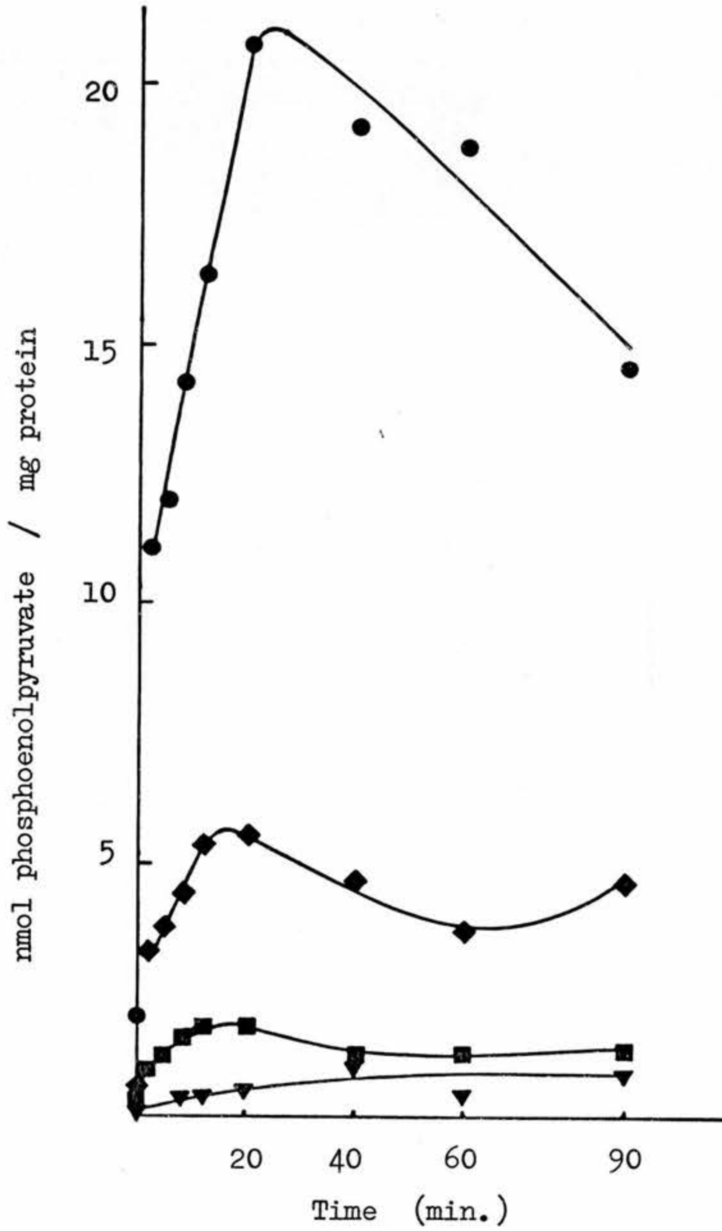
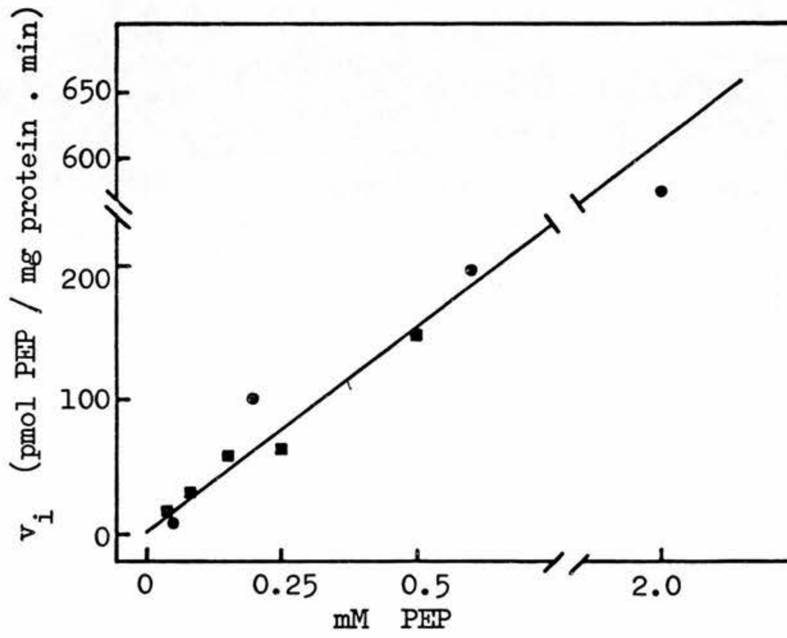


Fig.40: Uptake of PEP into ghosts at various PEP concentrations in the medium: 2mM (●), 600μM (◆), 200μM (■), and 50μM (▼); ATP (5mM) and $MgSO_4$ (2.5mM) were kept constant.



<u>Fig.41:</u> Effect of PEP concentration in the medium on the initial rates of PEP uptake into ghosts. Data are from two independent experiments (■) and (●).

in Fig.38, further experiments were done with agents which inhibit the energization of ghosts. In Fig.42 the uptake of 200µM PEP into chromaffin granule ghosts was measured in presence of 2.5mM MgATP and a two-fold concentration gradient was established within 30 min. In presence of the mitochondrial uncoupler FCCP (10µM) the uptake was reduced to 50% of the control and was similar to the uptake observed in absence of MgATP in Fig.38. This showed clearly that there is an unenergized equilibration of PEP plus an energized uptake across the ghost membrane. Uptake was also measured in presence of 10mM $(NH_4)_2SO_4$ or 10mM KSCN, and only the permeant anion SCN^- was inhibitory whereas ammonia ions had no inhibitory effect. This indicated that a part of the PEP uptake is indeed dependent on ΔP but not on ΔpH. The slight stimulation of ammonia might be due to a stimulation of the proton pumping activity of the ATPase which is observed when the pH-gradient is abolished and the electrical gradient increases which in turn could stimulate the PEP uptake slightly.

When the transport of PEP was measured in a standard medium which was supplemented with 5mM NaI and additional lomM Hepes neutralized with Tris-base to pH 7.2, a stimulated uptake was observed (Fig.43a). Within 60 min. at 37°C PEP equivalent to an internal concentration of 1.1mM was obtained with 200µM PEP in the medium. This stimulation was not observed when I^- was substituted by SCN^- (10mM KSCN), an other membrane permeant anion. Control experiments also showed, that the stimulation was not due to cations of the added salt. A smaller stimulation was observed

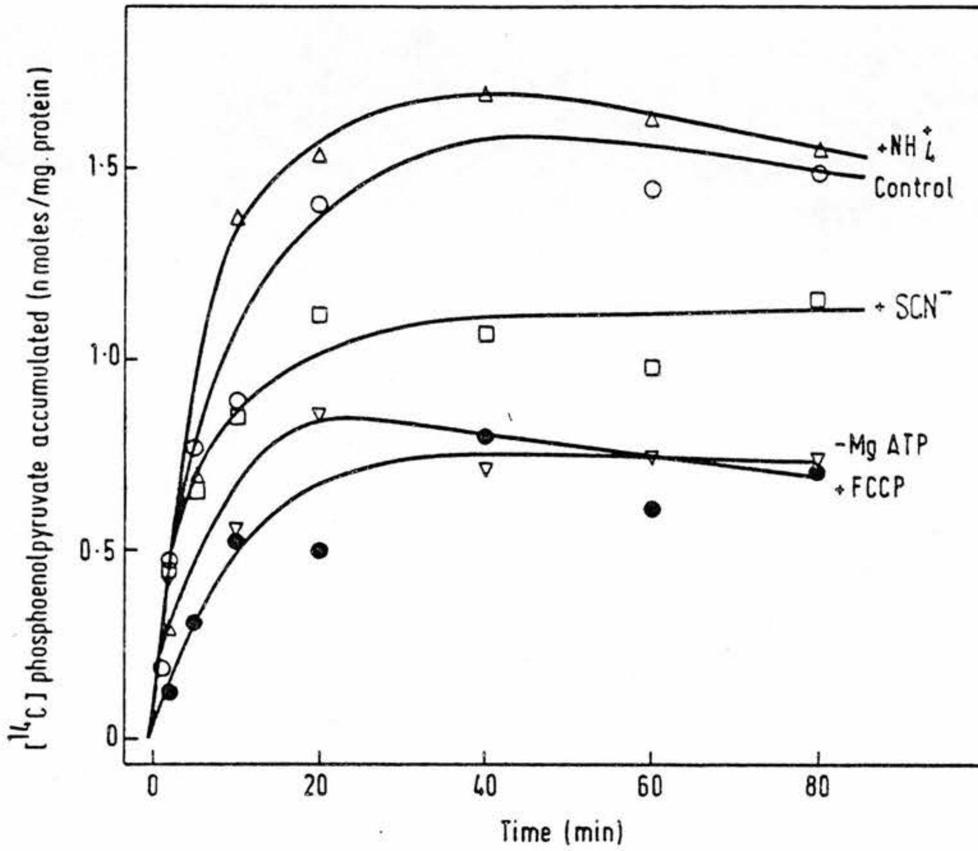
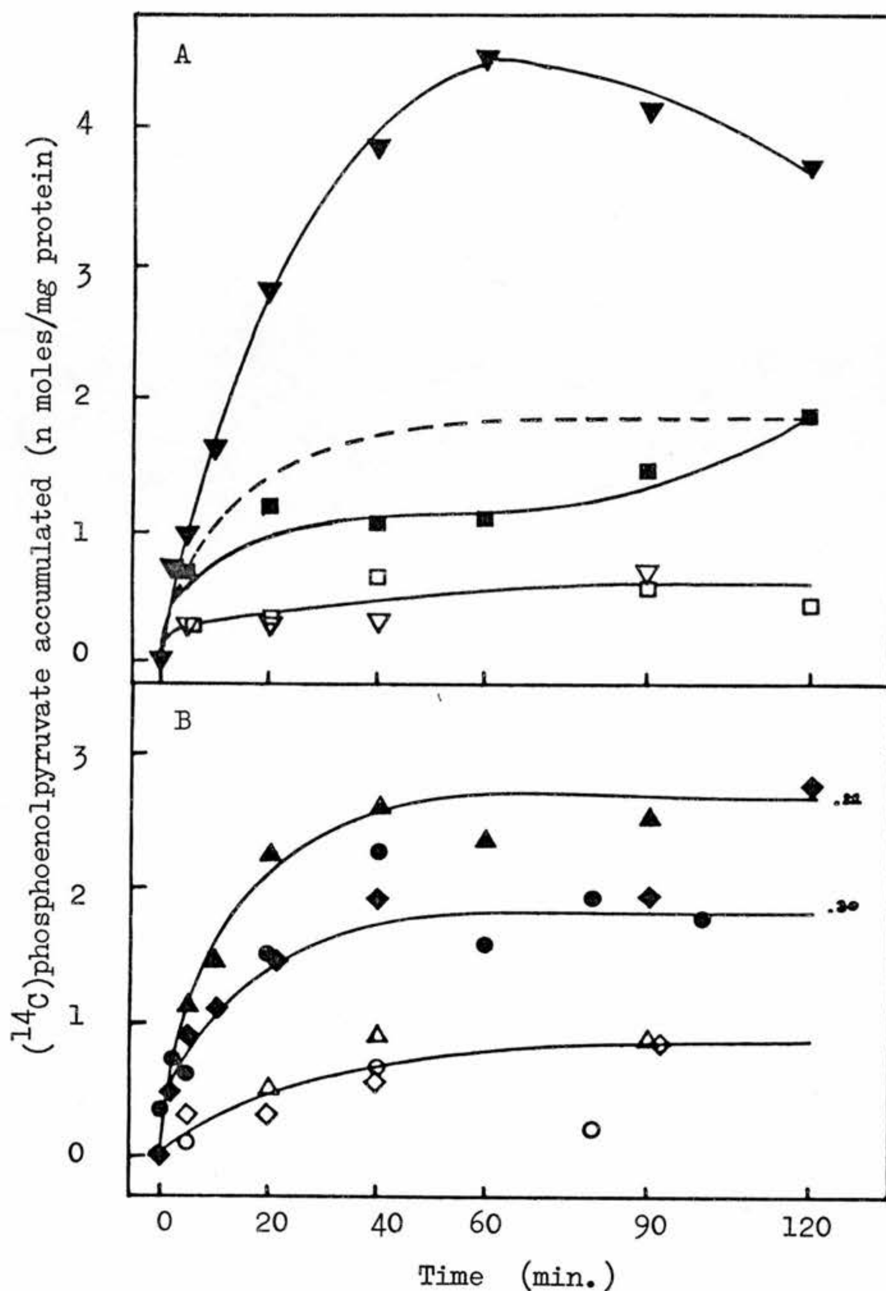


Fig.42: PEP uptake into ghosts in presence of uncoupling agents; 10mM $(NH_4)_2SO_4$ (△), 10mM KSCN (□), 10μM FCCP (▽), no addition (○); all media except one (●) contained 2.5mM MgATP.



<u>Fig.43a:</u> Effect of various ions on PEP uptake into ghosts at 37°C (solid symbols) and 0°C (open symbols). Standard incubation media contained additionally 10mM TrisHepes and were supplemented with 5mM NaI (▼,▽), or 10mM KSCN (■,□). The dotted line denotes a control curve under standard conditions as shown in Fig.43b.

<u>Fig.43b:</u> Stimulation of PEP uptake into ghosts incubated in media of lower osmotic density at 37°C (solid symbols) and 0°C (open symbols). The media contained sucrose at a concentration of 0.3M (●,○); 0.26M (◆,◇); 0.22M (▲,△).

in presence of higher concentrations of Tris – Cl (10mM, pH 7.2) and absence of NaI. A possible interpretation, that the uptake is due to swelling is unlikely, since these observations could not be made for the uptake of ATP and because KSCN did dot show the same effect. Also when the ghosts were swollen by suspension in a medium with a lowered osmolarity (0.22M sucrose), the stimulation of PEP uptake was much smaller and showed a different time course (Fig.43b). The reason for the stimulation by NaI remained unclear.

V. Discussion.

These kinetic experiments were performed to investigate the mechanism by which chromaffin granules obtain the high concentrations of adenine nucleotides found within the matrix in vivo. Lysed and resealed granule vesicles, ('ghosts'), were used as a model system in an attempt to eliminate transport processes which are either due to binding of the nucleotides to proteins (chromogranins) within the granules, or due to exchange of radioactively labelled adenine nucleotides in the medium with unlabelled endogenous material from the granule matrix. The aim was to investigate in ghosts the existence of an active uptake mechanism for adenine nucleotides, presumably powered by the electrical part of the electrochemical proton gradient, as had been proposed for intact granules by Aberer et al. (1978). The experiments were performed with a filtering technique in analogy to the investigations on the transport of catecholamines into chromaffin granule ghosts introduced by Phillips (1974a).

Initial experiments showed that radioactive ATP indeed crosses the chromaffin granule membrane although the passive anion permeability of the chromaffin granule membrane is low as reported by Phillips (1977). This permeability of the membrane to ATP had already previously been observed in ghosts (Phillips & Morton, 1978; Taugner et al., 1979a,b) but no further characterisations have been reported in the literature since.

At the beginning of the present investigations it was assumed that a large molecule with several negative charges, such as ATP, cannot permeate through a lipid bilayer membrane without the involvement of a facilitating protein. Therefore the parameters of an enzymatic transport activity were tested in the uptake experiments initially. Such an enzymatic process ought to show substrate-specificity and saturation kinetics and, in the case of an active uptake, an energy requirement. It should also be possible to inhibit the process by specific agents.

The presented results show that ATP is taken up into ghosts in a temperature-dependent process. The two other adenine nucleotides, ADP and AMP are taken up as well and to the same extent as ATP. The transport process appears to act on several adenine nucleotides, rather than on ATP only, although this is the major nucleotide in the matrix in vivo. The ability of the granule membrane to transport all three adenine nucleotides is in agreement with the observations made with intact granules (Aberer et al., 1978).

The data obtained in this study do not suggest that the phosphorylation state of the nucleotides changes during the uptake process to obtain a more membrane-permeant substrate; furthermore in none of the experiments was adenosine or adenine found either in the incubation medium or in the ghost interior. When AMP-transport was studied only AMP was found inside the ghosts. Under conditions where the medium contained exclusively ATP during the whole incubation period, ATP as well as ADP, but no AMP or adenosine were found inside the ghosts. The presence of ADP however might

be due to some hydrolysis during the analysis after the uptake. That the adenine nucleotides are transported into chromaffin granule ghosts and not into contaminating mitochondria was shown by analysis of the particulate material on sucrose density gradients after uptake experiments. ATP comigrates with the granule membrane enzyme DBH and with serotonin which is transported into the ghosts together with ATP in double labelling experiments. The absence of mitochondria in ordinary uptake assays was also shown. This absence of mitochondria is an advantage of the ghost preparation introduced by Phillips (1974a), in contrast with experiments done with intact granules. Granule preparations are not free of mitochondria during the incubation with radioactive transport substrates and have to be further purified after the uptake. It is not known what effect this procedure has on the kinetic studies: for example, hydrolysis by mitochondrial ATPase might be extensive under some conditions. Also in Taugner's investigations on adenine nucleotides and chromaffin granule membranes (Taugner et al., 1979a,b) it is by no means clear whether mitochondrial activities can be excluded from the observations made.

In most experiments the uptake into ghosts is observed in two phases. There is an initial rapid process which lasts for the first 5 to 10 minutes, and a subsequent slower process which lasts for about 3 hours. In some experiments the fast initial event is not observed and the slower uptake starts at much higher values of (^{3}H)ATP associated with the ghosts when the curve is extrapolated back to 0 min.

Representative curves of the two uptake patterns are drawn in Fig.44a and b. To simplify the curve in Fig.44a, it could also be drawn as a combination of two lines, the first representing the initial fast event, and the second the subsequent slow event which is also seen in Fig.44b. The fast initial event from Fig.44a can be interpreted as being in Fig.44b so fast that no resolution on the time scale is obtained. The higher 'background' therefore represents the fast initial event. From the effects temperature and inhibitors have on the two phases of the ATP uptake, and on the sensitivity of accumulated ATP to lysis in a hypoosmotic medium, it is suggested that both curves in Fig. 44 essentially represent the same process. The initial rapid uptake appears to be a temperature sensitive binding of ATP to the ghost membrane since it is not lysis sensitive. Only the ATP accumulated during the slow kinetic phase appears to be taken up into the ghost interior.

In several experiments ATP uptake was measured in presence of different ATP concentrations. The initial rates of the uptake always increased linearly with increased substrate concentrations. The measured rates probably represented a mixture of the rapid binding as well as the lysis sensitive uptake. It was not possible to measure initial rates of lysis sensitive uptake as a function of the ATP concentration on its own; nevertheless the time courses taken over two hours which describe mainly this uptake did not suggest a saturable process, for ATP concentrations between 50µM and 20mM. The uptake of phosphoenolpyruvate was also not saturable, for substrate concentrations between 50µM and 2mM.

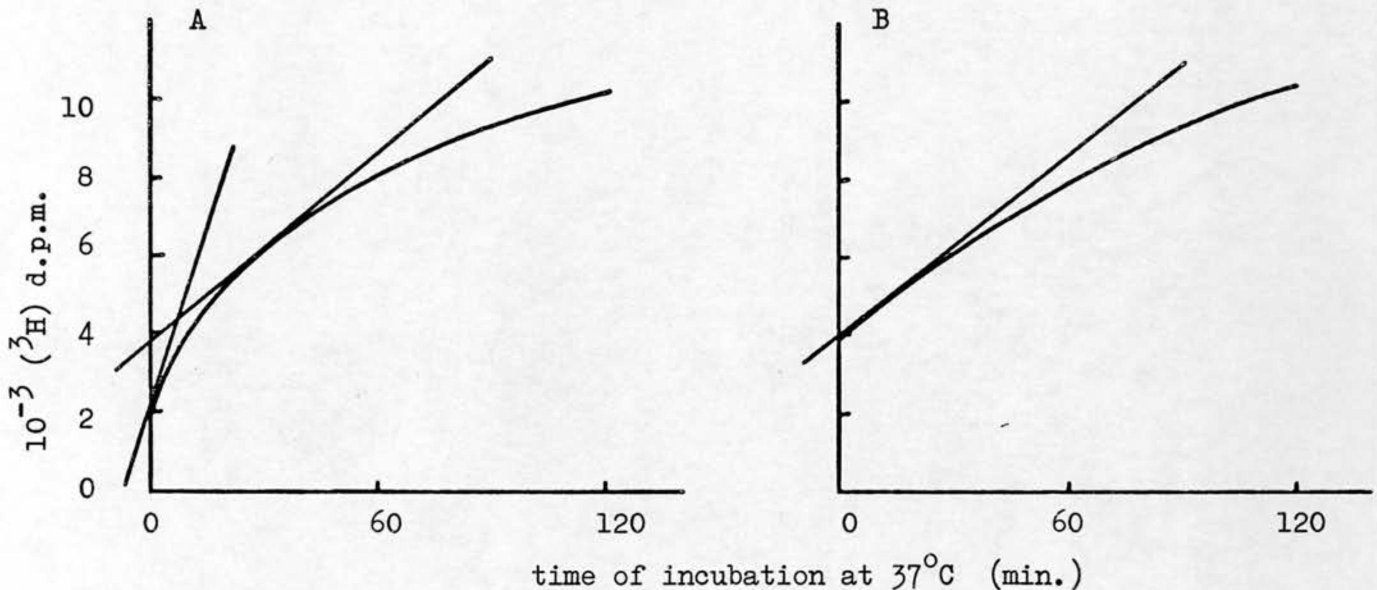


Fig.44: Representative curves of the normally observed (^{3}H)ATP uptake (in d.p.m.) into chromaffin granule ghosts. The left figure (44a) shows the two phases of uptake, the initial rapid, and the subsequent slower one. The right figure (44b) shows the second phase only, but that curve intercepts the y-axis at a higher d.p.m. value.

The total amount of ATP which was accumulated into the ghosts always accounted for 1-2 times the concentration present in the medium. For these calculations a ghost volume of 4µl/mg protein was assumed (Phillips 1977). Phosphoenol-pyruvate was taken up to two times the medium concentration in presence of MgATP and only to a concentration equilibrium in absence of MgATP or when agents were added which inhibit the energisation of the ghost membrane.

The two observations, that ATP uptake is not saturable and that no large concentration gradients are established, do not support the proposition that ATP is transported in an active, carrier mediated process. ATP might be taken up in an energy-independent mechanism, but if any protein is involved in the transport, saturation kinetics ought to be observed. Otherwise it has to be proposed that ATP diffuses freely through the granule membrane. Perhaps saturation kinetics could not be observed because the Km of the uptake process is very much higher than the concentrations of ATP with which the experiments were performed; this would mean the process has a Km of about 30mM or higher.

Experiments in presence of EDTA and variable concentrations of Mg++ suggested that the free ATP anion is the transported molecule since the ATP uptake was slightly increased when lower Mg++ concentrations were present in the medium. This is in contrast to the ATPase in the membrane which uses MgATP as a substrate.

Because the transport of ATP and PEP as well as ADP and AMP was not saturable and formed only a maximally two-fold concentration gradient, it is possible that they share the

same mechanism of uptake. The inhibition of ATP uptake by PEP could be explained if PEP has a higher affinity for the transport mechanism than ATP and therefore inhibits the uptake competitively; however the data could equally well be explained by separate uptake mechanisms, in which PEP inhibits ATP uptake, but not vice versa.

The lysis-sensitive uptake of ATP could be partially inhibited by atractyloside and protein-modifying agents like NEM and DCCD. Although an inhibition by the latter two compounds is sometimes used as an indication of an involvement of the proton pumping ATPase in a process, in as much as they inhibit this enzyme, ATP uptake is unlikely to depend on the ATPase. Uncouplers and other inhibitors of the electrochemical membrane potential which is formed by the ATPase had little or no inhibitory effect on the ATP uptake. Furthermore ADP and AMP are taken up by the ghosts like ATP under conditions where the membrane is unenergized, as shown in double labelling experiments with serotonin as a control for the energy-dependent catecholamine uptake. However, part of the phosphoenolpyruvate uptake can certainly be inhibited by uncouplers. By limiting the amount of MgATP, the rate and extent of PEP uptake are reduced. In contrast to catecholamine uptake where the membrane energisation achieves a large concentration gradient is the transport of PEP only slightly stimulated in presence of MgATP.

Because of the potential differences on either side of the energized membrane some uptake might occur due to electrostatic effects which would appear to be energized uptake. However if nucleotides would equilibrate according to the membrane charge potential $\Delta\Psi$, which is about 70mV in

energized ghosts (Apps et al., 1980a) a concentration gradient for ATP of at least 10^4-fold ought to be obtained, if the transported species is ATP^{4-} (Phillips & Apps, 1980).

Weber & Winkler (1981) have observed that nucleotide uptake into intact granules consists of a passive diffusion component and an energy-requiring component. They assessed diffusion by its insensitivity to the presence of a high concentration of the permeant anion SCN^- in the medium, the part of the ATP uptake which could be inhibited in this way being considered to be active, $\Delta\psi$-dependent uptake. In the present experiments it was not possible to inhibit ATP uptake into ghosts slectively with SCN^-, indicating that the uptake is not energized by $\Delta\psi$.

Strong inhibition of ATP uptake into ghosts was found in presence of cibacron blue and other polysulfonated aromatic compounds. They not only inhibited the lysis-sensitive uptake but also the rapid binding. The two processes showed different behaviour with respect to varying inhibitor concentrations or varying temperatures of incubation which adds to the evidence that the two are independent processes.

Atractyloside and carboxyatractyloside were only slightly inhibitory to nucleotide uptake in the ghosts. Weber & Winkler (1981) observed a significant inhibition in intact granules and concluded from this observation that the uptake of ATP into chromaffin granules is related to the adenine nucleotide exchange in mitochondria. When assessing observations of ATP uptake into intact granules it has to be remembered that they already contain high concentrations of ATP and that therefore the observed uptake is likely to be an exchange process. The amount of ATP taken up into intact

granules is only about 1% of the ATP which is already present within the granules. Under these circumstances it is probably misleading to speak of accumulation of ATP into intact granules, as is often done.

In ghosts it was shown with AMP that the transport of nucleotides is a dynamic process also when the apparent uptake has reached a plateau. At this steady-state the influx of AMP, which is only slightly lower than at the beginning of the time course equals the efflux of AMP. This suggests that AMP might also at the beginning of the time course be transported via an exchange process with residual endogenous nucleotides present in the ghosts.

However, since the amount of residual nucleotides associated with ghosts is low compared with the substrate concentrations used in the uptake experiments, a saturation of the uptake process ought to have been observed if ATP transport occured via exchange only.

Although therefore the experiments in ghosts and granules cannot really be compared, it is interesting to note that for instance the pH-profiles of ATP uptake are virtually identical for ghosts (Fig.20) and intact granules (Aberer et al., 1978).

All the results discussed so far put together do not give a coherent picture of the adenine nucleotide transport into chromaffin granule ghosts, and there are several controversial observations which are difficult to interpret. Serotonin uptake which was measured together with the ATP uptake into ghosts to assess the energisation and the integrity of the ghosts is clearly energy-dependent,

saturable, specifically inhibited by reserpine, and concentration gradients of several thousand can be obtained.

Obviously the situation is not as clear for the uptake of ATP. Although ATP passes through the ghost membrane such concentration gradients as occur in vivo are not achieved. Certainly linked with this observation is the fact that the described uptake seems not to require energy. There is of course the possibility that the ghost preparation is not adequate for ATP uptake, i.e. during the preparation the ghosts lose an active transport activity which granules hypothetically possess in vivo. The serotonin uptake served as a control for the integrity of the ghosts and that enzymic activities like that of the proton-translocating ATPase were present. The putative ATP transport activity would therefore have to be specifically lost during the ghost preparation which sounds at least unlikely.

Alternatively it could be argued that indeed no active transport exists in the ghosts. ATP was not found to equilibrate across the membrane in response to the ATP-dependent $\Delta\Psi$, with which a considerable concentration gradient ought to be reached at the membrane potential difference in energized ghosts.

ATP is already found inside the granules early in their development, and it might therefore not be essential to get it there as fast as catecholamines and therefore the granules might not have a similarly active transport system for ATP. Catecholamines are also stored at a much higher concentration gradient, so possibly their leakage rate is higher as well. Furthermore a specific membrane traffic for catecholamines is necessary due to the location of the DBH.

The gradient of ATP is likely to be only about 100-fold between cytoplasm and granule interior. Additionally, n.m.r. studies of intact granules suggest that ATP is not as free in solution inside the granules as the catecholamines. It might therefore be possible that ATP passes through the membrane and is removed from the equilibrium by binding inside the ghosts. In this case experiments would be useful in which ghosts are resealed with the essential binding substrate in their matrix. However, even if ATP is taken up in an unenergized process, but with the involvement of a facilitating protein, an uptake kinetic which shows saturation would still be expected, contrary to the results obtained in the present investigation.

RESULTS PART 2: AFFINITY LABELLING OF CHROMAFFIN GRANULE
& DISCUSSION
PROTEINS.

Introduction.

In order to identify proteins which might play a role in the transport of adenine nucleotides and other metabolites across the chromaffin granule membrane, labelling experiments with radioactive substances were performed. The substances were selected from the group of agents which had inhibited the transport activities of chromaffin granule ghosts in the kinetics experiments discussed in the last section. Only such inhibitors which could be expected to act at the transport-facilitating structures directly and bind irreversibly to the protein(s) in question could be considered as affinity labels. Furthermore, the inhibitors had to be available in a radiolabelled form, either through synthesis or from a commercial source. Such chosen substances were used to label chromaffin granule ghosts or membranes, lysate proteins and intact chromaffin granules under various conditions. The labelled proteins were then analyzed and where possible identified using gel-electrophoretic techniques.

Proteins were separated according to their molecular weight on SDS — polyacrylamide gels containing exponential gradients of 6-15 or 10-15% acrylamide to vary the pattern of separation. Gels were run in presence or absence of

reducing agents capable of cleaving cystine bonds. Proteins were also analysed in two dimensions, using an electrofocussing/electrophoresis system which separates proteins first according to their charge and then according to their molecular weight. A few proteins in the patterns obtained were identified using specific antibodies.

Labelling with chloronitrobenzofurazan.

In the kinetic experiments discussed in the last section, 7-chloro-4-nitrobenzofurazan (Nbf-Cl) inhibited a part of the ATP transport into chromaffin granule ghosts, but it was not possible to tell whether this happened via inhibiting the actual transport-facilitating protein. In published investigations Nbf-Cl has been used to label purified F_1-ATPase from various sources like beef-heart mitochondria, chloroplasts and bacteria (Gregory et al., 1981).

Detailed studies with beef-heart mitochondrial F_1-ATPase suggest that the enzyme is inactivated by a covalent modification with Nbf-Cl (Ferguson et al.,1975). The reagent is believed to bind stochiometrically to a single tyrosine residue of the ATPase β-subunit. This binding however is reversed by sulfhydryl reagents; in their presence the Nbf-group is transferred from the tyrosyl oxygen to the thiol. It has also been shown that labelling of the ATPase with Nbf-Cl is pH-dependent (Ferguson et al., 1975). When the modified protein is incubated at pH 9.0, the nitrobenzofurazan-group undergoes an intramolecular transfer from the tyrosyl-group to an amino-group of the enzyme. This new bond is not affected by sulfhydryl reagents. This behaviour of the label is important in view of analysing modified proteins on SDS-gels, which are

normally run at pH 8.8 in presence or absence of the reducing sulfhydryl reagent mercaptoethanol. Although only the β-subunit of isolated F_1 – ATPase is labelled in stochiometric amounts, the other subunits seem to get labelled as well, at least to some extent (Gregory et al., 1981). Although these findings were made during labelling of a single purified protein, they ought to give some ideas on how to perform and interpret the labelling of a mixture of membrane-bound proteins as in the case of chromaffin granules.

<u>Labelling of chromaffin granule membranes.</u> Freshly prepared chromaffin granule membranes (2mg in 2ml 50mM NaHepes, 10μM EDTA, pH 7.4), were incubated with 4μCi (^{14}C)Nbf-Cl (109mCi/mmol) at 4°C in the dark for 12 hours with gentle stirring to prevent a sedimentation of the membranes. After the incubation the volume was adjusted to 10ml with 50mM NaHepes, pH 7.4, and the labelled membranes were separated from the medium by centrifugation. The pellet was resuspended in a small amount of buffer by gentle homogenisation and split into two equal portions. These were suspended in 10ml of either 50mM NaHepes, pH 7.2, or 50mM NaBicine, pH 9.5, and incubated on ice for a further 6 hours. Membranes were pelleted again and resuspended in sample buffer for gel-electrophoresis (containing 5% SDS). About 50% of the radioactivity initially present in the labelling medium was associated with the protein prepared for electrophoresis. 40 to 150μg protein, equivalent to 100,000 – 250,000 (^{14}C) d.p.m., were applied to the gels, depending on the size of the wells. After electrophoresis

gels were either stained directly, or soaked in salicylate, autoradiographed for 4-14 days and stained afterwards.

Fig.45 shows a typical pattern of chromaffin granule membranes separated on a SDS-gel with a gradient of 10-15% acrylamide and stained with coomassie blue. The gel was run in presence of mercaptoethanol and membranes which had been incubated after the labelling at pH 7.4 (Fig.45, track 1) and pH 9.5 (Fig.45, track 2) were separated. The figure also shows the autoradiographs of the two experiments. As expected, Nbf-Cl is a rather unspecific label and many proteins become radioactive. The main 12 labelled bands are marked and identified as bands or regions on the stained gel. Because of shrinking effects during the handling of the gel (soaking in salicylate, staining, drying, etc.) the developed X-ray films are not exactly superimposable onto the stained and dried gels. The identification of radioactive bands with the stained patterns was therefore done by comparing the results from a number of experiments.

On films exposed for a few days, instead of 10 days as in Fig.45, only the stronger bands (1,2,3,4,8,11,12) were detected. Since most of these bands correspond to intensely stained proteins on the gel, it was concluded that a stronger blackening of the film is not necessarily due to proteins being labelled more specifically with Nbf-Cl but can be due to a higher abundance of these proteins. However, the most marked radioactive band was band 12 in membranes which were incubated at pH 9.5 after the labelling (track 2), a band which does not correspond to a major

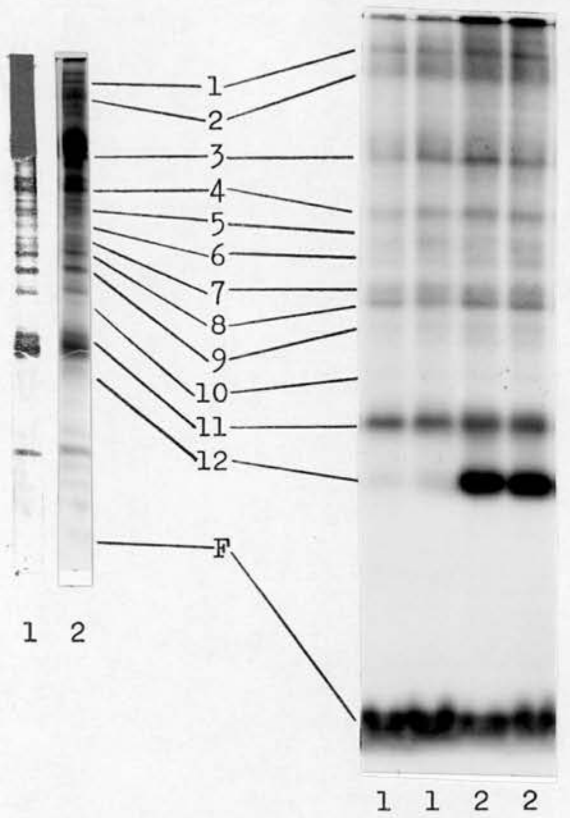


Fig.45

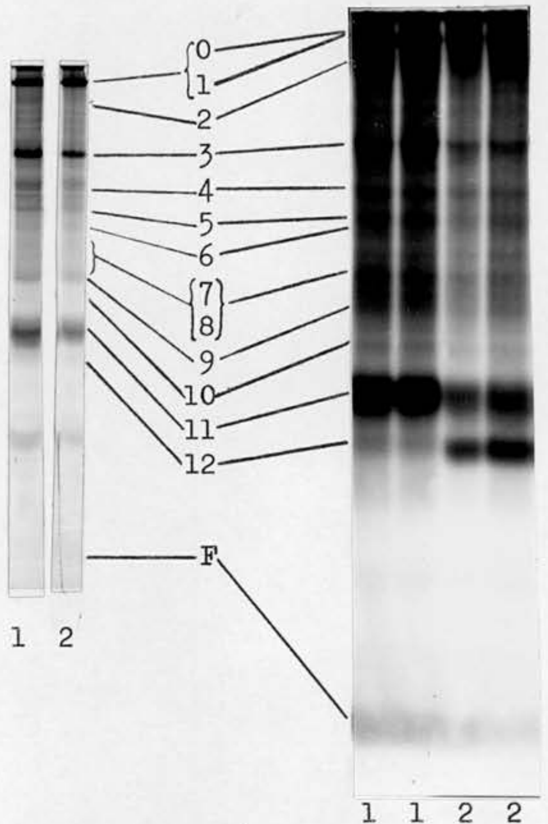


Fig.46

Fig.45: Analysis of chromaffin granule membranes labelled with (^{14}C)Nbf-Cl by reducing SDS acrylamide gel-elecrtophoresis. Membranes were labelled (and then incubated) at pH 7.4 (track 1) and pH 9.5 (track 2). The gel stained for protein with coomassie blue is shown on the left, the autoradiography on the right (with two tracks for each experiment). The main bands are numbered (1-12) and the front of the electrophoresis is marked (F).

Fig.46: As Fig.45, but electrophoresis was performed in absence of the reducing agent β-mercaptoethanol.

stained protein. The band was almost absent in samples incubated at pH 7.2 after labelling. The same effect, although to a minor extent, was the case for band 8 (this was more marked in other experiments not shown here).

In a gel run without reductant (Fig.46) labelling of essentially the same proteins was seen as in Fig.45 but with some changes in intensity. The following differences were observed: On top of the gel a heavily staining protein (0) is present which is strongly radiolabelled. The stained gel suggests that it is the dimer of DBH (MW=150,000) migrating in the reduced gel as a monomer (MW=75,000) to a position above band 3. Although this relationship seems to be clear for stained gels, the radioactive banding does not follow this pattern. Only little additional radioactivity above band 3 is seen in reduced gels compared with non-reduced ones. It seems that the strong radioactivity of band 0 is unaccounted for in the reduced gel. A possible interpretation is that DBH dimer might lose its label when it is dissociated into monomers.

In the non-reduced gel (Fig.46) there is also a strong new band observed on top of band 11 in the sample which was incubated at pH 7.4 after the labelling (track 1). In the sample incubated at pH 9.5, an equally strong band migrates as band 12 (track 2), like the corresponding sample of the reduced gel in Fig.45. It seems that the label hops from one position to the other according to the pH in which the labelled membranes were incubated before subjecting them to the electrophoresis. A possible interpretation can be made

considering the observations made by Ferguson et al.(1975) and discussed above. It is assumed that the labelling with Nbf-Cl at neutral pH takes place at protein 11, perhaps on a tyrosine residue which is sensitive to mercaptoethanol and therefore the label is lost on a reducing gel (Fig.46, track 1). If, however, samples are incubated at pH 9.5 before electrophoresis, Nbf-groups migrate from residues (probably tyrosine) in protein 11 to a nearby group on the different protein 12. The new bond is not sensitive to reducing agents and the label is therefore seen in both reduced and non-reduced gels.

This result makes it possible to speculate on the the local proximity of proteins 11 and 12, the hopping of the label from one to the other suggesting that they are in close contact. (The pattern of the protein-stained gel does not suggest that band 12 is a degradation-product of band 11.) As for the other labelled proteins, they seem not to be affected by the change of pH or the presence of mercaptoethanol, either because the label in those proteins binds to groups other than tyrosine or the rearrangement of the label takes place on the same protein.

Identification of labelled bands. Since the main purpose of these labelling experiments was to identify a possible adenine nucleotide transport protein in the chromaffin granule membranes, it was necessary to identify the labelled proteins as far as possible. The remaining proteins could then be considered as possible candidates, if of course, the putative transport protein is labelled at

all. One way of identification is by trying to inhibit the labelling of some proteins with appropriate agents: MgATP or DCCD might prevent the labelling of ATPase subunits whereas atractyloside might do this for an adenine nucleotide transporter. To investigate this, membranes at 1mg/ml in 50mM NaHepes, pH 7.4, were preincubated on ice for 4 hours with either 2mM ATP, 2mM ATP and 2mM $MgSO_4$, 100μM atractyloside, 100μM DCCD or nothing added. Labelling and separation on gels was then performed as before. All four conditions, incubation at pH 7.4 or 9.5 and electrophoresis in presence or absence of mercaptoethanol, were used but none of the agents under none of the conditions changed the labelling pattern of the proteins compared with that of Fig.45.

There are, of course, proteins in the chromaffin granule membrane which have already been identified. The radioactive band 0 in non reducing gels migrates like the dimer of DBH but as discussed above, no increased blackening is seen in reduced gels at the position of the DBH monomer. Band 3 might be chromogranin A, a contaminating matrix protein, labelling as a soluble protein much better than the membrane bound proteins. Band 5 is probably the β-subunit of the F_1-ATPase but it seems not to lose the label in presence mercaptoethanol in the pH 7.4 experiment as described by Ferguson et al.(1975). Band 11 is the cytrochrome b561, with the hopping band being on top of the cytochrome band (Fig.46, track 2). The cytochrome itself seems to run variably as a broad band or two separated darker stained bands. That region in the gel is therefore

referred to sometimes as 'cytochrome area'. Some of the bands in that region are related as shown in experiments with digesting agents (Abbs & Phillips, 1980; Hunter et al., 1982).

Separation of Nbf-labelled proteins in two dimensions.

For a further characterisation of the labelled proteins, analysis in two dimensions was performed by electrofocussing labelled membranes in a rod gel and then further separating on a SDS slab-gel (for details see the Materials and Methods-section). Samples prepared at pH 7.4 and 9.5 were analysed. The pH-range of the isoelectric focussing-gel was about pH 4.0 to 7.5 and the acrylamide concentration of the slab gel for the second dimension was 10-15%. Fig.47a shows chromaffin granule membrane proteins separated in such a system. The gel was stained for protein with coomassie blue and in the figure the proteins DBH, chromogranin A and cytochrome b561 which have been identified previously (Apps et al.,1980b) are shown. An autoradiograph of such a two-dimensional map of membranes labelled with (^{14}C)Nbf-Cl and incubated at pH 9.5 is shown in Fig.47b. A considerable amount of radioactivity seems not to enter the second dimension and remains at the top of the slab gel. This is probably the cause for the vertical streaky blackening of the film.

A very marked amount of radioactivity is seen a quarter down the gel to the right and corresponds to band 3 in the one-dimension gel in Fig.45. The radioactivity seems not to

Fig.47a: protein-stained gel

DBH (↙)

strongly radioactive protein (x), band 3

chromogranin A (↖)

cytochrome b561 (↙)

Fig.47b: autoradiography

DBH (↙)

band 3 (x)

band 4 (↙)

band 6 (↘)

band 8 (↘)

band 10 (↘)

band 11 }

Fig.47: Analysis of membranes, with (^{14}C)Nbf-Cl labelled (and then incubated) at pH 9.5, into two dimensions: isoelectric focussing (horizontally, acidic end on the right); SDS-electrophoresis (vertically). Some of the proteins are identified with the same numbers as in Fig.45 (for details see text). The strongly radioactive protein is marked with x.

stem from either DBH or chromogranin A, but from a protein migrating near DBH with a slightly more acidic pI and an apparent molecular weight between that of DBH and chromogranin A (CGA). But DBH and CGA are faintly labelled themselves as well. Other radioactive areas were identified with labelled bands from Fig.45 as far as possible, comparing gels and autoradiographs from six different experiments: The more strongly radioactive regions seen at the lower part of the gel in Fig.47b, correspond probably to band 11 rather than 12 in Fig.45, because most of the radioactivity was still present when samples were analysed which had been incubated at neutral pH after the labelling.

Because band 12 migrates on incubation at pH 9.5 from a position just above the cytochrome in a one-dimensional gel to one below it, it was investigated whether the radioactivity seen at the bottom of the gel in Fig.47b represented proteins which are immunologically related to the cytochrome b561.

Proteins separated in two dimensions were transferred to a nitrocellulose sheet and incubated with antiserum against cytochrome b561. Bound antibodies were identified with radiolabelled protein A. An autoradiograph of such an experiment is shown in Fig.48. This experiment was done with labelled membranes and a pattern of black spots as seen in Fig.47b is therefore superimposed on the blackening which stems from radioactive protein A and has to be subtracted. The cytochrome, with a pI of 6.2, is clearly seen in the lower left of the gel. It is also present as a dimer and

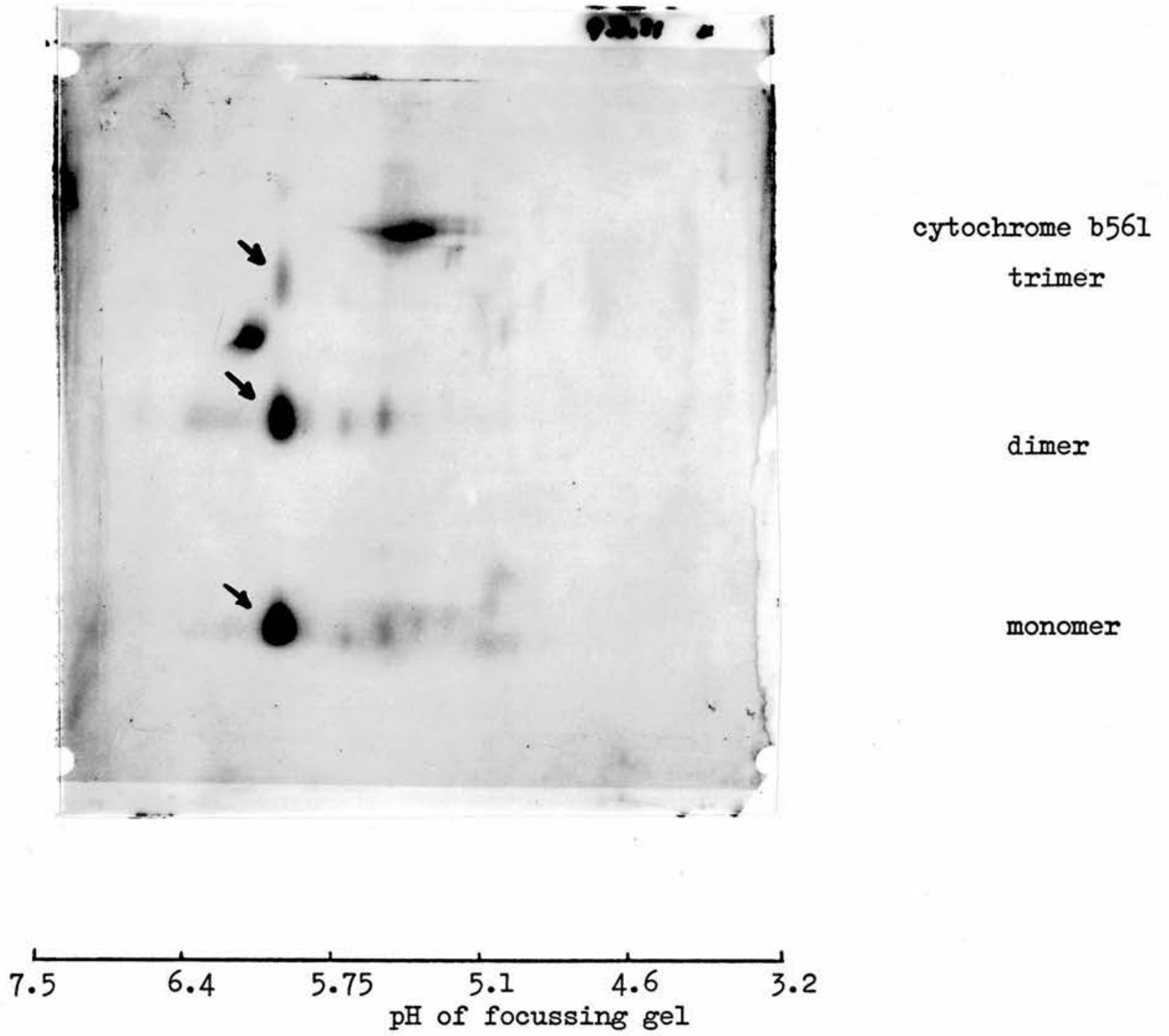


Fig.48: Antibody-blot with cytochrome b561-antibodies of a two dimensional gel as shown in Fig.47. The cytochrome b561 is seen as mono-, di- and trimer.

trimer, seen as smaller spots directly above the monomer. Additionally, there are 2 or 3 minor spots of about the same molecular weight as the cytochrome, focussing at lower pH-values; they are also observed as dimers. These spots seem to correspond with the left part of the Nbf-labelled protein seen in Fig.47b. However, most of the Nbf-labelled protein of a molecular weight close to that of the cytochrome but with a more acidic pI does not react with the antibodies and is therefore immunologically not related.

This antibody-blot method is very sensitive, but when interpreting results the following points ought to be considered: Firstly an antibody preparation is only as pure as the antigen with which the animal was immunized. Especially major proteins from a mixture might be still present in minute amounts in the purified antigen preparation. This is a particular problem for proteins such as DBH, which are strongly immunogenic. Safe interpretations are therefore made more easily in a negative sense, i.e. when excluding that a protein is immunologically related to the antigen rather than stating that a protein is related only because it does cross-react with the antibodies. Secondly, the transfer of proteins from the gel to the cellulose-nitrate filter is not absolute and some proteins transfer more easily than others. However an antibody reaction can still take place even if only very little protein has been transferred and the tracing with (^{125}I) labelled protein A is very sensitive as well.

Labelling with dialdehyde ATP.

2',3'-Dialdehyde ATP (open ATP) has been used as an affinity-label for ATP-requiring enzymes (Easterbrook-Smith, 1976; Kumar et al., 1979). In the kinetic experiments above it was shown to inhibit ATP-uptake into chromaffin granule ghosts (Fig.34). The transport of ATP was more strongly inhibited than that of serotonin. This suggests that the inhibitor acts more specifically at the mechanism facilitating the adenine nucleotide transport. Since dialdehyde ATP reacts covalently with proteins it can be used as an affinity label. It was prepared in a radioactive form as described in the Materials and Methods-section.

To label chromaffin granule membrane proteins, 1,5mg freshly prepared purified membranes in 1ml 10mM NaHepes, pH 7.2, and 20mM KCl were incubated with about 4µCi dialdehyde (^{3}H)ATP in a 10ml centrifuge tube with slight stirring, at 4° C, for 18 hours. After the labelling, the volume of the medium was adjusted to 10ml with 10mM NaHepes, pH 7.2. The membranes were pelleted by centrifugation and resuspended in 400µl 5% SDS electrophoresis sample-buffer. They were frozen at -20°C when not used on the same day. About 95% of the label originally added to the incubation was associated with the dissolved membranes.

For electrophoresis, SDS-polyacrylamide gels with an exponential gradient of 10 - 15% acrylamide were run in

presence or absence of reducing agents. Protein samples of 150µl (500µg), 100,000-200,000 (^{3}H) d.p.m., were applied to thick gels (1.5mm spacers) with wide wells (12mm). Under these conditions gels were loaded very heavily with protein and the staining with coomassie blue was therefore done in a solution containing a limiting amount of dye rather than excessive dye as used normally. Loading of so much protein was necessary in order to have enough radioactivity present for autoradiography. After fixing and washing the gels were soaked in a solution of salicylate (1M) and exposed to X-ray films for 2-3 months at -70° C. Some gels were pre-run, i.e. they were run overnight without a stacking gel or a sample. Only some sample buffer was added directly onto the main gel. After the pre-run a stacking gel was added and the gels were used as normal. This procedure had an effect on the overall running pattern of proteins on the gel and gave a better resolution of the proteins between molecular weights of 20,000 and 60,000.

In Fig.49 (track d), membrane proteins labelled with dialdehyde (^{3}H)ATP were separated on an ordinary (Fig.49a) and on a pre-run (Fig.49b) gel both of which were run under reducing conditions. Gels stained for proteins and their autoradiographs are shown. For comparison and identification the radioactive bands are numberd and the stained bands are grouped in an easily recognizable pattern of five regions (a,b,c,d,e), identified by the migrating-distance, in cm, from the top of the gel, as follows:

a.(0-2.0cm) The stained bands above the strongly stained

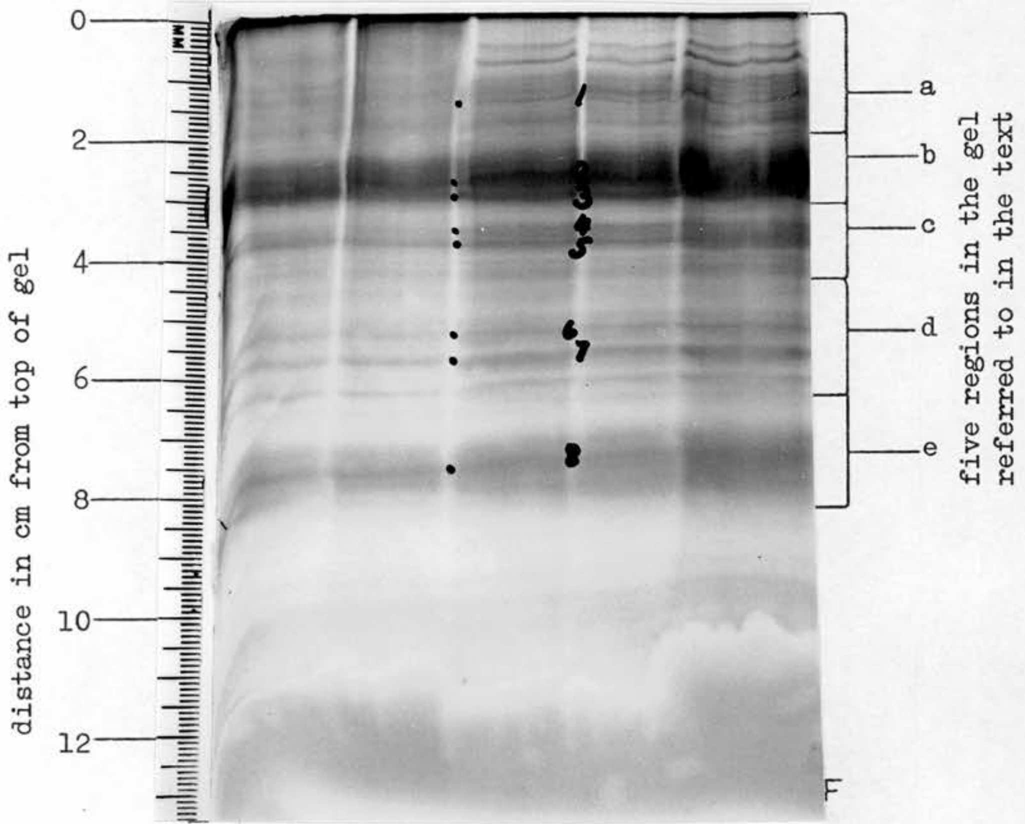


Fig.49a (protein stain)

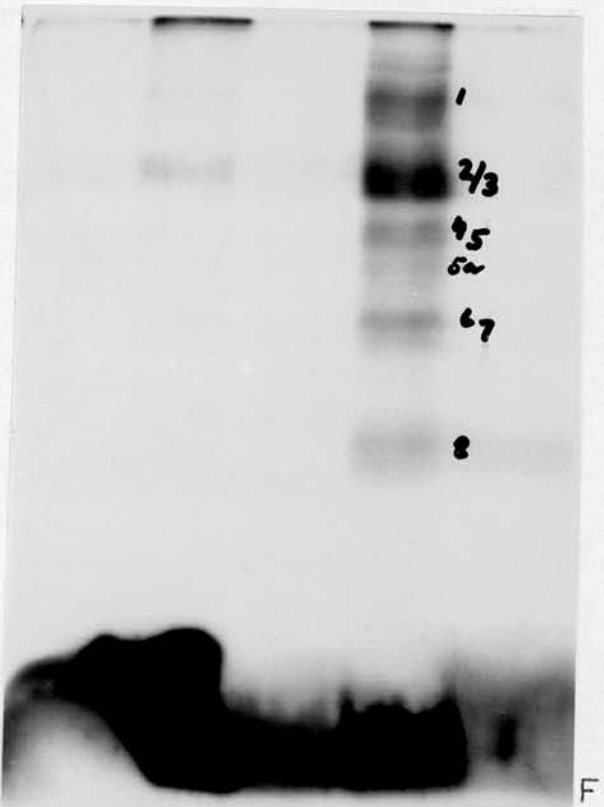


Fig.49a (autoradiography)

<u>Fig.49:</u> Proteins of chromaffin granule membranes labelled with various tritiated compounds separated on a directly run (Fig.49a, this page) and pre-run (Fig.49b, next page) 10-15% acrylamide SDS gel in presence of a reducing agent. In tracks d membrane proteins labelled with (^{3}H)dialdehydeATP are separated. Predominantly labelled proteins are numbered from 1 to 8 and are marked with dots on the stained gel. These numbers are <u>not</u> identical with the numbers of (^{14}C)Nbf-Cl labelled proteins in the previous figures.

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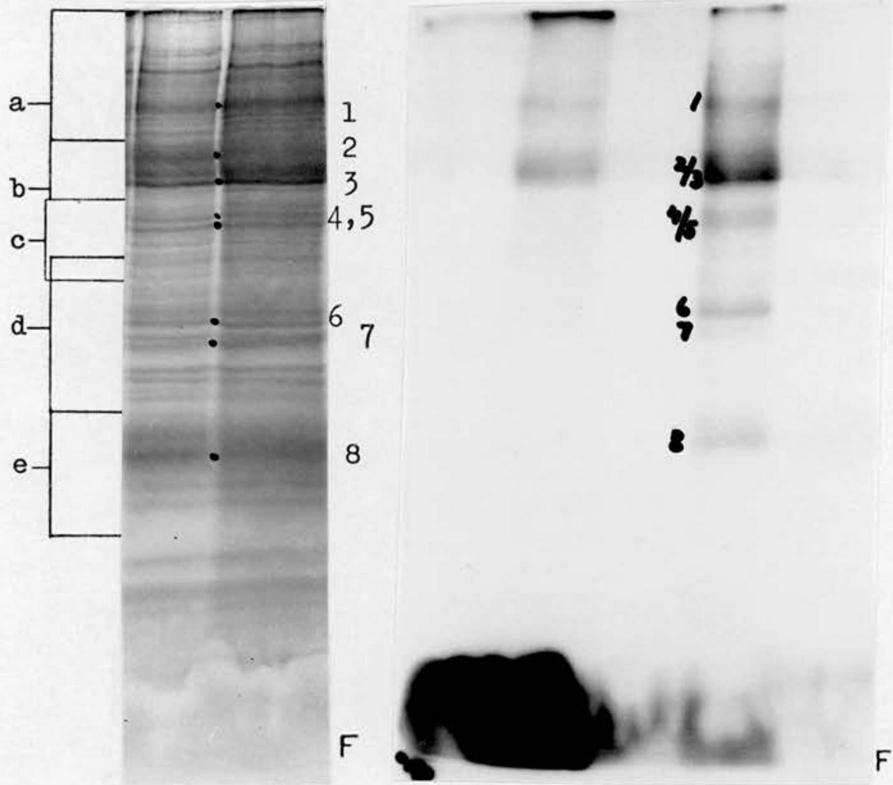


Fig.49b (protein stain) Fig.49b (autoradiography)

Fig.49: (cont'd) In tracks a, b and c the label is $(^{3}H)NaBH_4$ which was added to the membranes after preincubation with unlabelled dialdehydeATP for 30 min. (track a) or overnight (track b). In a control experiment label was added in absence of dialdehydeATP (track c).

Membranes labelled with (^{3}H)FSBA are analyzed in track e. F denotes the front of the chromatography.

bands of DBH and chromogranin A, with molecular weights of about 90,000 and higher; with several fuzzy one stronger radioactive band (1) at about 150,000 mol.wt. The radioactive band 1 might represent remaining dimers of DBH which are not dissociated in spite of the presence of the reducing agent mercaptoethanol during the electrophoresis.

b.(2.5-3.0cm) Area around DBH (75,000 mol.wt.) and chromogranin A (72,000 mol.wt.) with two strongly labelled bands (2,3). In pre-run gels bands 2 and 3 always ran more closely together and in autoradiographs looked like a single band.

c.(3.0-5.0cm) Bands below DBH and above the more strongly staining group of proteins, which are seen below about 5.5cm. This region contains two marked proteins of about 60,000 and 57,000 mol.wt. both of which are radioactive (4,5), and also the two larger subunits of the F_1-ATPase (α, 51,000; β, 50,000), one of which might be faintly labelled by dialdehyde ATP (Fig.49a, track 4), but the subunits are not readily recognized on stained gels.

d.(5.0-7.5cm) This area shows in Fig.49a three predominantly stained bands of molecular weights between 33,000 and 40,000, some of which are resolved at least into doublets in pre-run gels (Fig.49b). The upper two of these three bands appear to be labelled (6,7), the upper band more strongly than the lower. If they run as doublets, the lower band of each of them seems to get labelled.

e.(7-7.5cm and lower) The region around cytochrome b561

and below. A broad fuzzy band with several more strongly staining bands superimposed is seen in the cytochrome region. One or two bands seem to be labelled (8), representing probably the actual cytochrome protein of 26,500 mol.wt. The dark area on the autoradiographs towards the bottom of the gels represents labelled lipids and perhaps free label.

In Fig.50 an autoradiograph is shown of membranes labelled either with radioactive dialdehyde ATP (track a, b, c), dialdehyde ADP (track d, e) or dialdehyde AMP (track f), separated on an ordinary gel. The labelling with dialdehyde ATP and ADP was also done in presence of 8.5mM unlabelled ATP (track b) and ADP (track e), respectively. The same pattern of radioactive bands is obtained for all three analogues, dialdhyde AMP labelling all bands more weakly than the two other labels. Competition with unabelled nucleotides seems to have no effect on the labelling, apart perhaps from a faint band between the radioactive bands 5 and 6, probably ATPase β-subunit (50,000 mol.wt.), which seems to become slightly more labelled when ATP or ADP were present. It might be that these nucleotides, upon binding to a regulatory site of the enzyme, expose a further binding site to which the radioactive analogue then binds. When membranes are labelled in presence of 3.4mM cibacron, virtually no radioactive dialdehyde ATP at all is bound to the proteins (track c). Band 1 in this gel is very broad and seems to consist of several proteins.

A second example of membranes labelled with dialdehyde

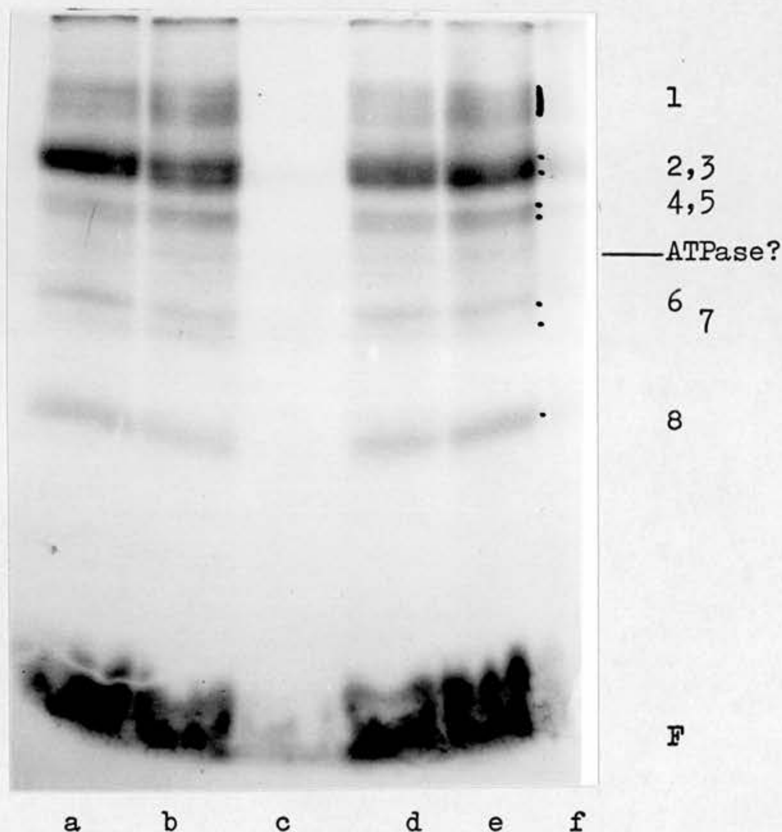


Fig.50: Autoradiography of membranes labelled with dialdehyde adenine nucleotides, separated on a directly run 10-15% acrylamide SDS-gel. Labelling was done with (^{3}H)dialdehyde -ATP (tracks a, b and c), -ADP (tracks d, e), -AMP (track f), in presence of unlabelled ATP (track b) or unlabelled ADP (track e) or cibacron blue (track c).

ATP is shown in Fig.51. The gel was pre-run and proteins were separated in presence of mecaptoethanol. Labelling was performed in presence (track b) and absence (track c) of 2mM $MgCl_2$. All proteins were more strongly labelled in absence of $MgCl_2$. Although the radioactive bands of tracks a and b are sharper than those in Figs.49 or 50, the overall labelling pattern is essentially the same. The bands are also darker because more protein was used and the gel was autoradiographed for a longer time *than* in Figs.49 and 50. The two pairs of bands 2,3 and 4,5 were not resolved and this was also the case to some extent in other pre—run gels (Fig.49). Bands 6, 7 and 8 are at similar positions as in other experiments but in this experiment band 7 is more strongly labelled than band 6. Band 1 which in other gels only migrates *as* one big fuzzy band is in this gel resolved into two bands (1a,1b). The upper one can be superimposed with the dimer of DBH (150,000 mol.wt.) in a non-reduced stained gel. But since it is present in this reduced gel where no major stained band can be attributed to it, it might represent either a protein different from DBH or a very small proportion of DBH which does not dissociate in presence of mercaptoethanol.

Reduction with radioactive sodium borohydride. The aldehyde groups of the dialdehyde analogues bind to amino groups of proteins by forming a Schiff's base. The formed C=N double bond can be reduced with borohydride. An alternative way of introducing radioactivity into such a modified protein is by reducing the bond with tritiated borohydride instead of using radiolabelled dialdehyde ATP.

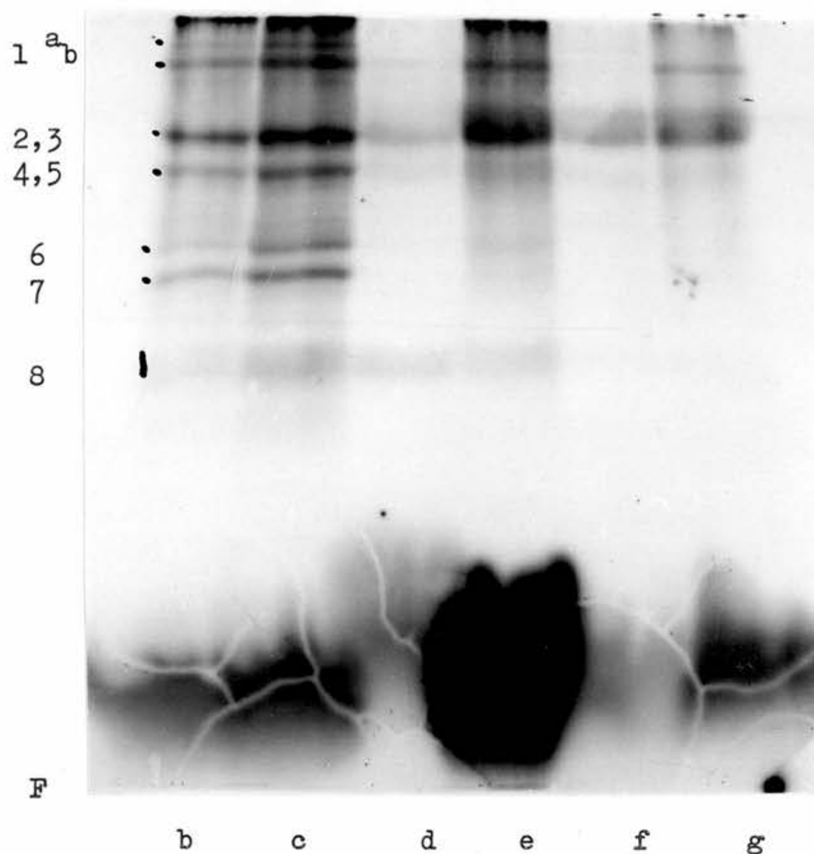


Fig.51: Autoradiograph of labelled membranes separated on a pre-run 10-15% acrylamide SDS gel in presence of mercaptoethanol. Samples were labelled with: dialdehyde(^{3}H)ATP in presence (track b) and absence (track c) of 2mM $MgCl_2$; (^{3}H)FSBA (track d); (^{3}H)$NaBH_4$ (track f), and after preincubation with unlabelled dialdehyde ATP for 30 min. (track g) and overnight (track e).

Membranes were labelled overnight as before, with the radioactive label replaced by 5mM unlabelled dialdehyde ATP and 1mM $MgCl_2$. In a second experiment membranes were incubated with dialdehyde ATP at 37°C for 30 min. After incubation the two preparations plus a third as a control, containing no inhibitor, were reduced by adding a few crystals of solid (^3H)$NaBH_4$ (100mCi/mmol). This step was done under a hood designed to handle radioactive gases since during the reduction radioactive hydrogen is released. The membranes were removed from the medium by centrifugation and dissolved in electrophoresis sample buffer. 100-150µg protein per well, representing 50,000- 300,000 (^3H) d.p.m., depending on the experiment, were separated on the same gels as before (Figs.49 and 51).

Tracks b, c and a in Figs.49a and 49b, and tracks e, f and g in Fig.51 show membranes incubated overnight in presence and absence, and for 30 min. in presence of dialdehyde ATP, respectively, before reducing with radioactive borohydride. The track f in Fig.51 shows unspecific labelling with (^3H)$NaBH_4$, a few proteins near DBH and lipids towards the bottom of the gel are radioactive. Long and short incubation with dialdehyde ATP seems to have only an effect on the actual amount of radioactivity being bound to the proteins but not on the labelling pattern itself. Prominantly labelled bands are identical to bands 1a, 2 and 3 in experiments with radioactive dialdehyde ATP (see Fig.51).

In most labelling experiments presented so far,

membranes were incubated with the label overnight. Such a long time was chosen to obtain proteins labelled with a high amount of radioactivity and which therefore minimizes the exposure—time required for the autoradiographs. However, if a protein is labelled specifically, one might expect this to be a quick process and that incubating over a long period rather increases unspecific labelling. However, preliminary experiments had shown that all proteins were only very weakly labelled after incubations for between 30 min. and 2 hours and that therefore extremely long exposure times were required to obtain a substantial blackening of X-ray films.

<u>Short time labelling of proteins.</u> Chromaffin granule ghosts, 0.5mg in 0.5ml 10mM NaHepes, pH 7.2, 0.3M sucrose were labelled at 37°C with 0.3µCi dialdehyde (^{3}H)ATP for 40 min. After the labelling the samples were reduced by incubating with 50µl freshly prepared 0.5M $NaBH_4$ on ice for 30 min. The ghosts were separated from the medium by centrifugation, lipids were extracted with ethanol/acetone and the protein was dissolved in electrophoresis sample buffer. In parallel experiments labelling was performed in presence of either 1mM $MgSO_4$, 4mM PEP and 1mM $MgSO_4$, 5mM ATP and 1mM $MgSO_4$ or 100µM atractyloside and 1mM $MgSO_4$. In the latter two experiments ghosts were preincubated with these substances at 37° C for 10 min. before adding the label. Samples of 100 — 150µg were separated on gels in presence or absence of 10% (v/v) of the reducing agent DTT. The equivalent of 28,000 (^{3}H) d.p.m. of radioactive protein was loaded per gel and after electrophoresis the gels were soaked in salicylate and autoradiographed for 7 months.

Fig.52 shows the labelled ghost proteins for these five experiments separated on 10-15% acrylamide SDS gels in presence (Fig.52a) and absence (Fig.52b) of DTT.

Overall labeling of proteins in absence of $MgSO_4$ (track b) was more intense than in its presence (track a). This agrees with the finding made in Fig.51 (tracks b and c). Excess of unlabelled ATP (track c) effectively prevented the labelling of most proteins. In other experiments (Fig.50) no competition of labelling was found but there adenine nucleotides were used in absence of Mg^{++}. In these experiments a band near the top of the gel which is the dimer of DBH is clearly only seen in the gel run in absence of reducing agent (Fig.52a), and in its presence an additional fuzzy band is seen above band 2/3. This indicates that at least part of band 1 in Figs.49, 50 and 51 might indeed be undissociated DBH.

There is one band (arrow) which seems to become labelled rather specifically, since other heavily labelled bands on top of the gel also represent high amounts of protein. On stained gels it is identified as a very faint band just above the cytochrome and well below what are in other gels (Figs.49, 50 and 51) bands 6 and 7. The labelling of this band seems to be effectively prevented by either MgATP, atractyloside or phosphoenolpyruvate. The labelling of all other bands is much less affected by these agents. This seems to be especially significant in view of the inhibitory effect phosphoenol pyruvate and atractyloside have on ATP transport into chromaffin granule ghosts. However

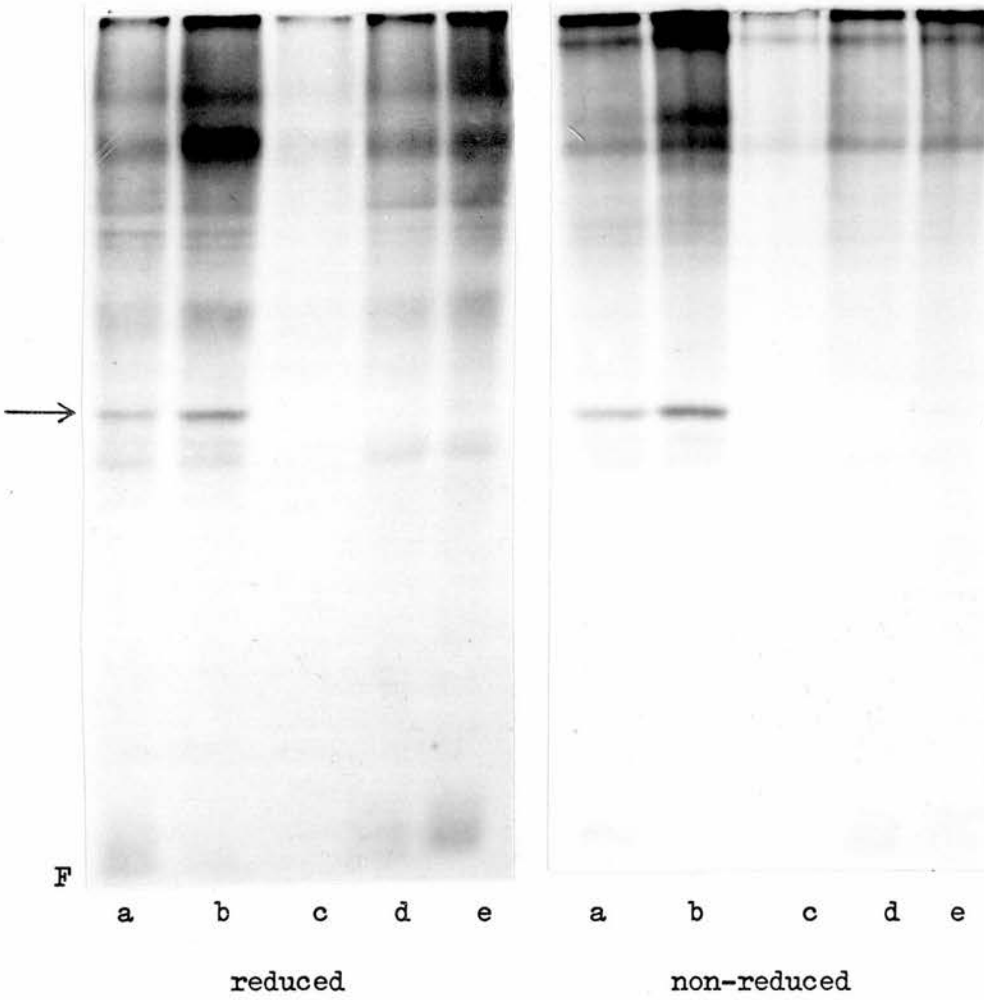


Fig.52: Short time labelling of chromaffin granule ghosts with dialdehyde(^{3}H)ATP in presence (tracks a) and absence (tracks b) of $MgSO_4$; additionally to $MgSO_4$, 4mM PEP (tracks d), 5mM ATP (tracks c) or 100µM atractyloside (tracks e) were present. The gels contained 10-15% acrylamide and were run in presence (left) and absence (right) of the reducing agent DTT.

comparison with experiments discussed below (Fig.54) revealed that this band is quite likely the mitochondrial adenine nucleotide transporter (30,000 mol.wt.) , which might be present due to mitochondrial contamination of this particular ghost preparation.

Labelling with FSBA.

Radioactive 5'-p-fluorosulphonylbenzoyladenosine (FSBA) was synthesized from (^{3}H)adenosine as desribed in the Materials and Methods-section. As discussed previously, it inhibits the transport of serotonin, but not of ATP, into chromaffin granule ghosts. The sulphonylfluoride substituent of FSBA is a sulphonating group of broad specificity and can react with lysine, tyrosine, histidine or serine-residues of proteins (Poulos & Price, 1974). To label chromaffin granule membrane proteins, 16mg freshly prepared membranes in 4ml 10mM NaHepes, pH 7.2, were incubated with 35.6µCi (^{3}H)FSBA (2,4mCi/mmol) dissolved in 40µl methanol, with gentle stirring at 4°C overnight. After the labelling the membranes were separated from the medium by centrifugation. About 50% of the added label was associated with the pelleted protein. The pellet was dissolved in electrophoresis sample buffer and portions of 80µg (180,000 (^{3}H) d.p.m.) were separated on the same gels as dialdehyde ATP - labelled membranes (Figs.49, track e, and 51, track d). As seen on the autoradiographs FSBA labels proteins less well than dialdehyde ATP. This might partially be due to its specific radioactivity which was 10 times lower for FSBA than for dialdehyde ATP. FSBA labelled

only proteins which had also been labelled by dialdehyde ATP, but not all of them. The main bands were 1, 2/3, 4/5 and 8; band 6 was labelled very faintly in Fig.49b but was absent in Fig.51.

Labelling with azido ATP.

8-Azido (γ-^{32}P)ATP was used to label proteins of the chromaffin granule membrane. After incubation of the proteins with the label in the dark, the azido-group was activated with UV light, by which it decomposes and forms a highly reactive nitrene group capable of binding covalently to proteins. Labelling of chromaffin granule ghosts with azido-AMP inhibited transport of both ATP and serotonin into the vesicles, as discussed in a previous section.

Chromaffin granule membranes were labelled at 4mg/ml protein in 10mM NaHepes, 2mM EDTA, pH 7.2. EDTA was added to inhibit ATPase activity which would cleave off the γ-phosphate from the affinity label. To portions of 125µl (500µg) protein, 2µl (4µCi) radioactive azidoATP (NEN, 29Ci/mmol) were added in the dark and the mixture was incubated in the dark on ice for 30 min. For the activation of the photolabel two sources of UV—light were used. The samples in quartz-cuvettes were either exposed at a distance of 3 cm to a high energy Xenon lamp (Wotan XBO, 250W, Osram, Berlin, Germany) in four intervals of 20s each. Between the intervals cuvettes were cooled on ice for 40s. With this treatment only little inhibition of the transport activities into chromaffin granule ghosts caused by UV-light alone was observed. Alternatively, samples were exposed on ice for 45 min. to a low energy UV — source (30W) normally used to visualize chromatograms. After the labelling samples were

either delipidated in 1ml ethanol/acetone and the precipitated protein washed in water before dissolving in electrophoresis sample buffer, or the suspension of labelled membranes were mixed directly with concentrated sample buffer giving a final concentration of 5% SDS.

Labelled membrane proteins are shown in Fig.53a separated on a 6-15% acrylamide SDS gel and stained for protein with coomassie blue. An autoradiograph of a similar gel soaked in salicylate and exposed for 3 weeks is shown in Fig.53b. Tracks 1 and 16 contain a mixture of unlabelled proteins as molecular weight marker, with 5μg of each in sample buffer loaded per track. They are, from top to bottom, BSA (68,000 mol.wt.), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (21,000), myoglobulin (17,300) and lysozyme (14,300). The samples in tracks 2 to 8 were flashed with the high energy UV light and delipidated before electrophoresis, the samples in tracks 9 to 15 were exposed to the low energy UV light and not delipidated. The lipids in these samples can be seen at the bottom of the stained gel, displacing some of the very low molecular weight protein-bands upwards. Protein samples labelled with azidoATP as described above were separated in tracks 8 and 13. They serve as controls for the other tracks containing membranes which were labelled in presence of potentially competitive agents. This was done as follows: membranes, 4mg/ml, were preincubated for 15 min. at room temperature in 10mM NaHepes at neutral pH in presence of either 20mM ATP (track 2), 19mM ADP (tracks 3 and 10), 14mM AMP (11), 50mM phosphate (4, 12), 400μM atractyloside (5, 9), 50mM PEP (6)

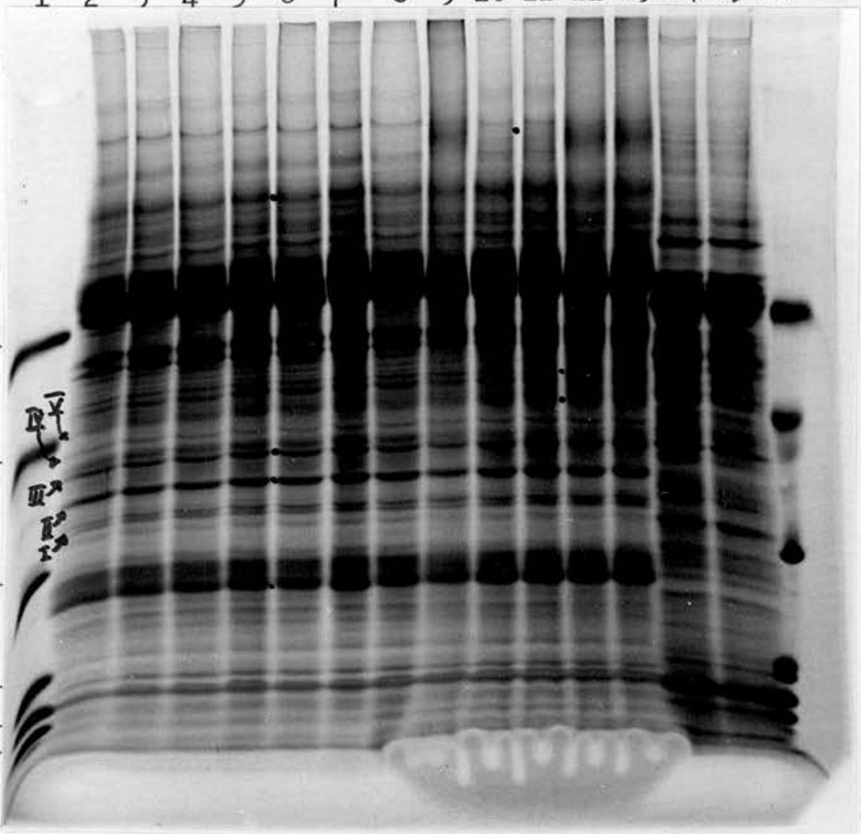


Fig.53a: Coomassie blue stained gel

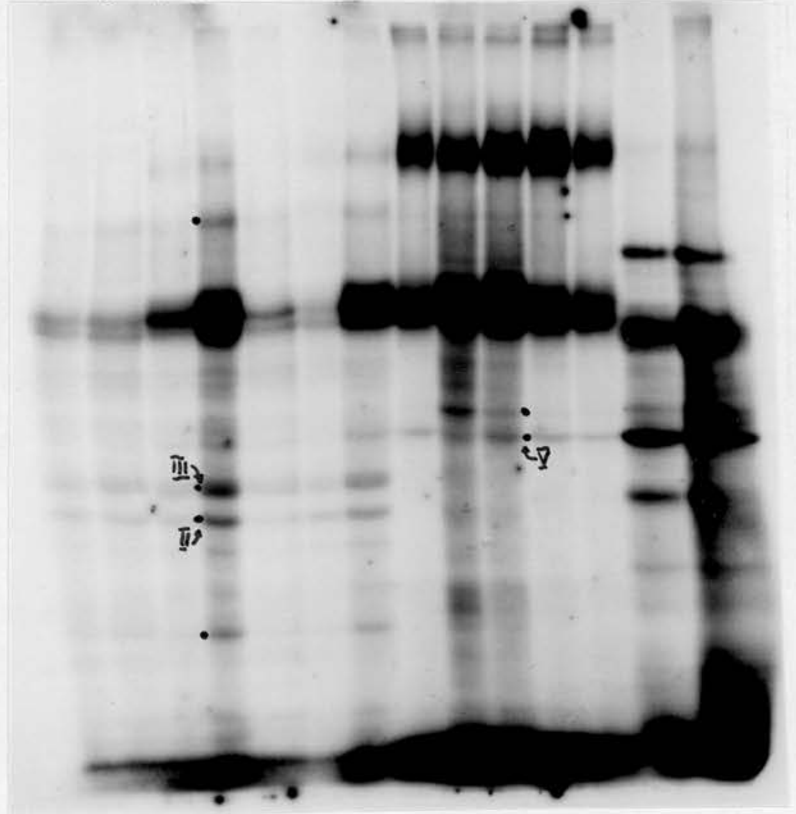


Fig.53b: Autoradiography

Fig.53: Chromaffin granule membranes labelled with azido(γ-^{32}P)ATP, separated on a 6-15% acrylamide SDS gel. A gel stained for proteins (Fig.53a) and an autoradiograph (Fig.53b) are shown from two individual experiments. The molecular weight of the marker proteins are indicated to the left. The labelling was performed in presence of:

20mM ATP	(tracks 2)	50mM phosphate	(tracks 4, 12)
19mM ADP	(" 3, 10)	50mM PEP	(" 6)
14mM AMP	(" 11)	400μM atractyloside	(" 5, 9)
no addition	(" 8, 13)	4mM cibacron blue	(" 7)

for experimental details see text.

Some bands referred to in the discussion are marked with a dot.

or 4mM cibacron blue (7) before adding azidoATP in the dark on ice. The labelling was then performed as described above.

The content of acrylamide in the gels in Fig.53 and 54 was different from that in former experiments, an exponential gradient beingof 6-15% instead of 10-15% acrylamide. Therefore the pattern of the separated proteins on the stained gel was somewhat altered. The strongly staining bands of the DBH region migrated more than a third down the gel, together with the 68,000 molecular weight marker. The upper third of this gel therefore separated proteins of molecular weights higher than 100,000. The cytochrome b561 migrated three quarters down the gel, and is seen just below the 30,000 molecular weight marker. The banding pattern between these two main proteins was quite similar to the pattern of the respective area in previous gels, and it was therfore relatively easy to identify corresponding proteins. The proteins migrating below the cytochrome, of molecular weights 25,000 and lower, were well resolved but on a smaller area than in previous gels. But major proteins could be identified on either gel system, like e.g. the dominant protein of about 18,000 mol.wt. which is seen in Fig.45 half way between the cytochrome and the bottom of the gel.

As seen on the autoradiograph in Fig.53, azidoATP labelled a whole range of proteins. In the two control experiments (tracks 8 and 13), proteins around DBH were predominantly labelled. An additional strong blackening was

seen at a very high molecular weight in the experiment which had been exposed to UV-light for 45 min. (track 13). In the experiments flashed with strong UV-light atractyloside (track 5) did not inhibit, but increased the labelling of all proteins observed in the control experiment (track 8) proportionally. The other substances inhibited the labelling of most proteins to some extent. Phosphate however (track 4), seemed to specifically inhibit the labelling of the lower of the two main bands in the DBH area when compared with membranes incubated with ATP or ADP (tracks 2 and 3). None of the other substances seemed to alter the labelling of one specific protein. As in the experiment with dialdehyde ATP (Fig.50), cibacron blue (track 7) inhibited the labelling of all proteins very strongly.

A characterisation of proteins which became labelled with azido ATP after a flash with strong UV-light is best done using the results from the experiment performed in presence of atractyloside (track 5). Apart from the labelled proteins in the DBH-region there are two strong bands below it, identical with two strongly stained bands of about 36,000 and 39,000 molecular weight. These two bands are probably identical with the two proteins in the same region labelled with dialdehyde adenine nucleotides (Figs.49-51, bands 7 and 6). As in Fig.49b, the 39,000 mol.wt. band runs as a doublet in this gel, of which the lower band appears to be labelled. The 36,000 mol.wt. band runs as a thick single band. The strongly labelled lower band in the cytochrome region, at about 26,000 mol.wt., is likely to be the actual cytochrome b561.

There were also several less heavily labelled bands observed. One, at a molecular weight of about 150,000, which is not stained very strongly, might perhaps be, as discussed before, a residual amount of the DBH-dimer which does not dissociate in the reducing gel. The two strongly stained bands below the DBH region (60,000 and 57,000 mol.wts.) were faintly labelled, and had also been labelled by dialdehyde adenine nucleotides (Figs.49-51). It was unclear whether the α and β subunits of the F_1-ATPase were labelled; some blackening of the film in the region around 50,000 mol.wt. was observed, but no definite identification could be made. A further radioactive band was at a molecular weight of 44,000. This band was also labelled in the membrane fractions exposed to the lower energy UV-light (tracks 9 to 13). One weaker band was superimposable with the stained doublet at 33,000 mol.wt..

The pattern of radioactive proteins from samples exposed to the low energy UV-light for 45min. was quite different, as seen in the control experiment in track 13. The presence of phosphate (track 12) and atractyloside (track 13) during the incubation gave no differences in the labelling pattern. ADP and AMP however, appeared to increase considerably the incorporation of label into several proteins. A very strong band at a high molecular weight (probably 150,000 or bigger) was only represented by a weakly staining protein band, which, however, reacts with antiserum against DBH. It might therefore represent DBH which was cross-linked upon exposure to UV—light. Apart

from this, additional radioactivity was also associated with different proteins in the gel region below the DBH-area. In the control experiment (track 11) only one protein at 44,000 mol.wt. was strongly labelled in that area. But AMP (track 11) and especially ADP (track 10) stimulated the labelling of this and other proteins. One strongly labelled protein was observed at about 51,000 mol.wt. and is probably the α-subunit of the F_1- ATPase. There were also two bands in the cytochrome region at 27,000-29,000 mol.wts. migrating above the major cytochrome band (26,500 mol.wt.) labelled in tracks 2-8. - In all , the effect of the various agents present during the labelling was very different depending on the exposure of the membranes to UV-light.

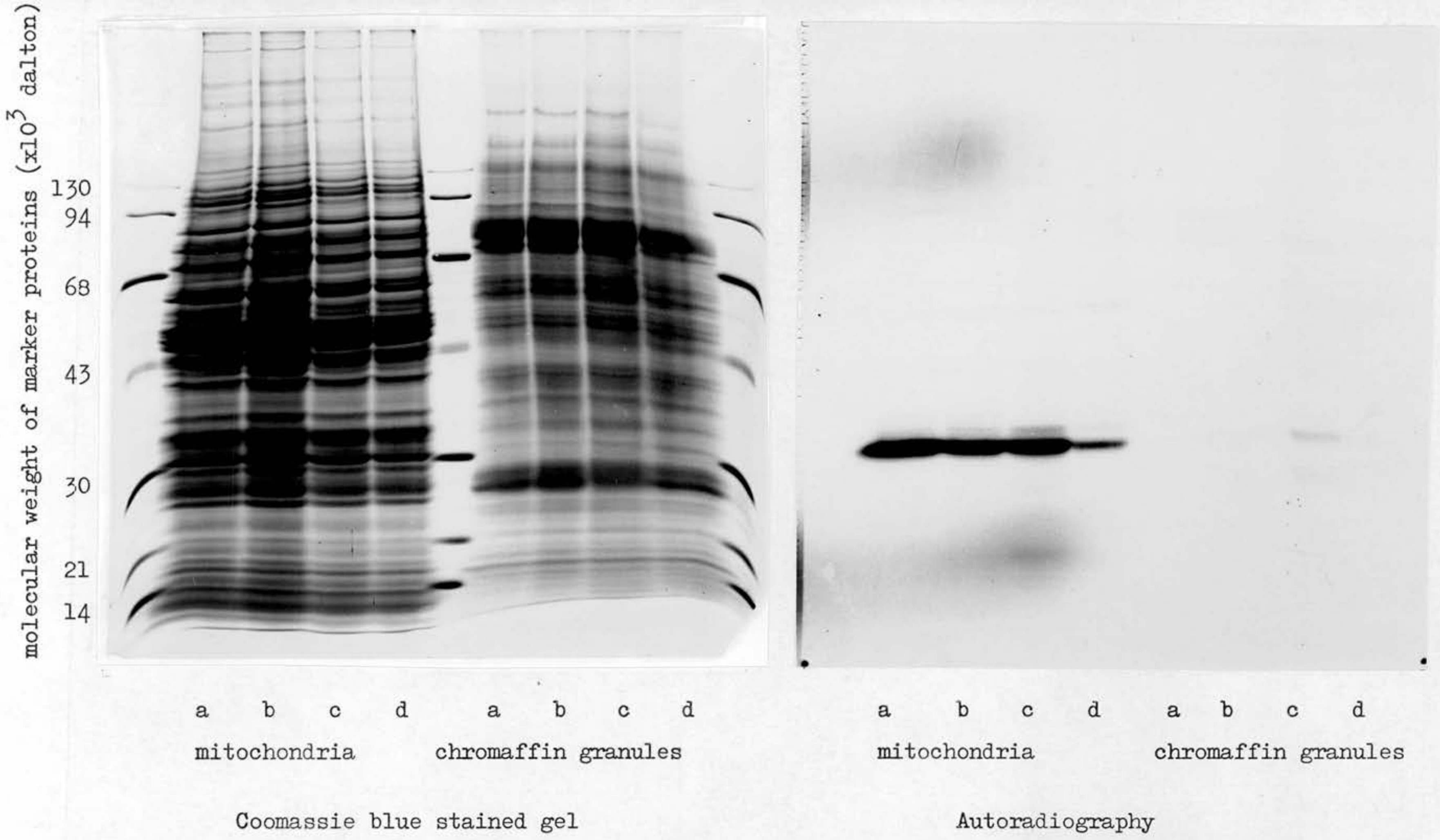
The determination of molecular weights could be made with some accuracy in these experiments because of the higher energy of the (^{32}P) radioactivity compared with tritium, which allowed to autoradiograph stained gels which had not been treated with salicylate. An exposure time of about 1 month was necessary. Such obtained films were exactly superimposable to the stained and dryed gels. Figures for molecular weights are calculated from the migration of standard proteins in the gels and ought to be understood as "apparent" molecular weights.

Since a large number of proteins became radioactive when membranes were labelled, and no major reduction of the labelling pattern was obtained with various substances present in the labelling media, a different approach was used to try to increase the specificity of labelling. An

experiment was designed to label chromaffin granule membranes only from that side which faces the cytoplasm in vivo. Since it has to be expected that as much as 45% of the membranes in a chromaffin granule ghost preparation are broken or sealed inside-out (Hunter et al.,1982), ghosts were not useful for such an experiment and only a preparation of intact granules could provide a correct orientation of the membrane.

A preparation of crude chromafin granules, 2mg in 2ml NaHepes, pH 7.2, was labelled with 5µl (10µCi) (γ-^{32}P)azidoATP. The sample was incubated with the label in the dark on ice for 30 min. and then given four flashes with a high energy UV-light as described above. The suspension of labelled crude granules, which was still contaminated with mitochondria, was purified by centrifugation through a 1.7M sucrose step. The pelleted granules were lysed in 10mM NaHepes, 1mM EDTA, pH 7.2, and the resulting membranes were pelleted and washed once. The contaminating mitochondria from the crude granule preparation could be collected from the interface of the step. They were lysed in 10mM NaHepes, 1mM EDTA, pH 7.2, and resulting membranes were pelleted and further purified by a centrifugation through 1M sucrose.

With this experiment labelled mitochondrial and chromaffin granule membranes were obtained at the same time. Both preparations were separated on a 6-15% acrylamide SDS gel as before (Fig.54, tracks a). On the same gel samples were also analysed which had been labelled in presence of



<u>Fig.54:</u> Labelling of intact mitochondria (left) and chromaffin granules (right) with azido(γ-^{32}P)ATP. Labelling was done at neutral pH in absence (tracks a) and presence of 400µM atractyloside (tracks b), or in presence of 50mM potassium phosphate at pH 6.0 (tracks c) or pH 8.0 (tracks d). For the separation a reducing SDS gel with 6-15% acrylamide was used, the molecular weight of the marker proteins is indicated.

either 400μM atractyloside (tracks b), 50mM potassium phosphate, pH 6.0 (tracks c), or pH 8.0 (tracks d). The autoradiograph of the gel is also shown in Fig.54, a somewhat different mixture of molecular weight markers was used and the proteins were, from top to bottom, (molecular weights): β-galactosidase (130,000), phosphorylase (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (21,000), lysozyme (14,000). The mitochondrial fractions showed one very strong radioactive band. It represents a protein of a molecular weight of 31,500 on the gel and is likely to be identical with the mitochondrial adenine nucleotide exchange protein of 30,000 mol.wt. (Kraemer & Klingenberg, 1977). The presence of atractyloside in the labelling medium did not inhibit the labelling of this band (track b), perhaps because the radiolabel had such a high specific radioactivity that only a few molecules, available even in presence of ATR, had to be labelled to give a strong blackening of the film.

None of the proteins of the chromaffin granules became labelled so strongly. This could mean that there is actually no protein in the membrane to be labelled with azidoATP, but then it would be not clear why azido ATP inhibits transport activities across the ghost membrane. Alternatively it could be argued that chromaffin granule membranes do not become labelled in a mixture with mitochondria because the mitochondrial protein has a higher affinity for the label and therfore acts as a sink, efficiently removing from the medium the small amount of

radioactive molecules available. In one track (c of the separated chromaffin granule membranes a faint band of radioactivity is seen at about the same position as the mitochondrial band. In a further experiment it was shown that this band runs to exactly the same position in the gel as the strongly labelled mitochondrial one. This was done by separating a mixture of labelled mitochondrial and chromaffin granule membrane proteins in one track in a gel, flanked by each a sample of mitochondria and granule protein. Since the radioactive band is not represented by a marked stained band in granule membranes and is also not seen as one of the major bands in other labelling experiments (Fig.53) it was considered to stem from mitochondrial contamination.

Obviously it would be desirable to label intact chromaffin granules which are purified from mitochondria, but every purification of whole granules involves centrifugation through concentrated sucrose, in which granules shrink first and then, when resuspended in 0.3M sucrose buffer, re-swell and burst. Therefore such a preparation would also yield labelled matrix proteins and that labelling of proteins from the inner side of the membrane could not be excluded.

Analysis in two dimensions of membrane proteins labelled with azidoATP.

The analysis of a mixture of proteins in two dimensions as introduced in Fig.47 has to be considered as a diagnostic

tool like a "fingerprint" of a digested protein. At the present stage of the development of the method it is not certain whether the proteins observed on a stained slab gel (Fig.55) represent all the proteins originally present in the mixture of solubilized chromaffin granule membranes. Much depends on the solubilisation itself and some of the proteins might not enter the first or the second gel. This is seen in Fig.55 where to the left a vertical array of proteins which did not enter the focussing gel are resolved on the slab gel. Especially integral membrane proteins which are very hydrophobic and are associated with lipids might not be solubilized readily and therefore not detected on the two dimensional maps. This was shown to be the case for the DCCD-reactive proteolipid of the chromaffin granule ATPase (R.Sutton, personal communication).

An advantage of the two dimension analysis is however, that it allows separation of mixtures of proteins of similar molecular weights, which migrate to the same area in an ordinary one dimensional slab gel. It makes it possible to associate radioactive bands to proteins of a strongly staining region as in the case of the DBH-area in Fig.53. But not all labelled proteins seen as bands on a one-dimensional gel can be shown as intensive black dots on an autoradiograph . Some proteins do not focus well, others do not even enter the focussing gel. Membrane proteins of higher molecular weights (above about 50,000) focussed generally better than smaller ones and labelled proteins of lower molecular weights were hardly ever seen on autoradiographs (also see Fig.47). The following results

can therefore not be expected to be complete and the discussion concentrates only on what could actually be observed.

A stained two dimensional map of chromaffin granule memrane proteins and an autoradiograph of proteins labelled with radioactive azido ATP and resolved in two dimensions is shown in Fig.55. Only a relatively small number of labelled proteins was seen compared with the autoradiograph of the one dimensional separation in Fig.53. — Several proteins of a molecular weight near that of DBH were labelled. One is probably DBH itself, one other strongly labelled protein is marked "A" in the figure. The strong darkening of the of the film around DBH and "A" is resolved into two distinct areas in films which were exposed for a shorter time. DBH is labelled very strongly and the blackening of the film seems to originate from the upper and lower streaky bands seen on the stained gel. Chromogranin A (CGA), which is probably a contaminant from the lysate, is however only labelled very weakly. These radioactive bands taken together explain the observation made in one —dimensional gels of two strong bands, DBH above "A", superimposed on a weaker fuzzy band, chromogranin A, (Fig.53). Chromogranin A seems not to contribute to the strong blackening of the DBH area in one dimensional gels. This is probably also true for labelled lysate proteins (Fig.56) where a strongly radioactive mark is seen in that area but stems from a different protein migrating near CGA. Similarly, in experiments with radioactive Nbf-Cl (Figs.45 and 46), another potein in that region of the gel was labelled apart from

B (↙)
A (↗)
C (↙)
D (↗)

7.5 6.4 5.75 5.1 4.6 4.2 3.2

pH of focussing gel

autoradiography

Fig.55: Two dimensional map of chromaffin granule membrane proteins. Horizontally the separation is according to the charge of the protein (isoelectric focussing) and vertically according to molecular weight (SDS gel-electrophoresis with 10-15% acrylamide). The proteins dopamine-β-hydroxylase (DBH), chromogranin A (CGA), and cytochrome b561 (CYT) are indicated. Additional four radioactive proteins are marked with A, B, C and D, respectively, on the autoradiography (right) and the protein stained gel (left).

DBH. It was marked "2" and is likely to be identical with "A".

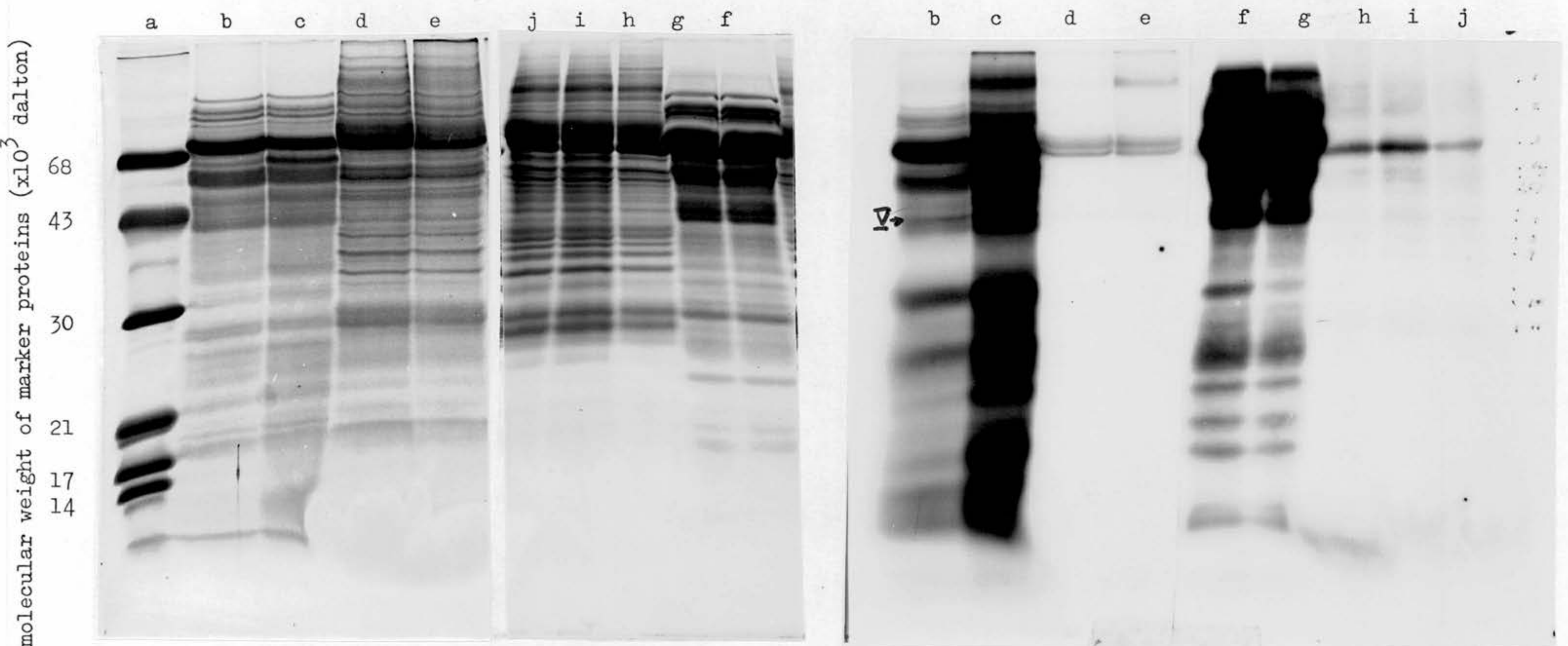
A further radioactive protein is marked with "B", and although it is very well focussed in the two dimensional gel, it seems not to be represented by a strongly stained band in a one dimensional gel, and might be one of the non-staining radioactive bands above DBH in Fig.53. The position of two fairly radioactive proteins, "C" and "D", was identified on the stained gel. But the position of other more strongly radioactive bands of lower molecular weights like the 39,000 and 35,000 mol.wt. proteins in Fig.53, and the cytochrome, could not be definitely identified in the two dimensional gel. Their radioactivity could only be marginally traced on X-ray films which had been sensitized by pre-exposing them to light. A possible location for the proteins is however suggested (III = 39,000; II = 35,000; I = 32,000 mol.wt.).

Labelling of lysate proteins with azidoATP.

Soluble proteins (lysate) from the chromaffin granule matrix, obtained by lysis of intact granules, were labelled with azido (γ-^{32}P)ATP. 250µl of 4mg/ml dialysed and concentrated lysate in 10mM NaHepes, 4mM EDTA, pH 7.2, were incubated with 4µCi azido ATP on ice in the dark for 20 min. The radioactive label was then activated by UV light in the two different ways described before. The labelled samples were mixed directly with concentrated electrophoresis buffer and analysed on an SDS gel with a 10-15% exponential

gradient of acrylamide. In Fig.56 a gel stained for proteins with coomassie blue and its autoradiograph is shown. Lysate proteins are separated in tracks b and c. Track b contains the sample which was flashed with strong UV light, track c the sample exposed for 45 min. to the low energy UV source. In track a molecular weight standards as in Fig.53 are run. Membranes labelled with azidoATP are shown as well.

The autoradiograph shows that a large number of the lysate proteins becomes labelled. Under similar conditions, matrix proteins (tracks b and c) are labelled much more strongly than membrane proteins (tracks d and e). Lysate proteins themselves are more strongly labelled when exposed to the low energy UV source than when flashed with the high energy UV light. One matrix protein which is predominantly labelled under both conditions migrates like chromogranin A, above the marker for 68,000 molecular weight. Below this protein there is a band observed which is much more abundant on the stained gel in track c and might therefore be a product due to the long exposure to UV radiation. It is present as a stained as well as radioactive band. A very sharp radioactive band (marked V) of about 48,000 molecular weight is seen both in the lysate and in the membrane proteins. It is also seen in labelled membrane proteins in Fig.53 (band V). Fig.56 also shows an autoradiograph of lysate proteins labelled with radioactive dialdehyde ADP (track f) and dialdehyde AMP (track g) separated on a similar gel together with membranes (track h) and ghosts (track i) labelled with radioactive dialdehyde ATP and



<u>Fig.56:</u> Chromaffin granule lysate proteins separated on a 10-15% acrylamide SDS gel. The proteins were labelled with azido(γ-^{32}P)ATP {tracks b, c (" d, e)} or with dialdehyde(^{3}H)ADP {track f (" h,i)} or -AMP (track g (" j)). Membrane proteins from parallel experiments are also shown (tracks d, e and h-j, respectively); for details see text.

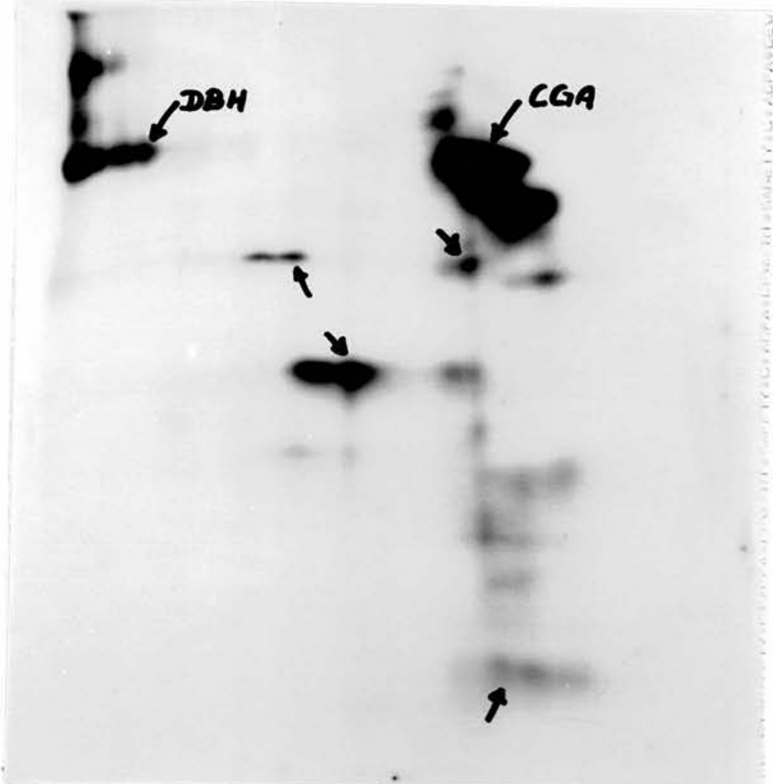
Note: tracks f-j are reversed in the left photograph

ghosts labelled with dialdehyde ADP (track j). Samples of membrane proteins were prepared as described for Fig.50. Dialysed and concentrated matrix proteins at 10mg/ml were incubated in in 320µl 2mM NaHepes, pH 7.2, 30mM NaCl, with 2-3µCi dialdehyde (^{3}H)adenine nucleotides at 4°C overnight. After the labelling the samples were mixed with concentrated electrophoresis sample buffer to give a solution of 5mg/ml of which 100µl were analysed per track on the gel.

More information about which proteins are labelled can be obtained if lysate proteins are analysed in two dimensions, as explained for Fig.55. Because the soluble proteins labelled very strongly and quantitatively entered both the the first- and second-dimension gels, it was easier to monitor them for radioactivity on the two dimensional gel as than it had been for labelled membrane proteins. To resolve the mainly acidic lysate proteins on a focussing gel, ampholines of a narrower pH-range were used (pH 4.0-6.0). The resulting pH— gradient of the gel was measured from eluted gel portions as described before and is shown in Fig.57. The autoradiograph of proteins labelled with azido (γ-^{32}P)ATP and exposed to a strong UV-light in four short intervals with cooling in between is also shown in Fig.57. For the second dimension a gel with an exponential gradient of 10-15% acrylamide was used as before. In the stained gel the vertical tail to the very left represents proteins which did not enter the focussing gel and are only resolved according to their molecular weight. They can be used to compare the two-dimensional map with one — dimensional gels. DBH and chromogranin A have

5.9 5.8 5.6 5.2 5.0 4.8 4.4 3.4

pH of focussing gel



autoradiography

Fig.57: Two dimensional map of chromaffin granule lysate proteins labelled with azido(γ-^{32}P)ATP. Horizontally the separation is according to the charge of the protein (isoelectric focussing; note the narrower pH range) and vertically according to molecular weight (SDS gel electrophoresis with 10-15% acrylamide). Dopamine-β-hydroxylase (DBH) and chromogranin A (CGA), as well as four other proteins are indicated on the coomassie blue stained gel (left) and on the autoradiography (right).

previously been identified on two dimensional gels of lysate proteins (Apps et al.,1980a).

DBH, which is only a minor protein in the chromaffin granule matrix, is labelled by azido ATP. This has already been observed in a similar experiment done with granule membranes (Fig.55). Chromogranin A (CGA) is labelled as well but a larger proportion of the the blackening of the film in that area seems to originate from the protein migrating directly beneath and to the right of chromogranin A. This is more clearly seen on autoradiographs exposed for a few days only. A few other labelled proteins are marked in both, stained gel and autoradiograph (Fig.57). Two pairs of strongly labelled proteins (arrows) are only very minor spots on the coomassie blue stained gel. These proteins are obviously labelled with a high specificity. Very minor proteins however might also represent some contamination from the cytoplasm of the chromaffin cell. — Several proteins on the stained gel form groups which have a similar molecular weight but a different charge and are probably charge isomers. Since the pH – gradient in this gel is shallow the analysis is very sensitive and proteins with only a slight difference in charge are separated. - Obviously most proteins cannot so far be identified on the gel. The experiments serve therfore mainly a purpose of listing proteins which get labelled with ATP— analogues. Some of them might be identified as ATP-requiring enzymes in the future.

Discussion.

The aim of the labelling experiments was to identify one of the proteins in the chromaffin granule membrane as the possible adenine nucleotide translocating protein. The affinity labels which were used varied in specificity but all of them labelled a series of proteins. Ideally, one of the analogues would have labelled a single protein only, thus identifying a very likely candidate. The feasibility of such an approach was convincingly shown for mitochondria in Fig.54 where of all mitochondrial proteins facing the cytoplasmic side in the inner membrane the adenine nucleotide exchange protein was the only one strongly labelled with azido—ATP. In a similar experiment with chromaffin granules, no protein was labelled. As outlined above it could have been for methodological reasons that no positive result was obtained; alternatively there might be none of the ATP-requiring granule membrane proteins facing the cytoplasm (F_1-ATPase, phosphatidylinositol kinase, putative adenine nucleotide carrier) capable of reacting with this ATP analogue.

In initial experiments little attention was given to the sidedness of the membranes and experimental conditions were established by which proteins were labelled as strongly as possible. Most of the analogues used labelled quite a number of proteins and many of the individual proteins became labelled by several analogues. All proteins which

were labelled by FSBA were also labelled by dialdehyde ATP and most of those labelled by dialdehyde ATP were also labelled by azidoATP, when the activation was done by flashing with strong UV light. As expected, the labelling pattern of the less specific label Nbf-Cl was fairly different from that of azidoATP.

Several ATP-requiring enzymes have been reported to be associated with chromaffin granule membranes. Apart from the F_1-type ATPase (Apps & Glover, 1978) another, vanadate-sensitive ATPase with an apparent molecular weight of 158,000, as determined by exclusion chromatography, has been reported (Apps et al., 1982). In an earlier study the ADP/ATP exchange activity present in the chromaffin granules (Apps & Reid, 1977) was reported to have an apparent molecular weight of 130,000. Both enzymes might be protein complexes and nothing is known about the size of possible subunits. Obviously the subunits would be observed on SDS gels. Dopamine β-hydroxylase has been reported to be activated by ADP (Tachikawa et al.,1979) and is therefore also likely to react with the affinity labels used. There is also a phosphatidylinositol kinase (Phillips, 1973) in the membranes.

These ATP-requiring enzymes are proteins which like the putative adenine nucleotide transporter, are expected to be labelled by the adenine nucleotide affinity analogues. The rather large number of proteins which were found to be labelled in the experiments is therefore not surprising. It was however not possible to inhibit individual proteins

selectively for further identification, with one exception in Fig.53 where the labelling of the band "3" with azido ATP was prevented by phosphate (track d). This protein is most likely to be identical with the protein termed "A" in the two dimensional gel in Fig.55 since it runs in both gels to a similar area below the strongly labelled band of DBH.

Rather little work has been published on the characterisation of proteins of the chromaffin granule membrane. Those few membrane-proteins which have at least been partially characterized have already been mentioned in the text. They include DBH, F_1-ATPase and cytochrome b561, actin and α-actinin; additionally there are activities such as catecholamine translocase which have not been associated with a protein so far. A more general approach to characterize membrane proteins was adopted by Abbs & Phillips (1980). In an extensive study they classified over 60 coomassie blue-stained proteins on SDS gels by their sensitivity to radiolabels and digestive agents. The proteins were numbered consecutively according to apparent molecular weight. At first sight the pattern of stained proteins on their gels and on the various gels presented here may look rather different, but employing the method of grouping the proteins as described for Fig.49 it is possible to identify similar proteins on the respective gels.

In Fig.58 the results of the experiments with the various affinity analogues are summarized. Membrane proteins separated on a representative 6 — 15% acrylamide gradient gel are as far as possible identified by the same

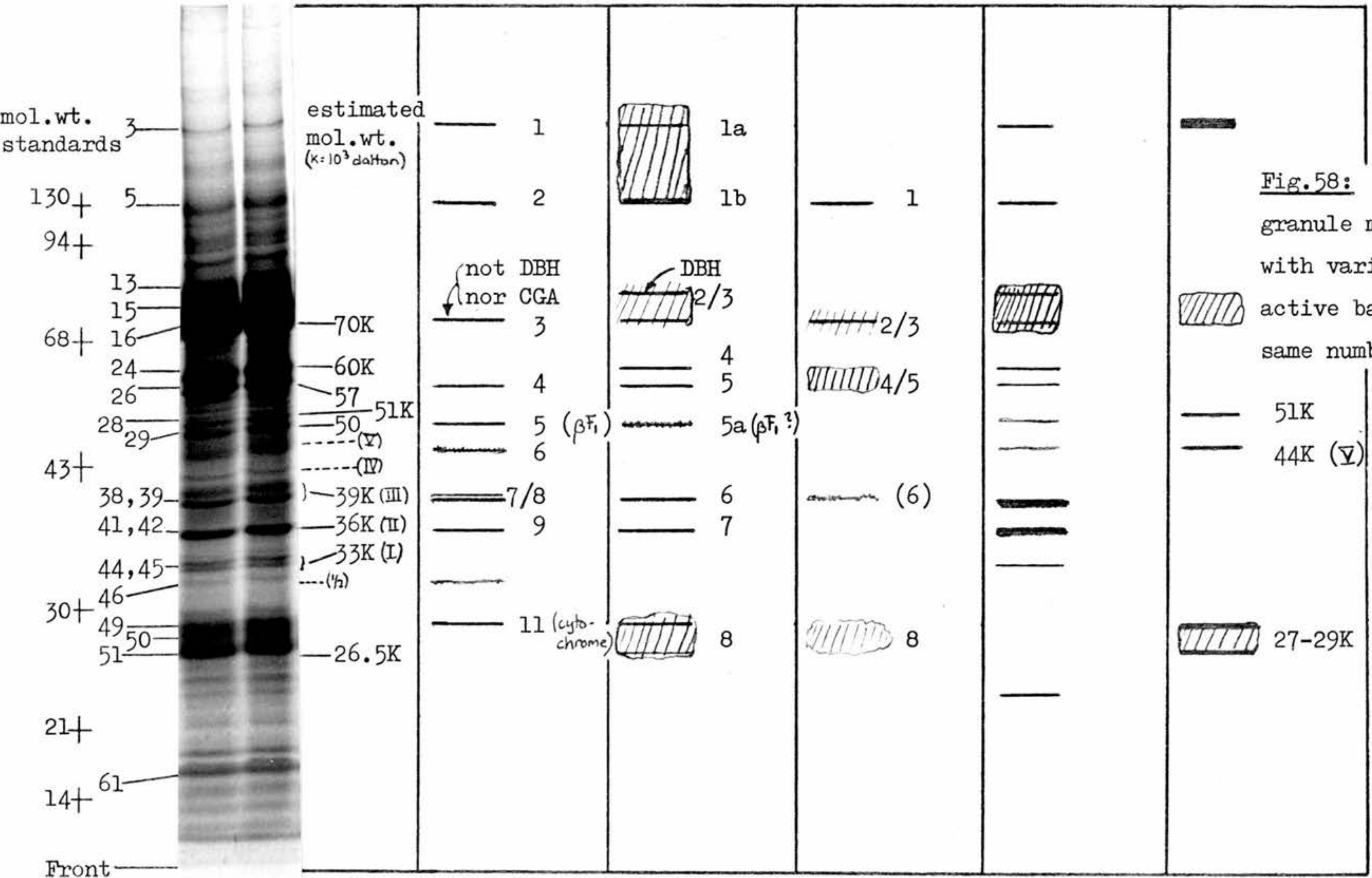


Fig.58: Comparison of chromaffin granule membrane proteins labelled with various affinity labels. Radioactive bands are identified with the same numbers as in the discussions of the respective experiments. The coomassie blue stained protein bands are numbered according to Abbs & Phillips, the gel contained 6-15% acrylamide. The position of molecular weight markers (in 10^3 dalton) is shown to the very left.

numbers as in the study by Abbs & Phillips (1980) . This was done by comparing the labelling pattern of each analogue from four or more experiments. In the subsequent discussion those numbers are used to name proteins. As explained before, several proteins tended to migrate as broad bands in one gel and as resolved doublets in the next; for simplicity these are identified as one band only in Fig.58. The radioactive bands were numbered as in the individual experiments with different affinity labels. Not all radiolabelled proteins could be identified with the same accuracy. Abbs & Phillips (1980) recognized only a few proteins above a molecular weight of 100,000, but in Fig.53 more proteins of that size can be seen, two of them were strongly labelled with azido ATP, and these might be identical with high molecular weight bands labelled by the other compounds as is indicated in the figure. The general appearance of these bands suggests an identity with bands 2 and 5 of Abbs & Phillips (1980); however our molecular weights (approximately 150,000 and 120,000, respectively) are higher than esatimated by these authors, so this identification is not conclusive.

All analogues labelled one or two proteins in the DBH-area strongly and one of them (band 16) was clearly labelled by both Nbf-Cl (band 3) and by azidoATP, and was shown to be different from DBH on two dimensional gels (Figs.47 and 55). It had an apparent molecular weight of

about 70,000 and was somewhat more acidic than DBH, which has a pI of 5.3. (Some further investigation of this strongly labelling protein would be worth doing). - Bands 24 and 26, of 60,000 and 57,000 mol.wt., which were always strongly stained with coomassie blue, could be easily recognized and were labelled by several agents, but nothing else is known about the nature of these two proteins. Further down in the gel in a molecular weight range between about 55,000 and 40,000 there are several more fainly staining bands of which some were radioactive in the different experiments. Since the F_1- ATPase subunits α and β (51,000 and 50,000 mol.wt.) migrate in that area, these were normally associated with the labelled bands, but this is only one possible interpretation. - The strongly stained bands 38/39 and 41/42 were clearly labelled by several agents. The doublet 38/39 is probably a glycoprotein and 39, at least, seems to span the membrane (Abbs & Phillips, 1980). The bands of the cytochrome area were variably labelled. Nbf-Cl seemed to label the upper band (49) in that area, dialdehyde ATP and FSBA rather the whole area and azido ATP the lower strong band (51). As outlined before the bands might be related to each other and one might be a product of the other, hence the variable labelling pattern.

There are six different proteins which were labelled by all the three substances Nbf-Cl, dialdehyde ATP and azido ATP (proteins 3,5,16,26,38/39,41/42). All these three agents also inhibited a part of the ATP transport into chromaffin granule ghosts (azidoAMP instead of azidoATP was used in kinetic studies), and these proteins reacting with

all the three labels are therefore the likeliest candidates for a possible adenine nucleotide transport protein. However there seems to be no reason to prefer one of these proteins as a more likely candidate above the other. Since FSBA does not inhibit the ATP uptake into ghosts , a possibility would be to discount the proteins labelled by this agent from the list of candidates, leaving essentially only the proteins 38/39 and 41/42.

The adenine nucleotide transporting enzyme might however not be a major protein in which case it was probably not considered for discussion in Fig.58, or it might be a protein only very weakly labelled with radioactivity. This is the case with the α- and β-subunits of the F_1-ATPase. Altough it is an enzyme which is vital for the chromaffin granule and was expected to label very strongly with the ATP analogues, the subunits could not be clearly identified as radioactive bands, probably because it is not a very abundant protein. There are probably only a few copies of this enzyme on in each granule (Apps et al., 1982). The same might be the case for the catecholamine transporter which has a very small specific activity in the membranes and is therfore not expected to be a very abundant protein.

Adrenergic synaptic vesicles have been shown to possess an active adenine nucleotide transport mechanism which might be facilitated by a 34,000 mol.wt. protein (Luqmani, 1981), and the mitochondrial ATP/ADP exchange protein has a molecular weight of 30,000. It is therefore tempting to look for a protein of a similar molecular weight in

chromaffin granules. This would favor proteins 38/39 or 41/42 with molecular weights of 39,000 and 36,000 respectively as candidates. Since there seems to be a close connection between the two F_1— ATPases from chromaffin granule and mitochondria (Apps & Schatz, 1979) a superficial conclusion could be made that the two adenine nucleotide transporters ought to be similar as well. But at this stage this is a speculation and at least some experiments with antibodies against the mitochondrial shuttle would be needed to make firmer comments on this. It has to be envisaged that a chromaffin granule carrier would have a very different task in filling empty vesicles with adenine nucleotides for the first time, in a transport mechanism which is likely to be unidirectional, whereas the mitochondrial protein facilitates an exchange reaction. A key to that problem might therefore lie in the question how mitochondria acquire their content of adenine nucleotide in the first instance (see e.g.Pollak & Sutton, 1979).

Using the discussed criteria of affinity labelling, the presented results suggest several proteins in the chromaffin granule as candidates for an adenine nucleotide transport protein, but they do not clearly favour one of them nor do they unequivocally prove the existence of such a protein. Therefore all proteins listed in Fig.58 ought to be kept in mind for future research which might usefully be done with "immature" chromaffin granules isolated from cultured adrenal cells.

Based on n.m.r. data (Sen & Sharp, 1981) and on kinetic and thermodynamic considerations (Phillips & Apps, 1980) there is good evidence that the catecholamines inside the chromaffin granules are in an unbound form and free in solution. An energy-dependent uptake mechanism exists to create initially, and then to maintain, the high catecholamine gradient.

The investigations presented in this report suggest that the situation for the uptake of ATP into chromaffin granules might be quite different. Chromaffin granule ghosts have been used as a model system and the transport of ATP across the ghost membrane has been studied. It was possible to show and partially characterize an ATP uptake process which is quite different from the uptake mechanism for catecholamines: The ATP uptake was not saturable and no significant concentration gradients could be obtained. Because of the dual nature of the transported metabolite which is transported substrate as well as energy carrier, it was very difficult to judge the energy dependence of the uptake mechanism, but it appears that most of the observed ATP uptake does not depend on membrane energization. During the uptake ATP did not equilibrate with the membrane potential as suggested in a series of reports from Winkler's group, and their proposition tht ATP uptake is dependent on the membrane potential, which was based on experiments performed with intact granules, is not supported by the findings made with ghosts.

The ATP uptake mechanism in ghosts showed a broad specificity - all adenine nucleotides were transported equally well, and phosphoenolpyruvate was also transported, quite possibly by the same process. Some substances were found which inhibited the uptake, the most striking effects being observed with polysulfonated aromatic compounds such as cibacron blue.

A likely process by which ATP is transported through the membrane is facilitated diffusion, for which, however, saturation kinetics ought to be observed. Perhaps the process has a very high Km (>30mM) which made it impossible to observe saturation in the experiments. If on the other hand the diffusion is not facilitated it is necessary to investigate how a large molecule with several negative charges passes through the membrane which is otherwise quite impermeable to a whole series of monovalent and divalent anions (Phillips, 1977). It might be informative to conduct uptake experiments with artificial vesicles which resemble the granule membrane in their lipid composition and kinetic experiments with reconstituted membrane vesicles might also yield some conclusions.

Winkler and coworkers have made considerable efforts to distinguish between carrier-mediated uptake and diffusion of ATP into intact chromaffin granules. Perhaps such a clear distinction cannot be made and it is probably wrong to exclude difffusion as an artefact. If diffusion is observed, why should it not be one of the means which is used by the granules in vivo? The fact that the nucleotide composition of the granule essentially reflects that of the cytoplasm certainly supports the view of an unspecific process.

With the results presented here some questions could be partially clarified but as many new questions could be asked in the following discussions. More is known now on how ATP might cross the chromaffin granule membrane, but the essential problem, of how the granules obtain the high concentrations of nucleotides found in vivo has not been solved.

The accumulation might be achieved indeed via binding of ATP to proteins in the interior of nascent granules. To investigate this it would be interesting to work with ghosts which contain only matrix proteins; such a preparation has not yet been achieved, and is likely to be difficult to make. Alternatively, 'young' granules might be obtained from suitable adrenal cells in culture with which such experiments could be performed. With such vesicles it should also be easier to investigate whether ATP is transported in an exchange process.

In experiments with radioactive ATP analogues several proteins of the chromaffin granule could be labelled. At this stage these results have to be used empirically, as information towards a further classification of the granule proteins. Not enough data are available at the moment to make firmer comments and to conclude that one of the small number of proteins labelled by the various ATP analogues is involved in facilitating the ATP transport. The labelled proteins are however likely candidates for the several ATP-requiring enzyme activities known to exist in the granule membrane. It may therefore be profitable to pursue this type of experiment, especially with more strongly radioactive

substances with which results are obtained more quickly. A possibility for such labels are (^{32}P)-labelled dialdehyde adenine nucleotides which could be relatively easily synthesized.

Since chromaffin granules are only one type of a group of neurotransmitter storing vesicles, the clarification of their structure might it is hoped, advance the knowledge of the other vesicles, resulting in a further understanding of the mammalian nervous system.

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