To the Graduate Council:

I am submitting herewith a dissertation written by James A. Wilkins entitled "Transport of 5-Hydroxytryptamine by Platelet Dense Granules." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

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TRANSPORT OF 5-HYDROXYTRYPTAMINE BY PLATELET DENSE GRANULES

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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James A. Wilkins

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This dissertation is dedicated to my wife and friend, Diane, and to Dr. John W. Greenawalt who taught me a real appreciation for scientific inquiry.

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ABSTRACT

Dense granules, the subcellular storage organelles for 5-hydroxytryptamine in the blood platelet have been isolated and were shown to transport 5-hydroxytryptamine via two mechanisms: (1) a carrier-mediated process perdominating at low substrate concentrations and (2) a diffusion-mediated process predominating at higher substrate concentrations. The apparent Km for the carrier-mediated process was 3.3μM and its Vmax was 0.79 nmole 5-hydroxytryptamine/min/mg protein. 5-hydroxytryptamine transport was sensitive to temperature and studies revealed an apparent activation energy of 14.9 kcal/mole for the mediated process. Transport was inhibited by several structural analogs of 5-hydroxytryptamine and was also inhibited by reduction of the osmotic pressure of the incubation medium below 300mOsM. 5-hydroxytryptamine transport was sensitive to changes in pH of the incubation medium. This effect was apparently due to a change in the transmembrane pH gradient (ΔpH). It was shown by direct measurements using [^{14}C]-methylamine that decreasing the pH of the incubation medium decreased ΔpH from a value of 1.1 pH unit (acid inside) at pH 7.0 to values near zero at pH 6.0 and produced a concomitant decrease in the initial rate of 5-hydroxytryptamine transport by intact granules.

Ghosts of dense granules were also prepared by hypotonic lysis. Measurements showed that ghosts were depleted of 95% of the original endogenous 5-hydroxytryptamine content. Ghosts prepared in the above

way transported exogenously added 5-hydroxytryptamine with a time course similar to intact granules when incubated in the presence of ATP and Mg++. Transport in the absence of ATP and Mg++ was low, reaching approximately 30% of the steady state levels found in their presence. ATP-stimulated transport was sensitive to addition of nigericin, an ionophore which catalyzes electroneutral exchange between K+ or Na+ and H+. It was found that pH gradients formed in the presence of ATP and Mg++ were also reduced substantially by such additions of nigericin. Ghosts were found to swell upon the addition of ATP and Mg++ as measured by light scattering techniques. This swelling was inhibited by agents which were shown to inhibit the Mg++- stimulated ATPase and was therefore attributed to inwardly-directed proton translocation by the ATPase. Swelling in the presence of ATP and Mg++ was stimulated by addition of nigericin or the combination of valinomycin and carbonyl cyanide chlorophenylhydrazone. This effect was apparently due to increased anion transport in the presence of these uncouplers. Addition of increasing concentrations of the anion transport inhibitor, SITS (4-acetamido-4′-isothiocyanostilbene-2,2′ disulfonic acid) inhibited swelling induced by nigericin in the presence of ATP and Mg++ however SITS had no effect on swelling in the presence of ATP alone. This effect suggests that SITS acts at anion transport sites on the ghost membrane and does not inhibit ghost ATPase activity. Transport of 5-hydroxytryptamine was stimulated by ATP in a concentration-dependent manner and was also stimulated by the establishment of an artificial ΔpH across the ghost membrane.

In summary, the results presented here demonstrate that isolated dense granules transport 5-hydroxytryptamine via a mechanism which is dependent upon the presence of a transmembrane ΔpH (acid inside). Ghosts prepared from dense granules also transport 5-hydroxytryptamine in response to ΔpH. The transmembrane ΔpH appears to be generated by an inwardly-directed proton-translocating ATPase in the granule membrane. The results presented here along with those obtained by others using amine storage organelles such as chromaffin granules from adrenal medulla and synaptic vesicles from brain suggest a generalized mechanism for amine transport by subcellular biogenic amine storage organelles.

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5-HT	5-hydroxytryptamine
HEPES	N-2 hydroxyethylpiperazine-N-2-ethanesulfonic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid
ATP	adenosine 5'triphosphate, disodium salt
BSA	bovine serum albumin
EDTA	disodium (ethylenedinitrilo) tetraacetate
Butyl-PBD	2-(4-t-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole
5-HIAA	5-hydroxyindole-3-acetic acid
NMR	nuclear magnetic resonance
ΔpH	transmembrane proton gradient
MES	2-(N-morpholino) ethanesulfonic acid
CCCP	carbonyl cyanide m-chlorophenylhydrazone
DCCD	N,N-dicyclohexylcarbodiimide
SITS	4-acetamido-4'-isothiocyanostilbene-2,2'disulfonic acid (disodium salt)
ATPase	adenosine triphosphatase
TRIS	TRIS(hydroxymethyl) aminomethane
$\Delta\Psi$	transmembrane electrical potential
$\Delta\mu_{H+}$	transmembrane electrochemical gradient of protons

CHAPTER I

INTRODUCTION

Many different mammalian cell types are known to transport and store biologically active amines (1-3). Foremost among them are the neuronal cells from the central and peripheral nervous systems which synthesize as well as transport and store biogenic amines such as norepinephrine, serotonin (5-hydroxytryptamine), and dopamine among others. One function of the amines in the brain appears to be the transmission of signals between neuronal cells. They thus play an important role in the complex integration of signals within the brain. Other cell types which store and transport biogenic amines are the chromaffin cell of the adrenal medulla, which stores epinephrine and norepinephrine (4,5); the mast cell which stores histamine; and the platelet which stores large amounts of serotonin. The function of these amines in the chromaffin and mast cells is better characterized than that of serotonin in the blood platelet. However, it has been proposed that serotonin, due to its action as a vasoconstrictor, participates in the hemostatic mechanism (6). Although it is released extracellularly during the platelet "release" reaction (see below), the role of serotonin in hemostasis still remains to be firmly established (7). The major focus of the present study is to examine the storage mechanism for serotonin in the blood platelet and to compare these findings with

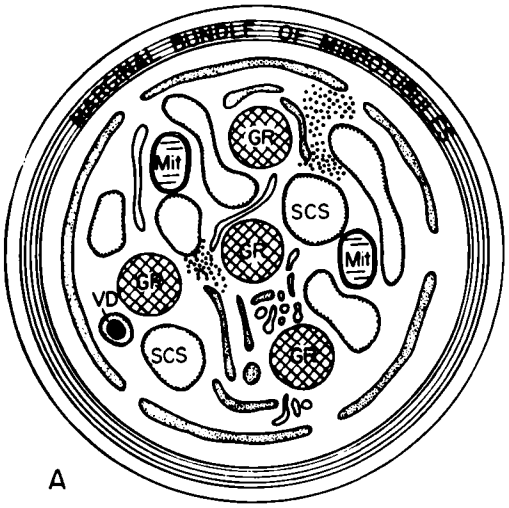
those of others both in platelets and in other tissues—most notably, adrenal medulla.

The Blood Platelet—General Features

In this section, it is intended to give only a very brief overview of the platelet and its functions. Several excellent reviews regarding the platelets' role in hemostasis and other physiological phenomena are available (8,9) and the reader is referred to these for a more thorough treatment of this particular subject. Blood platelets are disc-shaped with an average diameter of 2-4μ (10). Platelets, although they arise from differentiation of the megakaryocyte in the bone marrow and thus contain no nucleus, contain numerous subcellular organelles, some of which are easily identifiable upon examination in the electron microscope. Figures 1A and B give a diagrammatic representation of the blood platelet with its organelles shown. Figure 1C shows an electron micrograph of a typical blood platelet. As may be seen in this diagram, platelets contain many of the same organelles which are present in cells such as hepatocytes. In addition to mitochondria, microtubules, and an occasional Golgi apparatus, platelets contain numerous other organelles of varying electron densities. It has been shown by methods such as subcellular fractionation and secretion studies (see below), that these organelles consist of four basic types: (1) the organelles which store serotonin, ATP, ADP, and divalent metal ions—termed variously "bull's eye" granules, 5-HT granules, very dense bodies, and dense granules (these organelles will be referred to herein as "dense granules");

Figure 1. Diagrammatic scheme of an ultrathin section of a blood platelet.

The platelet appears in (A) equitorial section and (B) traverse section. GR-α-granules; VD—very dense bodies (dense granules); MT—microtubules; MIT—mitochondria; DTS—dense tubular system; GLY—glycogen granules. An electron micrograph of a human platelet is seen in (C). Abbreviations for subcellular structures are the same as in (A) and (B). PS—pseudopod; VAC—vacuole. Taken from Hovig, T. (1968) Ser. Haemat. 1 (2), p. 3.


PERIPHERAL BUNDLE OF MICROTUBULES
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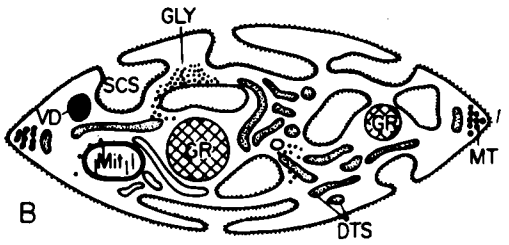
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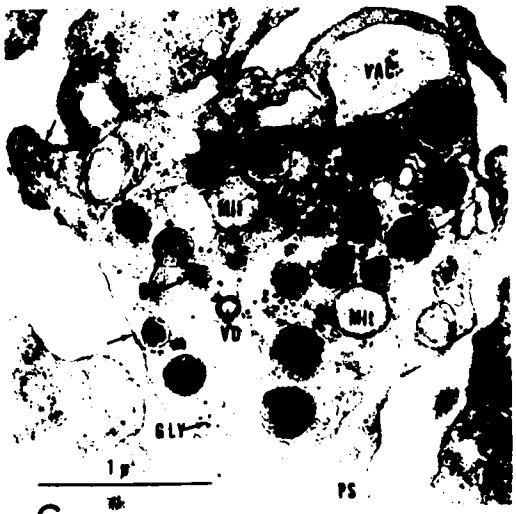
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Figure 1

(2) organelles which store platelet factor 4, growth factor, and fibrinogen, which are termed "α-granules"; (3 and 4) organelles which store acid hydrolases such as β-glycerophosphatase and thus are similar to lysosomes from other tissues. The differentiation of two different types of lysosomes is based mainly on secretion studies (see below). It should also be pointed out that it is impossible to distinguish between α-granules and lysosomes based upon morphological examination and therefore, these organelles have been referred to collectively as "α-granules." The"α-granules" and dense granules are, however, easily distinguished due to their differential electron density.

As implied above, platelets secrete the contents of the subcellular storage granules upon reception of the appropriate stimulus. These release stimuli are referred to collectively as "release inducers." These inducers set in motion a process known as the "basic platelet reaction." The steps in the basic platelet reaction are the following: shape change, aggregation of the platelets, dense granule secretion, α-granule secretion and acid hydrolase (lysosomal) secretion. The stage at which the above sequence terminates depends upon: (1) the strength of the stimulus and (3) the metabolic status of the platelet. Inducers which lead to the stage of dense granule secretion are termed "weak" inducers. Weak inducers are compounds such as ADP, epinephrine, and serotonin. Inducers which lead to secretion of lysosomal enzymes are termed "strong" inducers and are characterized by thrombin and collagen. The <u>in vivo</u> function of the platelet is to participate in the hemostatic process; in other words, to form an initial "plug" following vascular

injury. Although much work has been done, the functions of many of the components secreted from platelets during the basic platelet reaction remains unclear. This is an area which is currently under active investigation in many laboratories, however. As mentioned above, one function of serotonin in hemostasis may be to act as a local vasoconstrictor at the site of vascular injury.

Subcellular Fractionation—Isolation of Biogenic Amine Storage Organelles

In order to study the transport and storage of biogenic amines by subcellular storage organelles for biogenic amines, these organelles must be isolated from the cell in an intact state, avoiding damage during isolation, when possible. The classical techniques (and adaptations thereof) of Palade and DeDuve (12,13) have been applied with great success in separating the subcellular organelles of various cell types. Here, attention will be focused upon isolation of chromaffin granules from adrenal medulla and dense granules from blood platelets.

Chromaffin granules. Isolation of chromaffin granules generally involves the following steps: (1) homogenization of the tissue, (2) differential centrifugation in order to isolate a "crude mitochondrial" fraction, and (3) sedimentation on either continuous or discontinuous density gradients prepared from various materials. Homogenization generally is done in a Potter-Elvehjem type apparatus following mincing of the tissue and is not a problem for adrenal medulla since it is relatively soft tissue. Density

gradient centrifugation has involved several different methods. Smith and Winkler (14) introduced the method of using a single 1.6M sucrose step. A purer preparation of granules may be obtained by increasing the sucrose molarity to 1.8M and it was found that pure noradrenaline containing granules could be obtained by centrifugation through 2.2M sucrose (15) although yields were not as high as with 1.6M sucrose. Trifaro and Dworkind (16) introduced the use of Ficoll/D_2O gradients for isolation of chromaffin granules. The advantage in using Ficoll is that isotonic conditions are maintained on the gradient, whereas sucrose gradients are hypertonic and thus lead to shrinking of the granules. Chromaffin granules prepared by the Ficoll/D_2O method seem to suffer somewhat in purity, however, as pointed out previously (17).

Following isolation of gradient subfractions, fractions are analyzed using known marker components such as enzymes, or, in the case of amine storage organelles, the distribution of adenine nucleotides and amines may be followed.

Dense granules. The isolation of dense granules has been accomplished using similar methods to those given for chromaffin granules above. Several comments are in order with regard to differences in procedure, however. The homogenization of platelets is made difficult by their size and also by the apparent "toughness" of the plasma membrane. Therefore, one must resort to harsher methods of homogenization than those used for adrenal medulla. The most successful methods used so far for the homogenization and therefore the most successful subfractionation procedures have been

published by Pletscher et al. (18,19) using ultrasonication to disrupt rabbit platelets; by Salganicoff et al. (20,21) who used brief treatment with the proteolytic enzymes known as "Nagarse" followed by homogenization in a French pressure cell to disrupt porcine platelets," and by Fukami et al. (22) who employed the French pressure cell following treatment of human platelets with the metabolic inhibitors rotenone and 2-deoxy-glucose. In the case of porcine platelets, Salganicoff et al. also employed a modified Chapel and Perry medium (used originally by the latter authors to isolate skeletal muscle mitochondria (23)) containing KCl and ATP during homogenization in order to keep platelet actomyosin dissociated. The use of this medium was important since it was found that homogenization in sucrose, for instance, led to the formation of a gel which entrapped subcellular organelles and reduced yields substantially (21). Broekman et al. (24) appeared to be successful in using a nitrogen cavitation technique for platelet homogenization also, although no data for serotonin distribution in their gradients were given. An example of a poor homogenization technique which led to apparently erroneous conclusions is provided by the procedure published by Minter and Crawford (25). These authors used homogenization in a tight-fitting glass homogenizer and also in a blender to obtain a platelet homogenate from porcine platelets. The homogenate was centrifuged on discontinuous sucrose gradients and bands of membranes were collected. The authors concluded that 80% of the platelet 5-HT in porcine platelets is extragranular. This is clearly not the case as shown later in this study and in previous studies mentioned above and

shows that the homogenization methods used by Minter and Crawford were not adequate for the isolation of intact organelles.

Density gradient centrifugation of platelet homogenates was carried out by Pletscher et al. (19) using continuous urografin gradients (26%-36%). These authors found that the organelles corresponding to dense granules in morphology sedimented to the bottom of these gradients. Although no data on marker enzymes for other organelles were given, 5-HT and nucleotides were concentrated almost exclusively in the pellet fraction and electron micrographs indicated that the fraction was rather pure in dense granules.

As mentioned above, Salganicoff et al. (21) and Fukami et al. (22) have carried out subcellular fractionation of pig and human platelets respectively. These authors both employed discontinuous surcrose gradient centrifugation following homogenization and differential centrifugation to obtain fractions corresponding to the organelles found in the platelet. The fractions of both human and pig platelets were identified according to both biochemical and morphological criteria and it was shown that rather pure fractions of dense granules were obtained in both cases. The granules were found to pellet in gradients containing 2.0M sucrose as the highest density step.

Subcellular Storage of Biogenic Amines

All of the cell types mentioned so far appear to store their biogenic amines at least partially within membrane bound organelles located in the cell cytoplasm. This storage mechanism not only serves

to protect these amines from the action of metabolic enzymes in the cytoplasm such as monoamine oxidase (MAO), but also may serve to give a distinct package size to the amount of amine released upon proper cell stimulation (26). This may be especially relevant to the so-called "quantal" nature of neurotransmitter release in the nervous system (27). This quantal release also suggests that the membrane-bound storage organelles in the cell types under discussion release their contents extracellularly via exocytosis (i.e., fusion of the storage organelle membrane with the plasma membrane of the cell). The chromaffin cell has, in fact, been used as a model system for the study of exocytosis (reviewed in 28).

Composition of Storage Organelles

Chromaffin granules. Since their first isolation in 1953 (29), chromaffin granules have been analyzed extensively with regard to their constituent components. By far the most study has been done with chromaffin granules in this regard, probably since they are easily isolated relative to other amine storage organelles. The composition of chromaffin granules has been the subject of several recent reviews (e.g., 30), and therefore will only be briefly discussed here. In addition to a high content of epinephrine and norepinephrine, which together account for almost 21% of their dry weight, chromaffin granules contain a number of other components, including: (1) soluble and membrane bound proteins, (2) lipids (including a high content of lysolecithin), (3) nucleotides, which account for 15% of their dry weight, (4) mucopolysaccharides, (5) calcium,

and (6) ascorbic acid (31). Between 70 and 80% of the protein content of bovine chromaffin granules is soluble and is released upon hypotonic lysis of the organelles. There are three major components to the soluble protein content: (1) Chromogranins A and B which are both acidic proteins, as shown by the migration patterns on polyacrylamide gel electrophoresis and (2) Dopamine β-hydroxylase, the enzyme which catalyzes the conversion of dopamine into norepinephrine. Although the function of the chromogranins is not known, it has been suggested that they may play a role in storage of amines (32).

The membrane-bound enzymes include the following: (1) a membrane-bound form of Dopamine β-hydroxylase, (2) a divalent ion activated ATPase and (3) cytochrome b-561, a NADH: oxidoreductase. The function of the divalent ion activated ATPase will be discussed in more detail below. It is still unknown at present what the function of the b-type cytochrome is in this situation although Flatmark has studied this enzyme extensively (33,34). One proposal forwarded by Flatmark was that cytochrome b-561 acts as an electron donor in the dopamine β-hydroxylase reaction (35). Flatmark has recently abandoned this hypothesis, however.

The lipid composition of the chromaffin granule is remarkable in that the membrane appears to contain a relatively large amount of lysolecithin. According to Blaschko et al. (36), this component makes up almost 17% of the total membrane lipid phosphorous. This result has been confirmed by Winkler et al. (37). This is in contrast to other membranes which usually contain less than 5% lysolecithin. It has been

suggested (31) that this high content of lysolecithin might play a role in membrane fusion during exocytosis of granule contents. It must be cautioned that a high content of lysolecithin has also been found in pig pancreatic zymogen granules (38) and the presence of these lysophospholipids was found to be due to activation of phospholipases during the isolation procedure. Studies have not yet been carried out to determine whether this is the case for chromaffin granules, however. The functions of mucopolysaccharides and of ascorbate in the chromaffin granule have not yet been characterized. The possible function of nucleotides and divalent metal ions in the chromaffin granule will be discussed in a later section.

Dense granules. Dense granules from blood platelets have been studied much less than chromaffin granules and therefore, their constituents are much less characterized. The first investigators to isolate a fraction of organelles of sufficient purity for this purpose were DaPrada et al. (18) They showed, using dense granules isolated from rabbit platelets (see "Isolation Procedures"), that 5-hydroxytryptamine was stored in very high quantity (20%w/v) in these organelles along with ATP (25%w/v). In addition, significant quantities of Ca++ (2.1 mmole/g protein) and Mg++ (8.4 mmole/g protein) were detected. Minor components found in the organelles included norepinephrine, epinephrine; and the nucleotides adenosine diphosphate, guanosine triphosphate and uridine triphosphate. It was shown in later work that rabbit platelet dense

granules also contain a Mg++-dependent ATPase which is inhibited by Ca++ (39). In spite of the striking similarities between dense granules and chromaffin granules, very little soluble protein was found in dense granules by Pletscher et al. (40) in contrast to the high content of soluble protein found in chromaffin granules. Dense granules isolated from other species such as pig (Salganicoff et al. (21) and human (Fukami et al. (22)) demonstrate similar contents to those from rabbit. The major differences appear to lie in the following: (1) the content of divalent metal ions and (2) the ratio of ATP to ADP. In this regard human platelet dense granules appear to have more Ca++ than Mg++ whereas rabbit and pig dense granules are the reverse. The ATP/ADP ratio in human granules has been shown to be <1 whereas rabbit and pig granules have an ATP/ADP ratio of >1 (41). The significance of the latter observations regarding Ca++/Mg++ and ATP/ADP ratios is not yet clear, however.

Possible Role of Nucleotides and Divalent Metals in Amine Storage—The "Storage Complex" Hypothesis

As stated above, chromaffin granules from adrenal medulla and dense granules from platelets contain, along with amines, large quantities of nucleotides and divalent metal ions. This also appears to be true of amine storage organelles from brain and mast cells (42,43). It has been suggested by Pletscher and his colleagues (40) that biogenic amines, adenine nucleotides and divalent metal ions may participate in a storage complex which results in the ability of biogenic amine storage organelles to store massive amounts of amines. It was indeed shown by the above investigators that when ATP, 5-hydroxytryptamine and small amounts of

Ca++ were added together in the correct ratios *in vitro*, phase separation of the solution occurred (44). This phase separation was also shown to be pH and temperature dependent. The formation of a second, more dense phase was followed by measurement of the amounts of ATP and 5-HT in this dense phase. It was found that much higher concentrations of ATP and amine resided in the dense, viscous phase than in the top phase although crystalline structures were not formed. In addition, analytical ultracentrifugation indicated that the separation of this second phase was caused by the formation of aggregates which were stated to be "of indefinite size" (44). Berneis et al. (44) have propsed a hypothetical model for the formation of these aggregates; however, it remains difficult to either prove or disprove this proposed model since the system does not readily lend itself to rigorous structural analysis by methods such as X-ray crystallography. Clearly, however, much more work needs to be done in order to elucidate the nature of these high molecular weight complexes which, as stated earlier, may be important in the storage of amines in a wide variety of tissues.

Transport of Amines

Another important aspect which has received wide attention and may operate by similar mechanisms in the various tissues discussed previously is that of transport of amines across biological membranes. This function may be broken down into two different levels: (1) transport of the amine into the cell (i.e., across the plasma membrane) and (2) transport of the amine into the intracellular storage organelle following its entrance into the cytoplasm.

Transport of Amines across the Plasma Membrane

Transport of biogenic amines across the plasma membranes of the cells under discussion (e.g., brain and blood platelets) plays a very important role, of course, in determining the amounts of amine which will eventually be stored within the cytoplasmic organelles. The physiological functions of biogenic amine transport in the brain and in the blood platelet are different since the neuronal transport system, which is localized mainly in the nerve endings, probably functions mainly in the inactivation of neurotransmitter signals (e.g., by the reuptake of released biogenic amine); whereas, in the blood platelet, serotonin is transported across the plasma membrane during the circulation of the platelet through the body and is released during the platelet release reaction as described earlier. In spite of these and other differences in physiological function, the mechanism for biogenic amine transport in these two cell types is remarkably similar. For instance, transport of amines across the plasma membrane of platelets and neurons appears to be mediated by a carrier protein within the membrane and transport in both cell types also shows an absolute requirement for Na^+ (45,46). Other similarities between these cell types in this regard are discussed in the next section.

With particular regard to serotonin transport into the blood platelet, Sneddon (47) studied the Na^+ requirement for carrier-mediated 5-HT uptake in human platelets and presented data which showed that absolutely no 5-HT uptake occurred in a Na^+-free medium. On the other hand, increasing the Na^+ concentration in the external medium from 2 to 116mM dramatically reduced the Km for 5-HT transport thus producing a

marked stimulation in transport. This finding suggests that Na+ may bind to the 5-HT carrier of the plasma membrane and facilitate its movement of 5-HT into the cell. Sneddon (47) has proposed a model for 5-HT transport into the blood platelet which takes this Na+ requirement into account and indicates that Na+ and 5-HT bind to the carrier and cross the plasma membrane simulteneously. It is proposed that the driving force for active 5-HT accumulation (i.e., accumulation of 5-HT against its concentration gradient) is a gradient of Na+ ions such that the concentration of Na+ is greater outside the cell than inside. This concentration gradient of Na+ ions has been shown to be maintained in vivo by the Na+-K+ ATPase which resides on the plasma membrane. This type of model is very similar to those proposed by others (e.g., 48,49) for the transport of sugars and amino acids in other tissues. This type of model has also been propsed by Bogdanski et al. (50) for the uptake of norepinephrine by synaptosomes from brain. In support of such a model, Rudnick (51) in elegant studies using isolated blood platelet plasma membrane vesicles has verified that active 5-HT transport may be driven by artifically imposed gradients of Na+ and also has shown that membrane potentials induced by adding permeant anions such as SCN^- to the external medium may act as a driving force for active 5-HT accumulation. The effects of these two types of gradients on 5-HT transport (i.e., $Na^+_{out} > Na^+_{in}$ and induced membrane potential $\Delta\psi$, inside negative) were shown to be additive and were also shown to be reversed by the addition of appropriate ionophores which are known to deplete these types of gradients in other systems.

Another requirement for 5-HT uptake into the blood platelet which appears to be absolute is that for Cl^- as shown by Lingjaerde (52). Cl^- appears to bind to the 5-HT carrier and kinetic analysis of its requirement showed that it enhanced the Vmax of 5-HT transport without altering the affinity of the carrier for 5-HT. It has been suggested that the role of Cl^- in this context may be to act as a counter ion for Na+ although this has not been shown experimentally.

The Blood Platelet as a Model for Amine-Containing Neurons

It has been suggested by several investigators that the blood platelet might serve as a good experimental model for studying cellular storage of biogenic amines (53,54). Indeed, many similarities seem to exist between the uptake and storage of 5-HT in the platelet and in brain despite the fact that these two cell types arise from embryonically distinct regions. Aside from the obvious similarity that both cell types transport and store biogenic amines, one may cite other important similarities. As mentioned earlier, both cells show an absolute requirement for Na+ for the transport of biogenic amines such as norepinephrine and serotonin into synaptosomes and blood platelet plasma membrane transport of serotonin (reviewed in 54). Another important aspect of the similarity between these cell types is that both exhibit high and low affinity transport for certain biogenic amines (55) as shown by kinetic analysis. The "high affinity" component in both cases has been attributed to the presence of specific carriers in the plasma membranes for various biogenic amines. The affinities

(i.e., Km) of these carriers for their substrates are generally in the low micromolar range (56). The low affinity component has generally been attributed to passive diffusion across the plasma membrane since this component does not exhibit saturation kinetics and is much less sensitive to metabolic inhibitors such as ouabain, cyanide, or dinitrophenol (56). It was proposed by Shaskan and Snyder (57) that the high and low affinity uptake of serotonin by brain slices actually represents two different saturable uptake systems in the plasma membrane. This conclusion, however, appears to be incorrect as pointed out by Stahl et al. (58).

Another criterion which has led to the proposal of the platelet as a model for amine-containing neurons is that of inhibitor specificity. One class of compounds which is highly inhibitory to plasma membrane transport of serotonin in both platelets and in brain slices and synaptosomes is the tricyclic antidepressants, represented by compounds such as imipramine and its analogs chlorimipramine and desmethylimipramine. These compounds produce nearly complete inhibition of plasma membrane 5-HT transport in blood platelets at levels near 1μM (59). A similar pattern of inhibition has been found for the tricyclic antidepressants in studies on 5-HT uptake in brain slices (60) and synaptosomes (57).

Intracellular Fate of Biogenic Amines

As stated earlier, following transport of biogenic amines across the plasma membrane, the amines may follow two routes: (1) deamination by monoamine oxidase and subsequent further degradation or (2) storage

within intracellular organelles. The presence of these organelles, although they differ somewhat in morphology and size, is a common characteristic of all cells which store and release biogenic amines. By far the most transport studies have been directed toward the amine storage organelle of the adrenal medulla, namely the chromaffin granule. Early studies by Carlsson et al. (61) showed that chromaffin granules would accumulate catecholamines or serotonin when incubated in the presence of ATP and Mg++. These studies, in fact, showed that isolated chromaffin granules could accumulate a number of different biogenic amines in an ATP-dependent manner. Transport of these amines appears to involve a transmembrane carrier and although epinephrine and norepinephrine are the major amines found in the adrenal medulla, the chromaffin granule amine carrier accepts serotonin as substrate with higher affinity than the former amines (62).

Chromaffin granules contain a Mg++-stimulated ATPase activity and several attempts were made to demonstrate a direct stimulation of ATPase activity in the presence of catecholamines or serotonin. Such a stimulation would suggest direct coupling between ATPase activity and amine transport; however, such a stimulation has not been demonstrated (63). More recent studies, mainly by Radda et al. (64) and Johnson and Scarpa (65) suggest a different role for this ATPase: namely that of a proton-translocating enzyme.

When chromaffin granules are incubated in the presence of ATP and Mg++, a transmembrane pH gradient is generated such that the inside of the granule is approximately 1.5 pH units more acidic than the outside.

Several methods have been used to measure this gradient including distribution of the weak base methylamine (66), spectrophotometric measurement of bromthymol blue trapped within ghosts (67), fluorescence measurements using 8-anilino-naphthalene-sulfonic acid (ANS) (68), and ^{31}P NMR measurements (69). Phillips (70) has also shown ATP-dependent formation of a transmembrane ΔpH in chromaffin granule ghosts. In both cases, the pH gradients are sensitive to uncouplers such as nigericin or the combination of valinomycin, CCCP, and K+. Nigericin, for instance, mediates an electroneutral exchange of protons for either Na+ or K+ and thus depletes the pH gradients (65). The pH gradients are also sensitive to the pH of the external medium, a finding which is consistent with the idea that the pH gradient in these organelles does not arise from the establishment of a Donnan equilibrium of H+ across the membrane. Donnan equilibria, can in fact result in pH gradients (a phenomenon known sometimes as "membrane hydrolysis" (71)) as appears to be true for rat liver lysosomes (72).

The formation of a pH gradient in chromaffin granule ghosts has been shown by Phillips to be accompanied by the formation of a transmembrane electrical potential ($\Delta\psi$) (70). This $\Delta\psi$ is low, however, compared with the pH gradients formed, and this finding suggests that at least part of the proton translocation in chromaffin granules is not electrogenic (70). For example, if all H+ translocations were electrogenic, one would expect the formation of a $\Delta\psi$ of approximately 300mv (inside-positive) at ΔpH values of 1.2 units. In fact, Phillips finds

a $\Delta\psi$ of only 80mv based on equilibrium measurements of the distribution of radioactively labelled KSCN. This, along with evidence obtained from light scattering measurements, suggests that H+ translocation is accompanied to a large degree by movement of anions into the interior of the granules. Light scattering measurements also indicate that swelling due to H+ translocation results from entry of protons accompanied by anions. This swelling appears to be greater in media containing relatively permeant anions (e.g., SCN^-) than in media containing relatively impermeant anions (e.g., $SO_4^=$). However, marked swelling occurs even in solutions containing low concentrations of $SO_4^=$. The nature of this anion movement (i.e., whether it is facilitated by a carrier or simple diffusion across the membrane) remains to be determined.

Role of the Transmembrane ΔpH in Amine Transport

What role does the transmembrane ΔpH play in catecholamine or serotonin accumulation in chromaffin granules? This question has been investigated in some detail in studies by Johnson and Scarpa and by Phillips, using intact chromaffin granules (65) and chromaffin granule ghosts (73), respectively. A conclusion which is obviously based upon these studies is that the transmembrane ΔpH plays a very important role in amine accumulation by these organelles. When the ΔpH is dissipated by addition of uncouplers or NH_4^+ ions, amine accumulation is dramatically lowered. Similarly it has been shown that raising the pH of the external medium and thus increasing the magnitude of ΔpH increases

catecholamine accumulation. Another piece of evidence in favor of the hypothesis is that the transmembrane ΔpH drives amine transport is that addition of amines albeit at rather high concentrations was shown by Johnson et al. to decrease ΔpH which could be restored to approximately its original value by the inclusion of ATP (65). This restoration of the ΔpH was time-dependent as one would expect for ATP-driven H+ translocation.

On the basis of this concept Radda (74) and Johnson et al. (65) have proposed a model for amine accumulation. This model presumes that catecholamine or serotonin accumulation could occur in direct analogy to methylamine accumulation in response to a pH gradient (acid inside); i.e., the neutral species crosses the membrane and when inside the interior of the organelle combines with a proton. This protonation is favored in the interior of the organelle which is acidic in comparison with the external medium. Further, the protonated species of the amine is much less permeable to the membrane than its neutral form. This model will be examined in further detail in later sections.

Dense Granule Amine Transport

The subcellular amine storage organelles of blood platelets were first isolated by Pletscher et al. (from rabbit platelets) and, as stated earlier, were found to contain a high internal content of 5-hydroxytryptamine as well as adenine nucleotides and divalent metal ions. DaPrada and Pletscher (18,19) subsequently showed that isolated blood platelet dense granules would accumulate radioactively labelled amines, with serotonin being the preferred amine. When incubated in

plasma, rabbit platelet dense granules accumulated radioactively labelled amines in the following order of efficiency: 5-hydroxytryptamine > dopamine > epinephrine > norepinephrine > 5-hydroxydopamine = tyramine > tryptamine > histamine. These results suggested that a specific mechanism exists for the transport of 5-HT by dense granules of platelets which may discriminate between different amines on a structural basis. Uptake was also shown to be unaffected by inhibitors such as cyanide, fluoride, dinitrophenol or ouabain. The concentration dependence for amine accumulation was not studied by Pletscher et al.; however, these authors proposed a mechanism of 5-HT transport in dense granules based on the binding of this amine to the intraorganellar storage complex discussed earlier (40). More recently, Stahl and Meltzer (58) studied the uptake of 5-HT into a particulate fraction obtained from human platelets by sonication. The fraction was stated to contain "numerous" dense-cored organelles (i.e., dense granules) in addition to mitochondria and alpha granules, although no data on enzymatic markers or serotonin content in this fraction were given. It was found that 5-HT transport in this preparation did not exhibit saturation kinetics in the range of 5×10^{-8} to 5×10^{-5}M 5-HT. This result, however, is questionable in view of the comparative lack of sensitivity of 5-HT uptake in this system to inhibition by reserpine at concentrations as high as 10^{-4}M.

More recently, extensive studies have been carried out in order to determine the mechanism whereby 5-HT is accumulated by the dense granules of blood platelets. The present study represents the first

intensive study into the detailed mechanism of 5-HT accumulation by dense granules from blood platelets. The results presented may have importance not only in the understanding of amine accumulation in the blood platelet but also in other tissues. The results presented here will be discussed with regard to similarities and differences between amine storage in blood platelets and other cell types such as neurons and chromaffin cells.

CHAPTER II

CHARACTERISTICS OF 5-HYDROXYTRYPTAMINE TRANSPORT BY INTACT PORCINE PLATELET DENSE GRANULES

Summary

A method is described for the isolation of a homogeneous preparation of dense granules from porcine platelets. The purified dense granule fraction contained approximately 400 n mole of 5-hydroxytryptamine/mg protein and appeared to be homogeneous when examined by electron microscopy. Isolated dense granules transported exogenously added 5-hydroxytryptamine via two mechanisms: (1) a carrier-mediated process predominating at low substrate concentrations and (2) a diffusion-controlled process predominating at high substrate concentrations. Temperature studies revealed an apparent energy of activation of 14.9 kcal/mole for the carrier-mediated transport. Kinetic data yielded a K_m of 3.3μM and a Vmax of 0.79 n mol/min/mg protein for the mediated transport process. Steady state uptake was sensitive to changes in medium osmotic pressure and a decline in uptake below 300 mOsM was correlated with release of endogenous 5-hydroxytryptamine. The transport was inhibited by a number of structural analogs of 5-hydroxytryptamine. These results demonstrate the existence of a carrier-mediated transport system for 5-hydroxytryptamine in the membranes of the platelet dense granules.

Introduction

The uptake of 5-hydroxytryptamine into blood platelets has been extensively studied (45,46,74). This uptake occurs at two distinguishable compartments in the platelet: (a) at the plasma membrane level (50) and (b) at the level of a specialized storage organelle termed the dense granule or 5-HT organelle (75,76). Dense granules were first isolated by DaPrada et al. (18) who also provided strong evidence that 5-HT was stored as a complex with nucleotides and bivalent metals (40). Studies on storage organelles from other tissues such as the chromaffin granules of adrenal medulla (78) and synaptic vesicles from neurons (79) suggest that biogenic amines could also be stored as high molecular weight complexes in these organelles. The pharmacological properties of 5-HT uptake in platelets are similar to those in neurons (reviewed in 54). The platelet has therefore been proposed as a model for monoamine-containing neurons (53,54). Platelets offer a unique advantage in that, unlike preparations from brain tissues which may contain several types of storage organelles, the dense granules may be obtained in homogeneous form. Dense granules from platelets thus provide a model system to study the uptake of a biogenic amine into an intracellular storage organelle. Work presented in this chapter describes the isolation of the dense granules from porcine platelets and demonstrates a carrier-mediated transport system for 5-HT in these organelles.

Experimental Procedures

Methods. Sixteen liters of porcine blood was obtained from a local slaughterhouse and platelets were isolated as previously described (20). The cells were disrupted with a French pressure cell and the "crude mitochondrial" fraction was isolated from the homogenate essentially as described by Salganicoff and Fukami (20) with the following modifications: (a) the "incubation medium" was changed to 100 mM KCl, 50 mM HEPES, 2 mM EGTA, pH 6.5; (b) the "isolation medium" was changed to 100 mM KCl, 50 mM HEPES, 2 mM EGTA, 1 mM ATP and 0.5% (w/v) BSA, pH 7.4: (c) the crude mitochondrial pellet was resuspended in modified Tyrode buffer consisting of: 130 mM NaCl, 6 mM KCl, 2 mM EDTA, 1 mM NaH_2PO_4, 3 mM $NaHCO_3$, 0.5% (w/v) BSA, 111 mM glucose and 10 mM sucrose, pH 7.0. The protein concentration of the crude mitochondrial fraction was approximately 15 mg/ml.

Following overnight storage on ice, 5 ml of the crude mitochondrial fraction was layered onto each of three discontinuous sucrose density gradients. Gradients were prepared by layering successive 5 ml volumes of 2.5 M, 2.0 M, 1.8 M, 1.6 M and 0.8 M sucrose (all in 10 mM HEPES buffer, pH 7.2) into Beckman cellulose nitrate centrifuge tubes. The gradients were then centrifuged at 90,000 x g for 3 hrs at 3°C in a Beckman SW 25.1 rotor. Fractions corresponding to the different regions of the gradient shown in Figure 2 were collected by aspiration from the top of the gradient.

Cytochrome oxidase activity was measured polarographically by the method of Schnaitman et al. (80). Acid phosphatase activity was

Figure 2. Sucrose density gradient fractionation of crude mitochondrial fraction.

The crude mitochondrial fraction was layered onto discontinuous sucrose density gradients. The gradients were centrifuged and fractions collected as outlined in "Experimental Procedures." Molarities refer to sucrose concentrations. Brackets at right indicate the proportion of the gradient taken in each fraction.

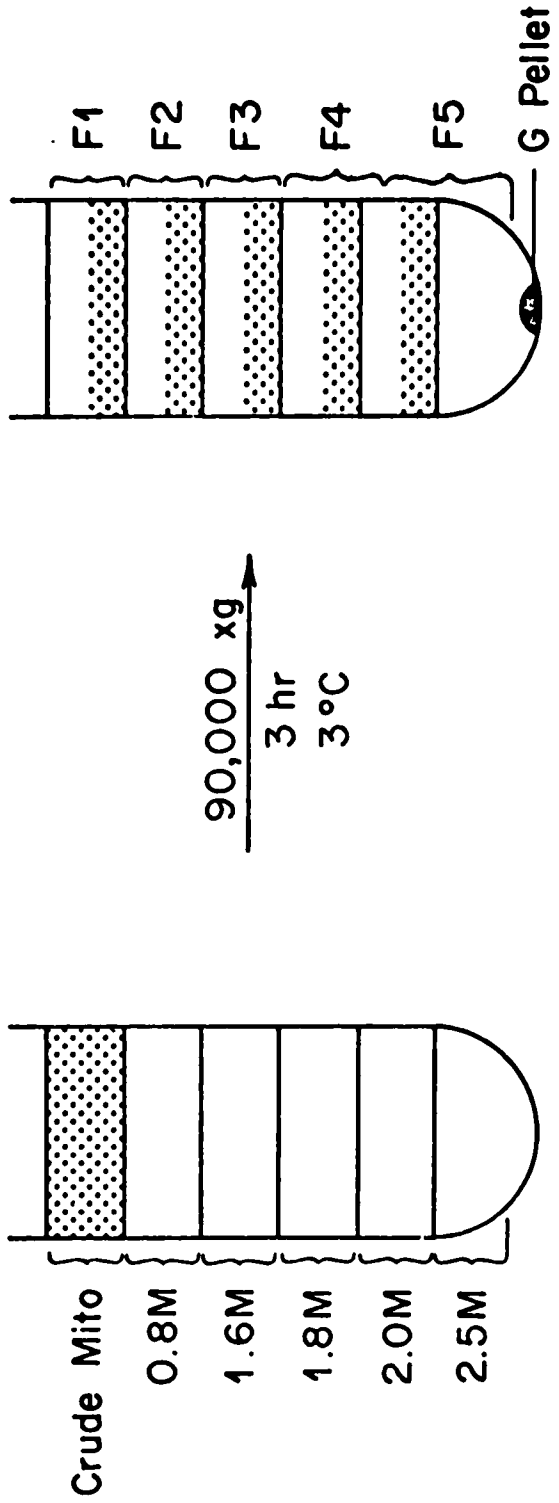
F1
F2
F3
F4
F5
G Pellet
90,000 xg
3 hr
3°C
Crude Mito
0.8M
1.6M
1.8M
2.0M
2.5M

Figure 2

determined by measuring the conversion of p-nitrophenyl phosphate to p-nitrophenol (81). Endogenous 5-HT was measured fluorometrically by the method of Udenfriend and Weissbach (82). Protein was determined by the method of Lowry et al. (83) using BSA as standard.

The pooled G. pellet fractions (Figure 2, page 28) from the three gradients were resuspended to a protein concentration of 4-5mg/ml in modified Tyrode buffer. The G. Pellet fraction, which was used exclusively for 5-HT uptake assays, was stored in ice. 5-HT uptake activity remained stable for at least 3 days under these conditions.

Routine assays of 5-HT uptake were performed by Millipore filtration as follows. 5-hydroxy[G-^{3}H]tryptamine creatinine sulfate (250 Ci/mol) was added to 1.6 ml of modified Tyrode buffer at a concentration of 5.7 µM. This solution was then equilibrated to the assay temperature. An aliquot of the G. Pellet fraction was then added at a final protein concentration of 200 µg/ml and incubated for various periods of time. Aliquots (0.5 ml) were removed after the desired incubation period and filtered through Millipore VCWP filters (0.1 µM, pore size), followed by a 2 ml wash with ice cold modified Tyrode buffer. A Millipore filtration manifold was used for all assays at a negative pressure of 20" Hg. Filtration and washing were complete within approximately 1 min. Filters were then placed in scintillation vials and solubilized by vigorous shaking at room temperature with 3 ml of 2-methoxyethanol for 30 min. Eight ml of a toluene-based scintillant containing 0.4% (w/v Butyl-PBD and 33% (v/v) Triton X-100 was then added. The vials were counted in a Beckman LS230 scintillation

counter at approximately 20% efficiency for [^{3}H] and 85% efficiency for [^{14}C]. All counts were corrected for quench and converted to dpm for calculation of results. Blank values (samples which contained no protein) were substracted from experimental values.

Electron microscopy was carried out as follows. Pelleted samples from the G. Pellet fraction were fixed for 2 hrs in 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, followed by washing and storing overnight in a scurose-phosphate buffer (84). This was followed by postfixation with 2% OsO_4 in 0.1 M phosphate buffer. Samples were dehydrated in a graded series of acetone and embedded in Epon 812 (85). Thin sections were obtained using an LKB ultramicrotome and stained with uranyl acetate followed by lead citrate (86). All specimens were viewed at 62KV in a Zeiss electron microscope, model EM9 S-2A.

Materials. 5-Hydroxy[G-^{3}H]tryptamine creatinine sulfate and 5-hydroxy[2-^{14}C]tryptamine creatinine sulfate were obtained from Amersham-Searle. Epinephrine, norepinephrine, and dopamine were purchased from Calbiochem; and 5-HT, tryptamine 5-HIAA and reserpine from Sigma. All other materials used were reagent grade obtained from commercial sources.

Results. The crude mitochondrial fraction was subfractionated by centrifugation on discontinuous sucrose density gradients (Figure 2, page 28). Table I shows the distribution of acid phosphates, cytochrome oxidase and 5-HT in the gradients. The mitochondrial

TABLE I

DISTRIBUTION OF MARKERS IN SUCROSE DENSITY GRADIENT FRACTIONS

Marker components were assayed as described in "Experimental Procedures." Cytochrome oxidase activity expressed as n atoms O consumed/min/mg protein. 5-HT content expressed as n mole 5-HT/mg protein. Acid phosphatase activity expressed as n mole p-nitrophenol produced/min/mg protein. Numbers in parentheses indicate percentage of total crude mitochondrial activity found in the various fractions. Results shown are the mean of duplicate experiments.

Fraction	Protein (mg)	Cytochrome Oxidase	5-HT	Acid Phosphatase
Crude Mitochondria	266.4 (100)	83.5 (100)	69.9 (100)	26.0 (100)
F1	94.0 (35.3)	0 (0)	30.4 (15.3)	40.5 (54.9)
F2	64.7 (24.3)	253.2 (73.6)	9.3 (3.2)	37.4 (34.9)
F3	53.7 (20.2)	5.9 (1.4)	24.1 (6.9)	4.0 (3.1)
F4	24.1 (9.0)	1.34 (0.1)	104.8 (13.6)	4.0 (3.1)
F5	18.2 (6.2)	2.3 (0.2)	401.4 (39.2)	4.0 (1.1)
G. Pellet	15.4 (5.8)	0 (0)	404.2 (33.4)	1.0 (0.2)
TOTAL RECOVERY	(101.4)	(75.3)	(111.6)	(95.6)

marker, cytochrome oxidase was localized almost exclusively in the F2 fraction with virtually no activity in the F5 and G. Pellet fractions. Practically no acid phosphatase activity was found in either the F5 or G. Pellet fraction. 5-HT, a marker for dense granules of the platelets (87,88) was found in relatively high amounts in both the F5 and G. Pellet fractions. There was an approximate 6-fold enrichment of 5-HT content in the G. Pellet fraction compared with the crude mitochondrial fraction. From these results, it is concluded that dense granules of highest purity reside in the G. Pellet fraction.

The G. Pellet fraction was further examined by electron microscopy. Numerous organelles identifiable as dense granules are seen in the micrographs (Figure 3a), many containing characteristic electron-dense deposits which are presumably due to reduction of OsO_4 by 5-HT (89). In the higher magnification view of the same preparation, granule membranes appear as "unit membrane" structures (Figure 3b). Virtually no other organelles such as mitochondria or α-granules were found in this fraction. On the basis of the combined biochemical and ultrastructural analyses, the G. Pellet fraction was shown to be essentially a homogenous dense granule preparation and was selected for 5-HT uptake studies.

The uptake of radioactive 5-HT by the dense granules was studied by a Millipore filtration method. Figure 4 shows the time course of such a process. At 37°C, uptake was rapid and linear for approximately 4 min. It reached approximately 90% of the steady state level (2.0 - 3.0 nmol 5-HT/mg protein) after 10 min. Practically no uptake

Figure 3. Electron micrographs of G. Pellet fraction.

Samples were prepared according to the method outlined in "Experimental Procedures." (a) Survey view of G. Pellet fraction (x 22,750). Bar is 0.5 μm. (b) High magnification view of the same preparation (x 71,250). Bar is 0.1 μm.

Figure 3

Figure 4. Time course of 5-HT uptake by dense granules.

Assays were performed as described in "Experimental Procedures." Uptake at 37°C (●); uptake at 0°C (▲).

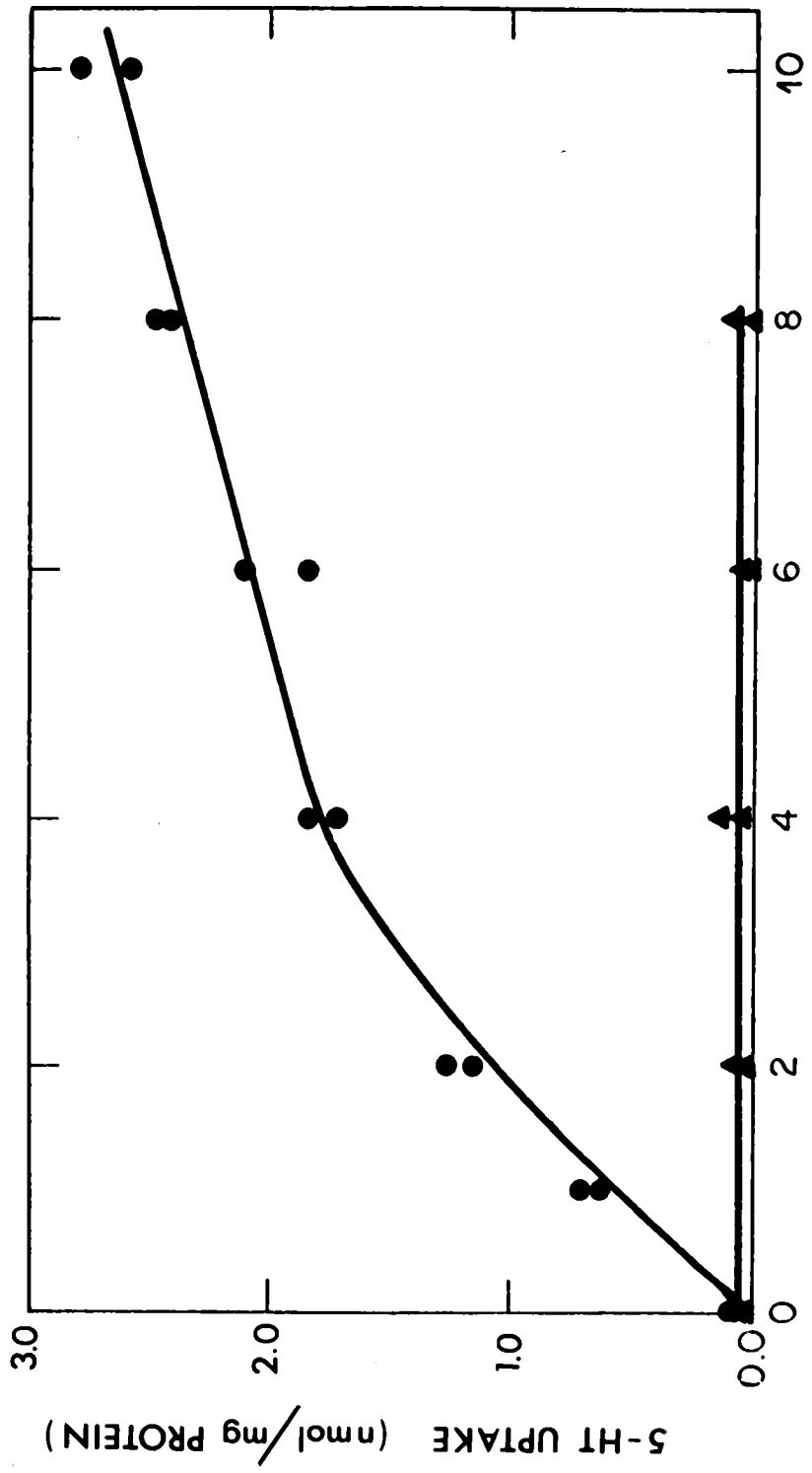

Figure 4

of 5-HT was measurable at 0°C. Figure 5 shows that 5-HT uptake is linear with protein concentrations up to 300 µg/ml. On the basis of these results, an incubation time of 4 min at 37°C and a protein concentration of 200 µg/ml were chosen as standard conditions for studying the initial velocity of 5-HT uptake.

The kinetics of 5-HT uptake was studied with various substrate concentrations (Figure 6). Data were analyzed using a computer program which is designed to produce a least-squares fit of nonlinear data. The program was developed by Dr. Stuart Hawkinson of this department and was an adaptation of published method (90). It was found that the data points in Figure 6 are adequately described by the following equation which represents the kinetics expected for a transport mechanism which consists of both saturable and nonsaturable components:

$$V_0 = k[S] + \frac{Vmax\ [S]}{K_m + [S]} \qquad (1)$$

where V_0 represents the initial velocity of 5-HT uptake, [S] represents the concentration of 5-HT. k is a first-order rate constant for the nonsaturable component of the uptake. Vmax is the maximum velocity and K_m is the Michaelis Constant of the saturable component. The values obtained by the computer for Vmax, k and K_m were the following: Vmax = 0.79 n mol/min/mg protein, k = 0.064 ml (min-mg protein)$^{-1}$ and K_m = 3.3 µM. The curve in Figure 6 was drawn by computer representing equation 1 with these values as parameters. The excellent fit of the actual data points to the curve is obvious. Clearly the presence of two uptake components in this system makes it difficult

Figure 5. Linearity of 5-HT uptake with protein concentration.

Assays were carried out at 37°C for 4 min as described in "Experimental Procedures."

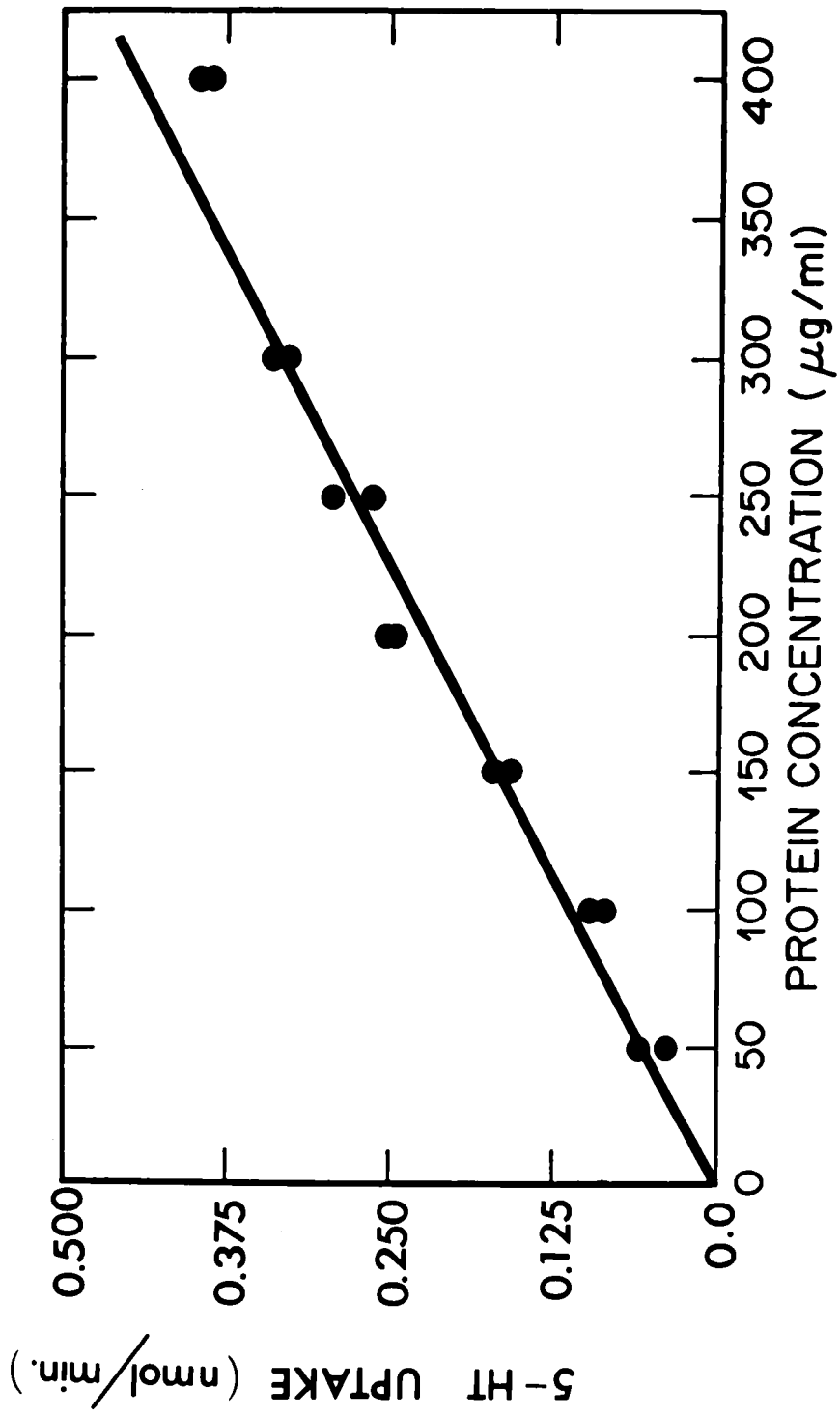
5-HT UPTAKE (nmol/min.)
0.500
0.375
0.250
0.125
0.0
0
50
100
150
200
250
300
350
400
PROTEIN CONCENTRATION (μg/ml)

Figure 5

Figure 6. Kinetics of 5-HT uptake.

Assays were carried out at 37°C for 4 min at the desired 5-HT concentrations as described in "Experimental Procedures." Data were plotted and analyzed by computer (see text).

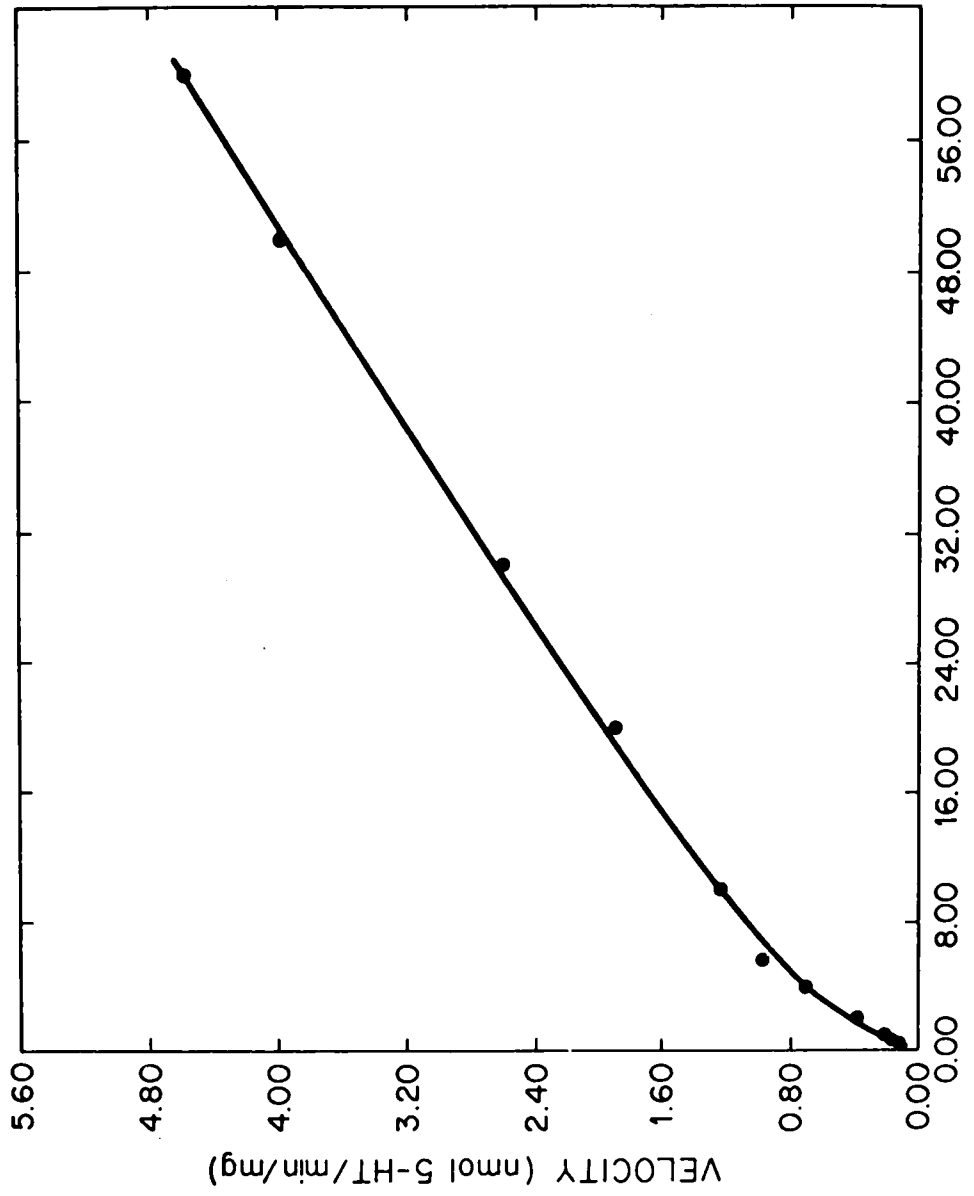
VELOCITY (nmol 5-HT/min/mg)
0.00
0.80
1.60
2.40
3.20
4.00
4.80
5.60
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
[5-HT] μM

Figure 6

at any given substrate concentration to measure the effects of the saturable component in isolation. A 5-HT concentration of 5.7 μM represented a concentration which was above the K_m of the saturable component and yet the nonsaturable component still only contributed a relatively minor proportion (approximately 30%) of the initial uptake velocity. This concentration therefore proved convenient for subsequent experiments investigating the effects of temperature, osmolarity and various inhibitors on the uptake.

The temperature dependence of 5-HT uptake by dense granules was also studied. The data are represented as a straight line in the Arrhenius plot (Figure 7). The apparent energy of activation calculated from this line was 14.9 kcal/mole.

The purified dense granules were apparently sensitive to low osmolarity of the medium. Dense granules (200 μg protein) were suspended in 1 ml of 0.01 M HEPES pH 7.0 containing various amounts of KCl to vary the medium osmolarity from 50 to 1,000 mOsM. This mixture was incubated 2 hrs at 0°C. Samples were then centrifuged in an Eppendorf model 3200 centrifuge for 2 min and the amount of 5-HT which leaked from the granules was measured fluorometrically (82). Above 300 mOsM, no detectable leakage of 5-HT was observed; whereas increasing amounts of 5-HT were released from the dense granules where medium osmolarity was decreased below 300 mOsM (Figure 8a).

Taking advantage of the instability of the dense granules at low medium osmolarity, several experiments were then undertaken in order to show that the observed uptake of 5-HT represented transport into the

Figure 7. Arrhenius plot of initial velocities of 5-HT uptake.

The assay mixture containing 5-HT was equilibrated for 5 min at the desired temperature. Uptake was then initiated by adding G. Pellet fraction into the assay mixture and was terminated after 4 min. Other details are given in "Experimental Procedures."

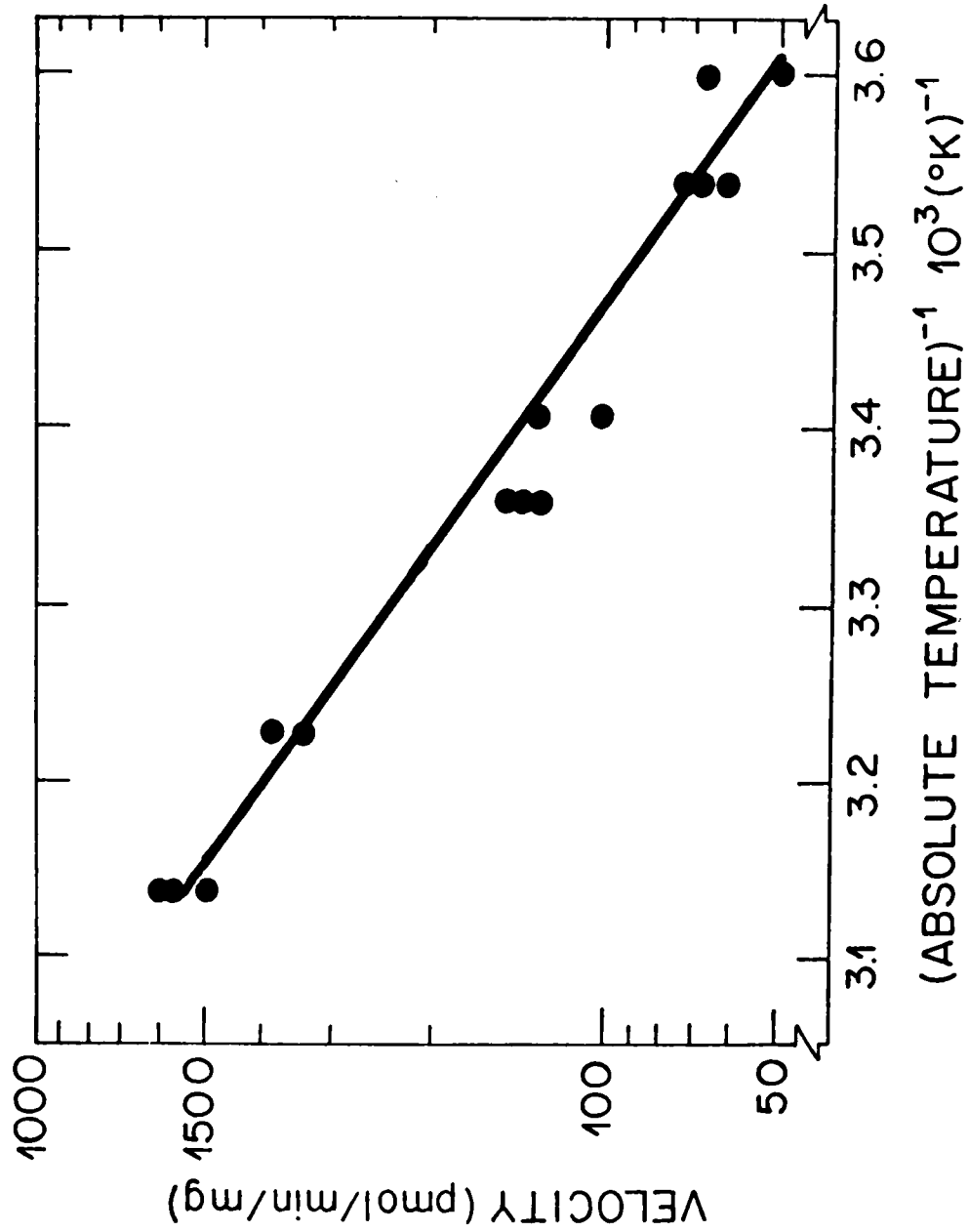
VELOCITY (pmol/min/mg)
1000
1500
100
50
3.1
3.2
3.3
3.4
3.5
3.6
(ABSOLUTE TEMPERATURE)$^{-1}$ 10^3(°K)$^{-1}$

Figure 7

Figure 8. Effect of osmotic pressure on release and uptake of 5-HT.

(a) Release of endogenous 5-HT from dense granules incubated in media of various osmolarity. (b) Steady state uptake of exogenous 5-HT by intact dense granules. Osmolarity varied by addition of NaCl (●) or sucrose (Δ). (c) Steady state uptake of exogenous 5-HT by granule "ghosts." Details of experiments are given in "Results."

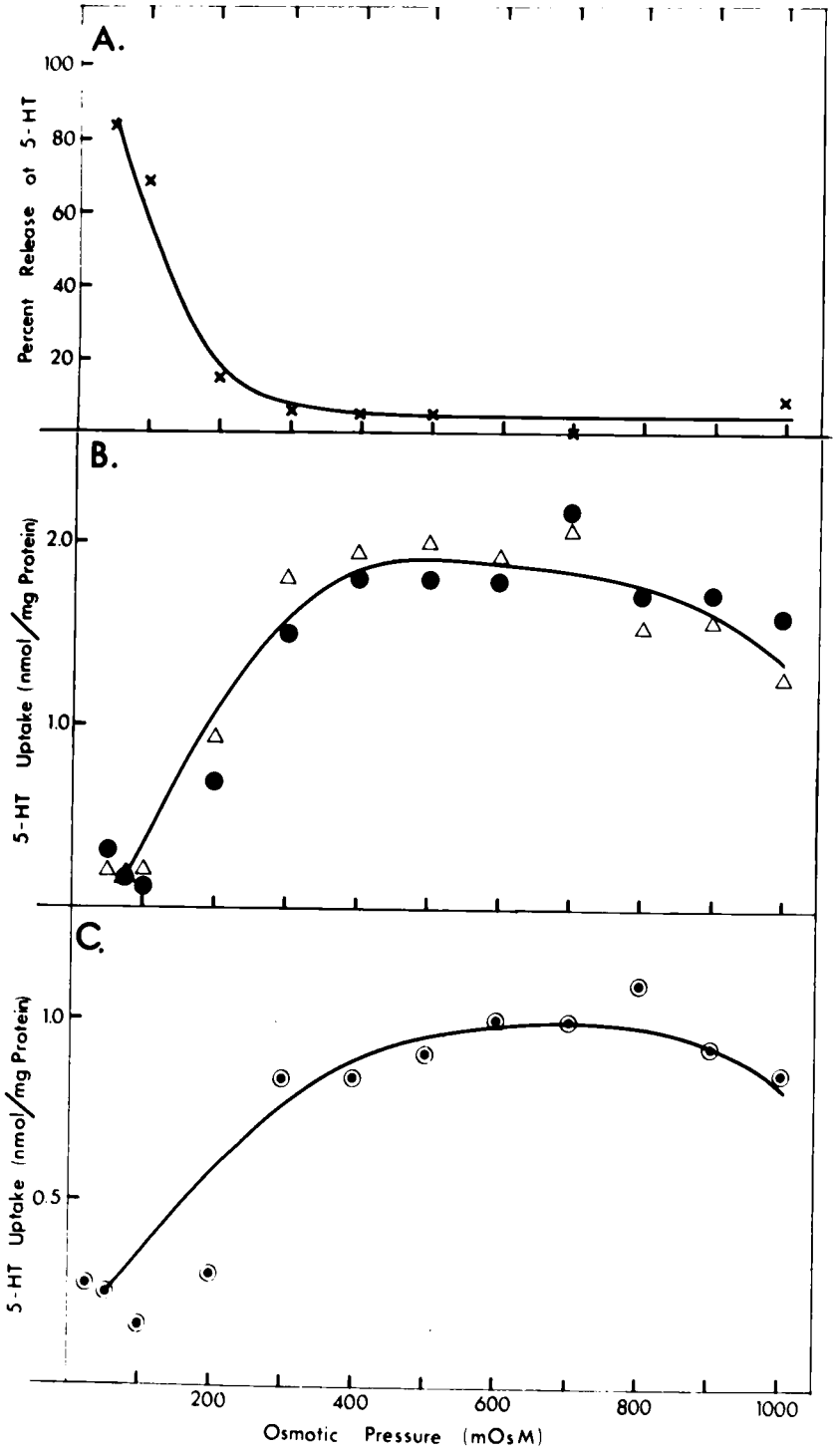
A.
Percent Release of 5-HT
100
80
60
40
20
B.
5-HT Uptake (nmol/mg Protein)
2.0
1.0
C.
5-HT Uptake (nmol/mg Protein)
1.0
0.5
200
400
600
800
1000
Osmotic Pressure (mOsM)

Figure 8

dense granules and not binding to their membranes. In one experiment, the steady state uptake of 5-HT was studied as a function of the osmolarity of the incubation medium. Glucose and NaCl were deleted from the modified Tyrode buffer (400 mOsM) which was usually used in uptake experiments. The osmolarity of the resulting solution was 36 mOsM. Proper amounts of NaCl or sucrose were added to obtain solutions of the desired osmolarity. Dense granules were allowed to equilibrate in these solutions at a protein concentration of 200 µg/ml for 2 hrs at 0°C. The incubation with radioactive 5-HT was then performed at 37° for 10 min to approach a steady state level of uptake. The results of this experiment are shown in Figure 8b. The steady state uptake of 5-HT was found to be independent of whether NaCl or sucrose was used to vary the osmolarity. 5-HT uptake was found to increase with increasing osmolarity. A maximum uptake of approximately 2 nmole/mg protein was obtained at osmolarities between 400 and 700 mOsM, followed by a slight decrease above 700 mOsM.

Since the ednogeneous 5-HT leaks out at low medium osmolarity, the possibility exists that the observed low steady state uptake of radioactive 5-HT is due to a dilution of the specific radioactivity by the released endogeneous 5-HT. In order to test this possibility, the G. Pellet fraction was resuspended directly from the gradient into 8 ml of ice cold 0.01 M HEPES 1.0 mM EDTA pH 7.0 and incubated on ice for 10 min. The suspension was then centrifuged at 200,000 x g for 20 min and the pellet was resuspended in modified Tyrode buffer to yield a "ghost" preparation. Treatment by this procedure releases approximately

50% of the endogeneous 5-HT from the granules as measured fluorometrically. The time course of 5-HT uptake into granule "ghosts" suspended in 0.2 M KCl, 0.01 M HEPES pH 7.0 (410 mOsM) is similar to that shown by the intact granules suspended in modified Tyrode buffer (see Figure 4, page 36). The steady state uptake of 5-HT versus osmolarity was then studied in the "ghost" preparation by the same method as that given for intact granules except that KCl media, all buffered with 0.01 M HEPES pH 7.0 were used for the incubations. As shown in Figure 8c, results qualitatively similar to those shown in Figure 8b were obtained. Therefore, the possibility stated above was ruled out.

In order to provide further evidence that uptake of 5-HT represented transport as opposed to binding, the following experiment was performed. Two aliquots of dense granules were incubated in modified Tyrode buffer at 37°C; one contained 40 μM unlabelled 5-HT and the other contained no added 5-HT. After 30 min equal volumes of modified Tyrode buffer were added to each tube. Both additions contained ^{14}C 5-HT and unlabelled 5-HT so that the final concentration of 5-HT (labelled and unlabelled) in each tube was 5.7 μM. The initial rates of uptake in both samples were then determined. As shown in Table II, the initial rate of 5-HT was unaffected by preincubation with a large amount of unlabelled ligand.

Several aromatic amines were tested to determine their effect on the initial rate of 5-HT uptake. As shown in Table III, the catecholamines norepinephrine, epinephrine and dopamine inhibited 50% of the activity at concentrations near 10^{-6}M. Tryptamine, an analog of 5-HT

TABLE II

EFFECT OF PRELOADING ON INITIAL RATE OF 5-HT UPTAKE

Uptake was assayed at 37°C as described in "Methods." Dense granules were preincubated for 30 min as described in "Results" before the addition of [^{14}C] 5-HT. Data shown are the means and standard errors of three separate experiments.

5-HT Concentration Preincubation (μM)	^{14}C 5-HT Concentration in Incubation (μM)	Initial Rate of ^{14}C 5-HT Uptake (nmol/min/mg Prot.)
40	5.7	0.75 ± 0.16
0	5.7	0.66 ± 0.07

TABLE III

INHIBITION OF INITIAL RATE OF 5-HT UPTAKE

Transport assays were carried out at 37°. 5.7 μM 5-HT and inhibitors at the desired concentration were added to 1.6 ml modified Tyrode buffer. An aliquot of the G. Pellet fraction was then added to a final protein concentration of 100 μg/ml and was allowed to incubate for 4 min. Filtration, washing, and counting were then performed as described in "Experimental Procedures." I_{50} represents the concentration of inhibitor required to cause 50% inhibition of control values.

Inhibitor	I_{50}
Epinephrine	1.0 μM
Norepinephrine	1.8
Dopamine	1.2
5-HIAA	3.5
Tryptamine	2.5
Benzylamine	1000
Reserpine	2.9

lacking an aromatic hydroxyl group, and 5-hydroxyindole-3-acetic acid, a deaminated metabolite of 5-HT, inhibited at concentrations similar to those of the catecholamines. Reserpine, an alkaloid which depletes granular amine stores in platelets (91) also inhibited 5-HT uptake by 50% at approximately 10^{-6}M. Benzylamine, a phenylalkylamine lacking aromatic hydroxyl groups, displayed 50% inhibitory activity only at concentrations 1,000-fold higher than those of the other aromatic amines tested.

Discussion

On the basis of the biochemical evidence obtained by measuring markers in the various fractions from sucrose density gradients, the G. Pellet fraction was judged to contain dense granules with highest purity. One possible source of contamination in this fraction would be α-granules, which are similar in many respects to lysosomes from other tissues (92). Only 7% of the crude mitochondrial β-N-acetyl-glucosamidase activity (a marker for α-granules) was found at a density greater than 2.0 M sucrose (d 1.25) by Salganicoff et al. (26). In the present study, a 2.5 M (d 1.32) sucrose layer was included in the gradients in order to further reduce α-granule contamination in the G. Pellet fraction. The purity of the dense granules in the G. Pellet is further substantiated by ultrastructural evidence (Figure 3, p. 34). Examination of a large number of preparations indicated virtually no contamination by α-granules or mitochondria in this fraction.

Uptake studies have been performed by other investigators on both dense granules from platelets (19,93) and on chromaffin granules from

adrenal medulla (61). However, these studies employed long incubation time and used a centrifugation method for removal of the organelles from the assay medium. These methods did not allow measurements of initial velocities and therefore kinetic analysis of the transport was not possible. Millipore filtration, the method used in the present study, allows a rapid separation of organelles from the assay medium and thus makes such measurements feasible.

The possibility exists that the observed uptake of 5-HT represents binding of this ligand to some sites on the dense granule membranes. However, several experiments suggest that 5-HT is actually transported into the organelles and not merely bound. The finding that the steady state level of 5-HT uptake varied with the osmolarity of the medium in both intact granules and in granule "ghosts" (Figure 8b and c, p. 46). indicates that 5-HT is accumulated in a membrane bound space. The dense granule membranes are apparently unstable at low osmolarities, thus showing very little steady state 5-HT accumulation at osmolarities below 300 mOsM. Support for this view comes from the experiment in which the release of endogenous 5-HT was monitored at different osmolarities (Figure 8c, p. 46). Release of biogenic amines from chromaffin granules has been shown to occur at osmolarities below 300 mOsM (66). Another line of evidence in support of 5-HT transport as opposed to binding comes from the experiment in which dense granules preloaded with a large amount of unlabelled 5-HT accumulated labelled 5-HT with approximately the same initial velocity as granules which were not preloaded. One would expect binding of labelled 5-HT to be blocked

under these conditions. Accumulated 5-HT is also completely released by small concentrations (0.1%) of Triton X-100 (data not shown). Taken in sum, these results strongly suggest that the observed 5-HT uptake is a transport rather than a binding phenomenon.

Kinetic analysis of transport revealed two mechanisms (a) a saturable component obeying Michaelis-Menten kinetics with a relatively low K_m (3.3 μM). and (b) a nonsaturable component which appeared to be diffusion-limited (Figure 6, page 41). The diffusion of 5-HT across the dense granule membrane at high substrate concentration is not unexpected due to the hydrophobic nature of this molecule. The presence of a saturable component demonstrates the existence of a carrier-mediated uptake into platelet dense granules. This conclusion is further supported by the data which show a relatively high apparent activation energy which is similar to those found for other enzyme-mediated processes. However, it cannot be concluded from the data presented in this chapter whether the 5-HT uptake is an active (i.e., energy-requiring) transport or a facilitated diffusion.

Uptake of 5-HT at a concentration of 5.7 μM, which is due to a large extent to the saturable component is sensitive to several structural analogs of 5-HT. A number of implications on the recognition on 5-HT by the transport system may be derived from these results. Thus the amino group of the molecule does not appear to be important, since 5-HIAA, a deaminated analog of 5-HT, is a potent inhibitor. The fact that dopamine, norepinephrine and epinephrine but not benzylamine are effective inhibitors at low concentrations suggests that the aromatic

hydroxyl groups of the molecule are probably involved in this process. Although aromatic hydroxyl groups may be important, they are not essential since the indole compound tryptamine which lacks the hydroxyl group of 5-HT is a potent inhibitor of uptake. The involvement of aromatic hydroxyl groups in the uptake of biogenic amines by the dense granules of rabbit platelets has also been suggested by Pletscher et al. (19). However, the mechanism whereby the amines tested in the present study inhibit 5-HT uptake is unknown. Reserpine, which induces the release of biogenic amine stores from platelets as well as other tissues (91), also inhibits the initial velocity of 5-HT uptake into dense granules. It is known that reserpine binds to the membranes of the dense granules (94). However, whether it directly binds to the 5-HT transport system remains to be elucidated.

In summary, the results presented in this chapter demonstrate the existence of a carrier mediated transport system for 5-HT in the membrane of porcine platelet dense granules. The present work provides the basic information necessary for further biochemical and pharmacological characterization of the mechanisms for intracellular storage of 5-HT in the platelet.

CHAPTER III

ROLE OF A TRANSMEMBRANE ΔpH IN 5-HYDROXYTRYPTAMINE TRANSPORT BY DENSE GRANULES AND DENSE GRANULE GHOSTS

Summary

Dense granules, the storage organelles for 5-hydroxytryptamine in blood platelets, have been isolated from porcine platelets and are shown to transport 5-hydroxytryptamine in response to a transmembrane ΔpH. Transport in the absence of ΔpH is minimal and it is shown that a dramatic increase in transport takes place as ΔpH increased. Direct measurements using [^{14}C]methylamine show a pH of 1.1 units (acid inside) for intact granules. Osmotically active ghosts of dense granules from which 95% of the endogenous 5-hydroxytryptamine content has been released have also been prepared. Ghosts swell in the presence of ATP and Mg++ and this swelling is shown to be due to the entry of protons via a process linked to ATP hydrolysis. ATP-induced swelling is relatively insensitive to the addition of the uncoupler CCCP suggesting that proton entry may be due in part to a nonelectrogenic mechanism. In addition, SITS (4-acetamido-4′-isothiocyanostilbene-2,2′disulfonic acid) was found to have differential effects on swelling induced by ATP alone as opposed to that induced by ATP in the presence of nigericin, suggesting the presence of two anion transport mechanisms in the ghosts. Steady state 5-hydroxytryptamine transport in ghosts is stimulated approximately three fold upon the addition of ATP to the incubation medium. The stimulation of 5-hydroxytryptamine transport

in ghosts also correlates with the formation of a transmembrane ΔpH. As measured by [^{14}C]methylamine distribution, ghosts generate a ΔpH of 1.1-1.3 pH units (acid inside) in the presence of 5 mM ATP, 2.5 mM $MgSO_4$. This ΔpH is generated within 2 min at 37°C and is dissipated by uncouplers such as nigericin. It is shown that a Mg++-stimulated ATPase activity is present on the ghost membrane and inhibition of the ATPase leads to a corresponding reduction in 5-hydroxytryptamine transport. The results presented support the idea that 5-hydroxytryptamine transport into platelet dense granules is at least partially dependent upon the presence of a transmembrane ΔpH and, together with previous findings by others, suggest a generalized mechanism for biogenic amine transport into subcellular storage organelles.

Introduction

Transport of biogenic amines by intracellular storage organelles has received a great deal of attention in recent years (62,64,65,95). Most of the work has been directed toward chromaffin granules from adrenal medulla which may be isolated in comparatively large quantities in homogeneous form. Studies by Bashford et al. (68,74) and Johnson et al. (65) using intact granules and studies by Phillips using chromaffin granule ghosts have dealt with the determination of the energy source for amine transport in these organelles. These investigators have found that the intact chromaffin granule maintains an internal pH of approximately 5.5 as measured by distribution of [^{14}C]methylamine (66) or by ^{31}P NMR (68) and that chromaffin granule

ghosts demonstrate a low internal pH following incubation with ATP (70). This low internal pH may be raised by addition of appropriate ionophores and appears to be maintained by a membrane bound ATPase which acts as an inwardly directed proton pump. In addition to these findings, Johnson et al. (65) have provided convincing evidence that epinephrine and dopamine transport in intact chromaffin granules are closely correlated with the magnitude of the transmembrane ΔpH. Phillips has also shown that 5-hydroxytryptamine (5-HT) transport in chromaffin granule ghosts is intimately related to the proton gradient (73).

It was shown previously (Chapter II) that the transport of 5-HT into porcine platelet dense granules occurs predominantly via a carrier-mediated process at low substrate concentrations (96). Recently, Johnson et al. reported the existence of a transmembrane ΔpH (acid inside) in dense granules of pig platelets of approximately one unit at an external pH of 6.9 (97). In this report, we confirm and extend this observation and its implications. 5-HT transport into dense granules is examined with relation to the transmembrane ΔpH. In addition, a method for the preparation of osmotically active resealed ghosts from dense granules is described. This preparation allows the study of the generation of ΔpH and of the relationship of the proton gradient to 5-HT transport. The ghost preparation also provides the advantage that the bulk of intragranular components (e.g., 5-HT) has been removed, thus excluding the possibility that these components might interfere with transport and other assays. In addition, the role of ΔpH with regard to a generalized mechanism for amine accumulation by biogenic amine subsellular storage organelles is discussed.

Experimental Procedures

Methods. Dense granules were isolated by a method described previously (20) using the modifications introduced (96). Briefly, washed platelets isolated by differential centrifugation from 16L of porcine blood were treated with the proteolytic enzyme Nagarse (Enzyme Development Corp., New York) for a brief time and then washed and resuspended in a medium containing KCl and ATP in order to keep platelet actomyosin in a dissociated state. The platelets were then subjected to homogenization in a modified Aminco French press at an internal pressure of 1500 psi (20). Following two complete homogenization steps in the French pressure cell, the suspension was centrifuged at 3,000 g for 5 min in a Sorvall SS-34 rotor to remove unbroken cells and large particulate matter. The pellets were discarded and the supernatants were recentrifuged at 12,000 g for 15 min in the same rotor to yield the "crude mitochondrial" fraction which contains the granules. This fraction was resuspended in modified Tyrode buffer, pH 7.0, and then centrifuged on discontinuous sucrose gradients (Chapter II, p. 28). Modified Tyrode buffer consisted of the following: 130 mM NaCl, 6 mM KCl, 2 mM EDTA, 1 mM NaH_2PO_4, 3 mM $NaHCO_3$, 0.5% (w/v) albumin, 111 mM glucose and 10 mM sucrose. The pellets from these gradients were resuspended in modified Tyrode buffer. This preparation was designated the "intact" granule preparation.

"Ghosts" were prepared from dense granules which had been isolated as above except for the omission of the 2.5 M sucrose layer from the

gradients (Figure 2, p. 28). The amounts of the other four layers (i.e., 2.0 M, 1.8 M, 1.6 M and 0.8 M sucrose) were increased to 6.0 ml each in these gradients. We deleted the 2.5 M layer since by doing so, we obtained a better recovery with little sacrifice in purity. In eralier work, we found that 5-HT was present in significant quantity in a fraction (designated F5) sedimenting immediately above 2.5 M sucrose (96). However, only 0.2 and 1.1 % of the marker enzymes cytochrome oxidase and acid phosphatase respectively were present in the latter fraction. Also, examination of electron micrographs indicated very little increase in contamination of the dense granules by other organelles when the F5 and pellet fractions were combined by deleting the 2.5 M sucrose layer. For preparation of ghosts, the crude mitochondrial fraction was layered onto three of these gradients in the same manner as described above. The gradients were centrifuged in a Beckman Model L2-65B centrifuge at 90,000 g for 3 hrs at 3°C. Following centrifugation, the pellets from these gradients were resuspended immediately into 10 ml of a solution containing 10 mM HEPES, 1 mM $MgSO_4$, and 0.1 mM dithiothreitol, pH 7.0 ("lysis buffer") at 37°C. After gentle homogenization, the suspension was centrifuged at 27,000 g for 15 min at 4°C. The supernatant (SI) was removed and saved for determination of 5-HT and the pellet was resuspended again with gentle homogenization in lysis buffer at 4°C. The latter suspension was allowed to stand overnight on ice. The following day, the suspension was centrifuged at 27,000 g for 15 min. The supernatant (SII) was removed and saved. The pellet was resuspended with gentle

homogenization in lysis buffer containing 0.25 M sucrose to yield the dense granule "ghost" preparation. The release of endogenous 5-HT was followed using the fluorometric assay of Undenfriend and Weissbach (82). Protein was determined by the method of Lowry et al. (83) using BSA as the standard.

Determination of ΔpH. The transmembrane ΔpH in intact granules or granule ghosts was determined by measuring the distribution of [^{14}C]methylamine. This method has been used extensively to determine ΔpH in organelles in which the pH of the internal space is lower than that of the external medium (e.g., 72). The principles underlying this method have been discussed in detail elsewhere (e.g., 98). Samples of intact granules (0.3 mg/ml) or granule ghosts (0.6 mg/ml) were incubated with ^{3}HOH (1 mCi/ml) and [^{14}C]methylamine (58 Ci/mol) at a fixed temperature for the desired period of time. In routine experiments, ^{3}HOH was added at a final concentration of approximately 900 CPM/μl and [^{14}C]methylamine was added at a final concentration of 8 μM (500 CPM/μl). Either halving or doubling the methylamine concentration in the incubations had a negligible effect on the ΔpH values measured in control experiments, thus ruling out a significant effect of binding on the measurements. Following incubation, samples were centrifuged in an Eppendorf Model 3200 centrifuge at 12,000 RPM for 2 min. The supernatants were carefully and completely aspirated with a Pasteur pipette. Pellets were resuspended in 0.2 ml of 2% (v/v) Triton X-100. Samples of supernatants (10 μl) and pellets (100 μl) were then added

to 5 ml of a scintillant consisting of 0.4% (w/v) butyl-PBD, 33% (v/v) Triton X-100 in toluene and counted for radioactivity in a Beckman Model LS-330 liquid scintillation counter.

Since pellets contained both intra-and extravesicular spaces, the size of the extravesicular space (i.e., trapped volume) was determined by incubating samples of intact granules or granule ghosts with ^{3}HOH (900 CPM/µl) and either [^{14}C]polydextran (600 CPM/µl final concentration) or [^{14}C]sucrose (800 CPM/µl final concentration). These samples were then centrifuged and treated exactly as samples in which methylamine distribution was measured. All values were corrected for crossover by counting samples containing [^{14}C] or [^{3}H] alone. ΔpH was then calculated by determining the ratio of the methylamine concentration inside divided by the methylamine concentration outside the organelles (i.e., C methylamine in/C methylamine out). From this ratio, one may calculate the ΔpH. For amines of high pKa,

$$\Delta pH = \log (C \text{ methylamine in}/C \text{ methylamine out})$$

Light scattering. Light scattering was measured at 540 nm in a 90°C angle mode at 37°C. A rectangular cuvette (1 x 1 cm, 4 sides clear) was used. The contents were stirred continuously by means of a small magnetic bar coupled to a motor and reducer located below the cuvette assembly. Scattering changes were amplified differentially with respect to a fixed voltage in an expanded scale mode and were recorded using a Brush Datapoint recorder. Protein concentration was selected for a linear region of response (see osmotic responses).

ATPase Activity. ATPase activity was determined by measuring the amount of inorganic phosphate released from ATP, using the Malachite green method (99). This method depends upon the formation of a complex between phosphomolybdate and malachite green at low pH and is sensitive to values of less than 1 nmol of inorganic phosphate. The standard incubation buffer for ATPase assays contained 50 mM HEPES, 2.5 mM $MgSO_4$, and 5 mM Na_2ATP at pH 7.0. Ghosts were incubated in this buffer at a final protein concentration of 100 µg/ml at 37°C. Samples (0.1 ml) were withdrawn after the desired time intervals and added to 1.0 ml of ice cold 5% (v/v) trichloroacetic acid (TCA). TCA-treated samples were incubated for 5 min on ice and then centrifuged in an Eppendorf Model 3200 centrifuge at 12,000 RPM for 2 min to remove precipitated material. Samples of ghosts which had been boiled or treated with 5% TCA prior to ATP addition served as blanks and were treated exactly as stated for experimental samples. All measurements were made in duplicate or triplicate.

Transport of 5-hydroxytryptamine. Transport of 5-HT by intact granules or by granule ghosts was assessed by Millipore filtration assay essentially as described previously (Chapter II). Intact granules or granule ghosts were incubated at a final protein concentration of 200 µg/ml in the desired buffer at 37°C for various times. All assays contained [^{14}C]5-hydroxytryptamine (50 Ci/mol) at a final concentration of 5.7 µM. Following the desired incubation time, 0.1 ml aliquots were removed and filtered through 0.1 µ pore size Millipore filters followed

by washing with 2.0 ml of ice cold incubation buffer. Samples containing no protein were filtered and washed in the same way as experimental samples in order to correct for binding of 5-HT to the filters. Filters were dissolved in 3 ml of 2-methoxyethanol and counted for radioactivity as described previously (Chapter II).

Electron microscopy. Pelleted samples of ghosts were fixed overnight at 0°C with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.2. This was followed by postfixation with 1% OsO_4 for 5 min and staining with 7.5% uranyl acetate for 10 min. Samples were then dehydrated in a graded series of ethanol followed by dehydration in 100% propyleneoxide. Samples were then embedded in Epon 812 followed by thin sectioning using an LKB III-8800 ultramicrotome. Thin sections were stained with uranyl acetate followed by lead citrate. All specimens were viewed in a Philips model 300 electron microscope.

Materials

5-hydroxy [2-^{14}C]tryptamine creatninine sulfate was obtained from Amersham/Searle. ^{3}HOH, [^{14}C(U)]Sucrose, [^{14}C]methylamine HCl, and [carboxyl-^{14}C]Polydextran were purchased from New England Nuclear. Nigericin was generously supplied by Dr. Robert Hosley, Lilly Research Laboratories, Indianapolis, IN. Valinomycin, CCCP, DCCD, Oligomycin and ouabain were purchased from Sigma Chemical Co., St. Louis, MO. Reserpine was supplied by Ciba Pharmaceutical Co. SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid) was obtained as the

disodium salt from Nutritional Biochemicals, Inc. All other materials used were reagent grade obtained from commercial sources. All ionophores were dissolved in absolute ethanol. DCCD, oligomycin and reserpine were dissolved in dimethylformamide.

Results

Transport of 5-hydroxytryptamine and ΔpH in intact granules. It has been suggested that a transmembrane ΔpH (acid inside) may play a role in 5-HT accumulation by dense granules (97). Initial experiments therefore focused on determination of 5-HT transport in intact granules as a function of the pH of the external medium. As shown in Figure 9, a dramatic increase in the initial velocity of 5-HT transport into intact dense granules took place as the external pH was increased from 6.0 to 10.0. This increase in 5-HT transport was completely abolished by the addition of nigericin, an ionophore which has been shown to mediate the electroneutral exchange of K+ or Na+ for H+ and, as a consequence, to disrupt ΔpH (100,101) (data not shown). In order to directly measure the transmembrane ΔpH, the distribution of [^{14}C] methylamine was monitored. This technique has been used previously to measure pH gradients in dense granules (97) and in chromaffin granules (66). Figure 9 also shows the behavior of ΔpH as the pH of the medium is changed. Clearly, an increase in 5-HT transport correlated with a rise in ΔpH from values near zero at an external pH of 6.0 to values greater than one unit at an external pH of 7.0. As shown in Table IV, dense granules isolated on sucrose gradients exhibited a pH gradient

Figure 9. Transport of 5-hydroxytryptamine and ΔpH in intact granules as a function of external pH.

Initial rates of 5-HT transport were assayed after 4 min incubation at 37°C as described under "Experimental Procedures." ΔpH was measured using the distribution of [^{14}C]methylamine as described under "Experimental Procedures." All assays were done in .175 M KCl containing 50 mM of one of the following buffers: pH 5.0 and 5.5, citrate; pH 6.0 and 6.5, MES; pH 7.0, 7.5, 8.0, HEPES; pH 9.0, 9.5, 10.0, glycine. All solutions were adjusted to the desired pH with 5N KOH. ●, 5-HT transport; x, ΔpH.

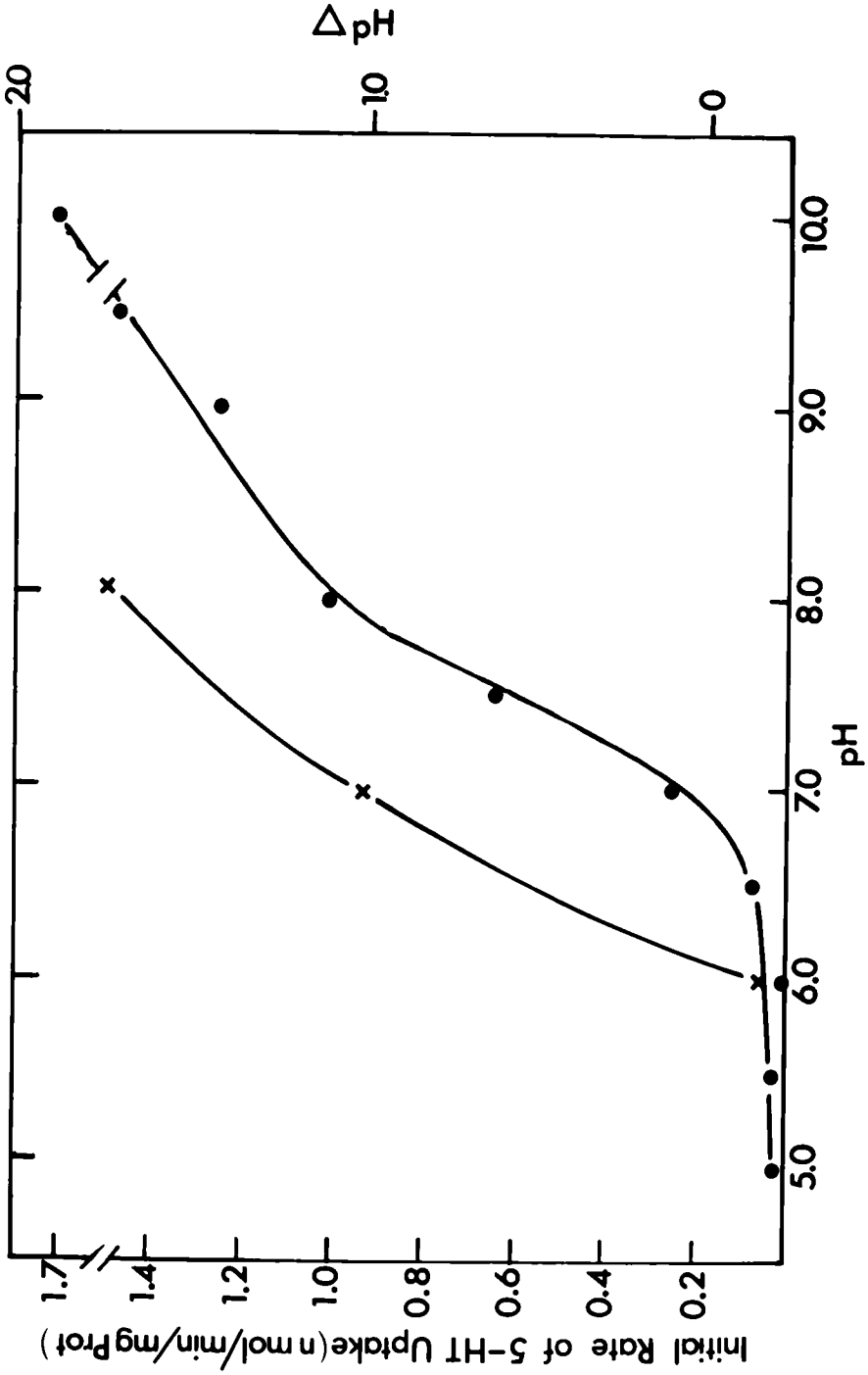
ΔpH
20
10
0
10.0
9.0
8.0
7.0
6.0
5.0
pH
1.7
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Initial Rate of 5-HT Uptake (n mol/min/mg Prot)

Figure 9

TABLE IV

ΔpH IN INTACT DENSE GRANULES

Intact dense granules prepared as described in "Experimental Procedures" were incubated at a final protein concentration of 0.3 mg/ml with ^{3}HOH and either [^{14}C]methylamine or [^{14}C]polydextran in order to determine ΔpH. Incubations were for 5 min and were initiated by adding the intact dense granule suspension to the complete incubation mixture which contained nigericin or NH_4^+ where indicated. Samples were then centrifuged and treated as described in "Experimental Procedures." Values given represent means of three determinations. Data in experiments A and B represent two separate preparations of granules respectively.

Medium	[^{14}C]methylamine (in)/ [^{14}C]methylamine (out)	pH = log ((in)/)out))
Experiment A		
0.4 M Sucrose 10 mM HEPES pH 7.0	13.9	1.14
0.175 M KCl 50 mM HEPES pH 7.0	9.7	0.99
0.4 M Sucrose 10 mM HEPES pH 7.0 20 mM KPO4 + 1 μg/ml Nigericin	2.6	0.41
0.4 M Sucrose 10 mM HEPES pH 7.0 + 20 mM NH_4Cl	1.4	0.15
Experiment B		
0.175 m KCl 50 mM MES pH 6.0	0.56	-0.25
0.175 M KCl 50 mM HEPES pH 7.0	14.1	1.14
0.175 M KCl 50 mM HEPES pH 8.0	50.5	1.70
0.175 M KCl 50 mM HEPES pH 7.0 +1 μg/ml Nigericin	0.50	-0.30

of 1.14 pH units (inside acid) at pH 7.0 (Experiment A). Raising or lowering the external pH produced a concomitant increase or decrease in the ΔpH (Experiment B). Also tested was the effect of various media on ΔpH. It is shown that ΔpH remained at approximately 1 unit regardless of medium composition (as long as isotonicity was maintained). These latter observations indicate that ΔpH in the granules did not arise from the establishment of a Donnan equilibrium. Again, addition of either nigericin (1 μg/ml) or NH_4Cl (20 mM) greatly reduced the magnitude of the observed pH gradients (Table IV).

Preparation and characterization of dense granule "ghosts." In order to further study the transport of 5-HT by dense granules and its energy requirements, we decided to prepare a resealed ghost preparation from dense granules. Such a preparation allows the study of 5-HT transport under conditions where the level of endogenous amine, which may interfere with measurements by leaking from intact granules during transport assay, has been greatly reduced. Further, the ghost preparation allows the study of the conditions necessary for the generation of a transmembrane ΔpH in the dense granule. Lysis of the dense granules was accomplished by suspension of pellets from sucrose gradients in a hypotonic medium as described in "Experimental Procedures." We found that two lysis steps, the first at 37°C and the second at 0°C were required to release the bulk of endogenous 5-HT. As shown in Table V, approximately 95% of the endogenous 5-HT was released by the procedure outlined, as measured fluorometrically. Figure 10 shows

TABLE V

RELEASE OF 5-HYDROXYTRYPTAMINE FROM DENSE GRANULES

Dense granules were lysed hypotonically to give a "ghost" preparation as described in "Experimental Procedures." Release of 5-HT was followed fluorometrically by the method of Undenfriend and Weissbach (14). Data shown are the means and standard errors from three separate preparations of ghosts. SI and SII represent supernatants from the first and second hypotonic lysis steps respectively (see "Experimental Procedures").

Fraction	% Endogenous 5-HT Present
SI	50.2 ± 3.4
SII	44.3 ± 5.7
Total Released	94.5 ± 4.1
Typical Preparation:	
Intact Granules - 534 nmol 5-HT/mg prot.	
Ghosts - 15 nmol 5-HT/mg prot.	

Figure 10. Electron micrograph of ghost preparation.

Samples were prepared according to the method outlined in "Experimental Procedures." Final magnification: 28,500X.

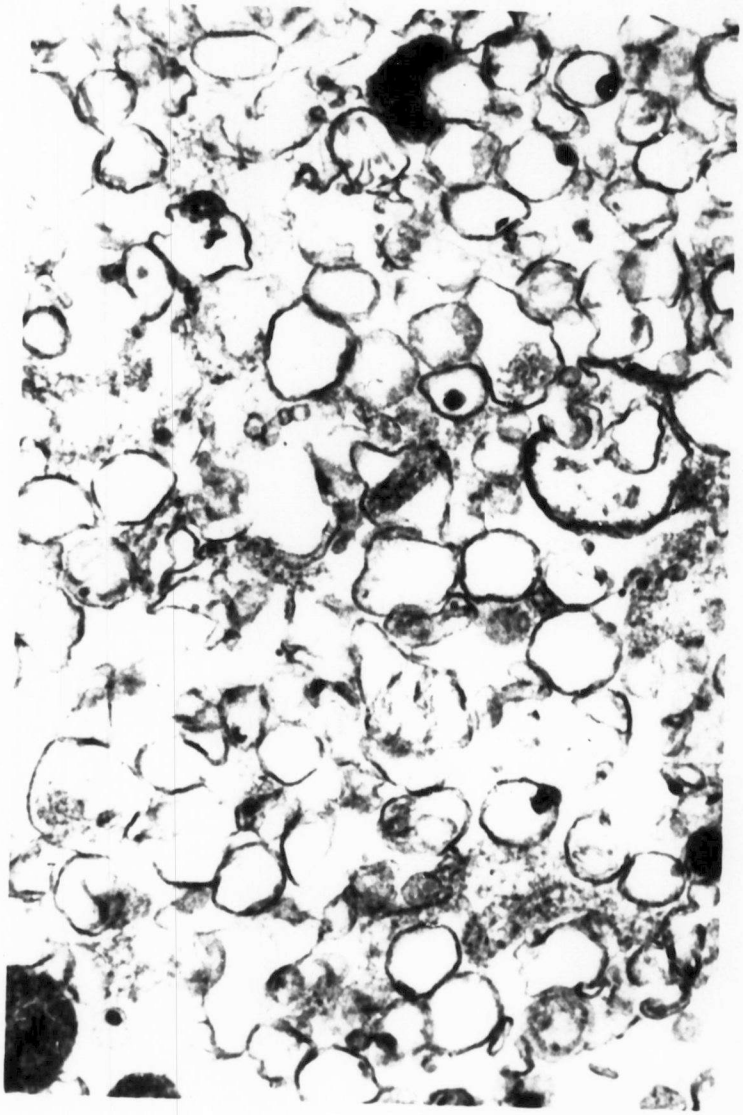

Figure 10

an electron micrograph of a typical ghost preparation. Most of the structures in these preparations appeared to be resealed dense granule membranes and some contained what appeared to be a small amount of residual core material.

Dense granule ghosts were assayed for 5-HT transport activity by Millipore filtration assay as described in "Experimental Procedures." Figure 11 shows the time course of 5-HT transport in the presence and absence of 5 mM ATP and 2.5 mM $MgSO_4$. Transport in the presence of ATP and Mg++ which was similar to that shown previously for intact granules (Chapter II), remained linear for approximately 4 min and reached near steady state levels after 10 min of incubation. 5-HT transport in the absence of ATP, however, was low and typically reached only about 30% of the steady state levels attained in the presence of ATP. Also shown in Figure 11 is the effect of nigericin addition on the steady state level of 5-HT transport. Nigericin, which disrupted the transmembrane ΔpH in intact granules also caused release of 5-HT accumulated by ghosts in response to ATP addition.

Osmotic behavior of ghosts—light scattering measurements. In order to study the osmotic behavior of the ghosts, light scattering experiments were performed. Ghosts were found to behave in a predictable way when suspended in media of various osmotic pressures as shown in Figure 12. The latter finding indicates that ghosts are osmotically active and obey the Boyle-Van't Hoff law for osmometers over a range of osmolarities (50-260 mOsM).

Figure 11. Time course of 5-hydroxytryptamine transport by dense granule ghosts.

The assay mixtures consisted of the following: 0.25 M sucrose, 50 mM KPO_4, 2.5 mM MgSO4, 5.7 µM [^{14}C] 5-HT, plus (x) and minus (●) 5.0 mM Na_2ATP^4 at pH 7.0. The solutions were equilibrated for 5 min at 37°C.^{2} Following equilibration, ghosts were added to a final concentration of 200 µg protein/ml and aliquots taken at the appropriate times as described in "Experimental Procedures." In addition, one sample was incubated for 5 min with [^{14}C] 5-HT and ATP at 37°C as described above and after 5 min (arrow), nigericin was added at a final concentration of 1 µg/ml(▲).

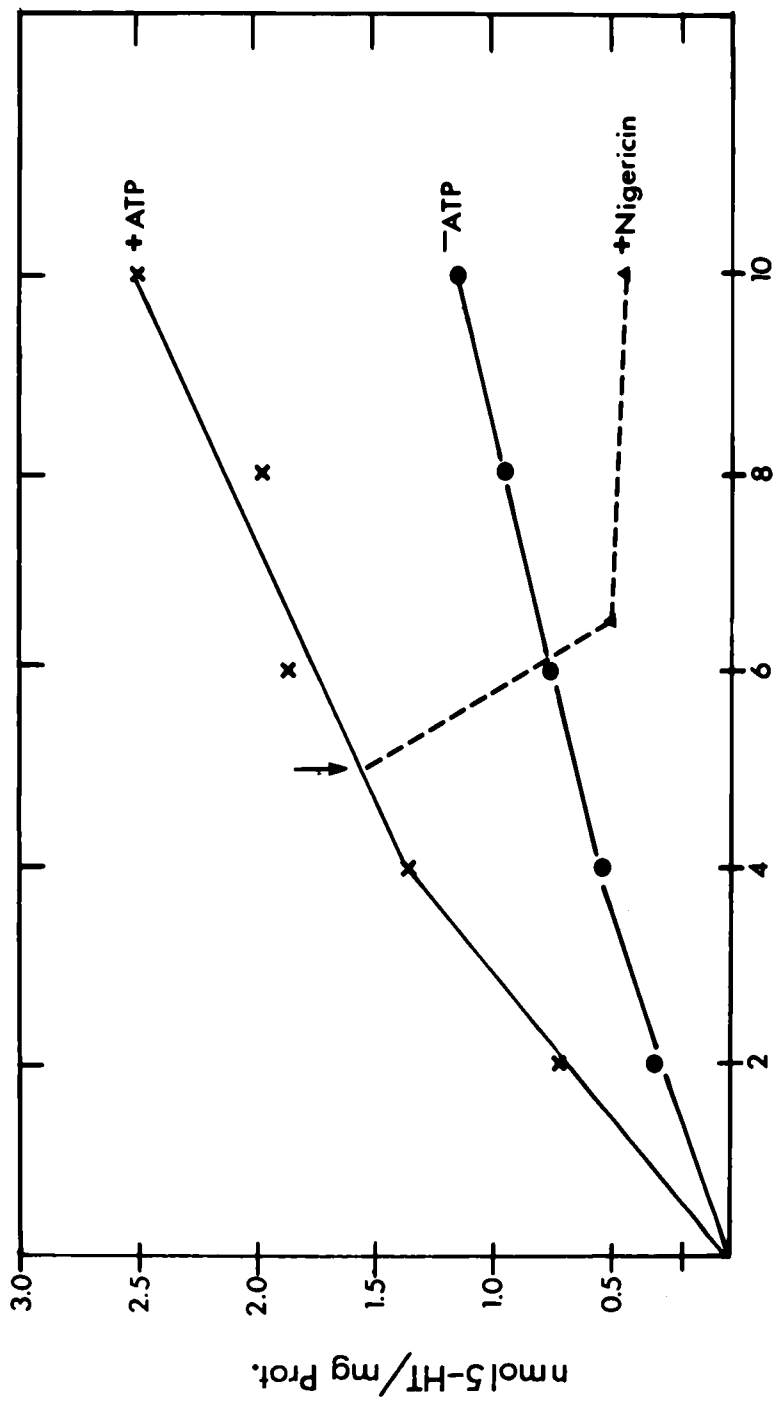
+ATP
−ATP
+Nigericin
3.0
2.5
2.0
1.5
1.0
0.5
2
4
6
8
10
nmol 5-HT/mg Prot.
Time (min)

Figure 11

Figure 12. Linearity of light scattering with osmotic pressure.

Ghosts were suspended in sucrose solutions containing 1 mM $MgSO_4$ buffered with 10 mM HEPES, pH 7.0 and these suspensions were allowed to equilibrate for 10 min at 37°C. Following this, light scattering of the suspensions was measured as described in "Experimental Procedures." "% Scatter" represents the percent of scattering obtained at an osmotic pressure of 262 mOsM. Results shown are the means of duplicate determinations.

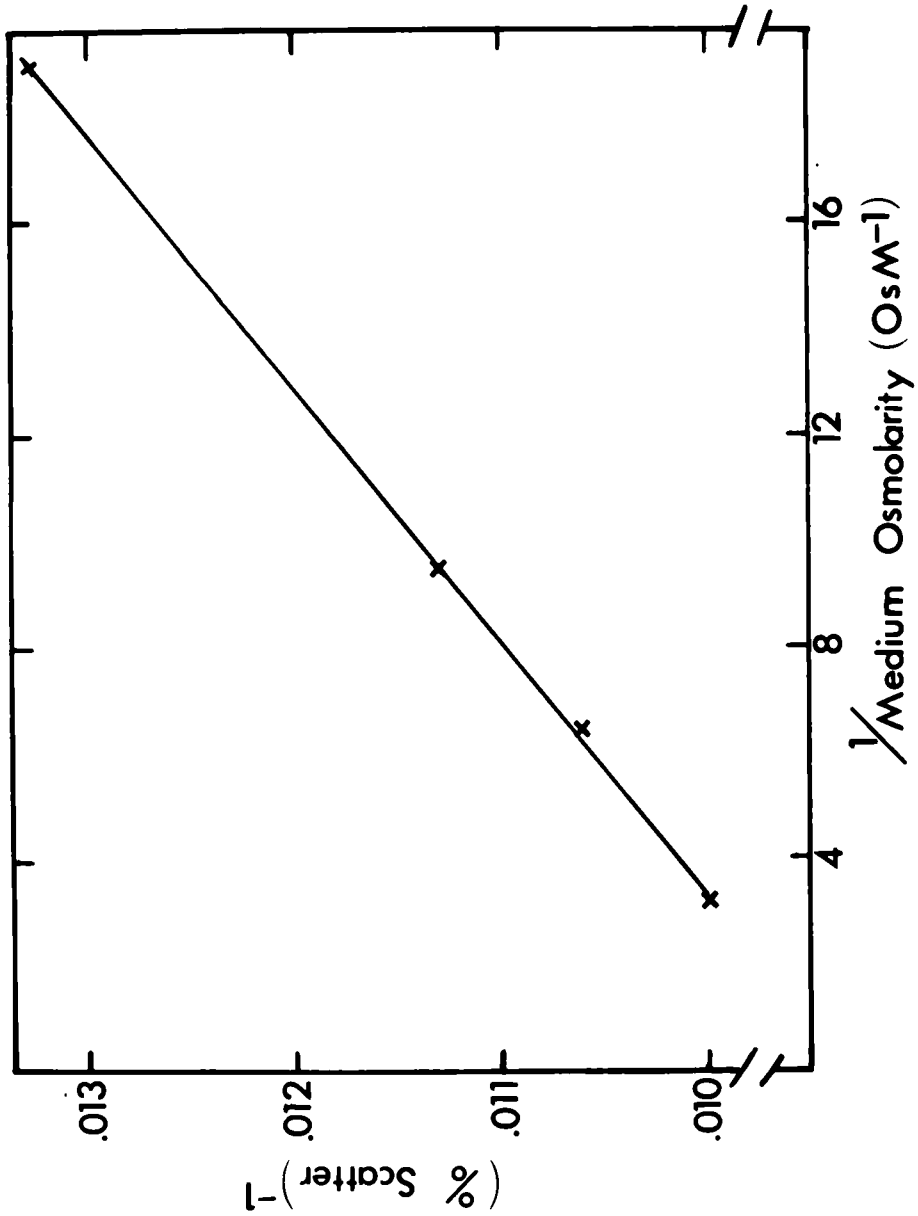

Figure 12

ATP-induced ghost swelling. A well-known property of chromaffin granules from adrenal medula is that they swell upon addition of ATP to their medium (70, 102-104). This swelling is time dependent and is thought to be due to entry of protons via a proton translocating ATPase in the chromaffin granule membrane. In order to determine whether dense granule ghosts behave in a similar manner, ghosts were suspended in 0.25 M sucrose, 10 mM HEPES, pH 7.0 containing 1 mM $MgSO_4$ at a final protein concentration of 100 µg/ml. Following stabilization of the recorder trace, Na_2ATP was added to a final concentration of 3 mM. As shown in Figure 13a, rapid swelling of the ghosts was followed by a slower phase of swelling. The slower phase was dependent upon the concentration of ATP added, being half maximal at 2 mM ATP (data not shown). The time dependent swelling of the ghosts on the addition of ATP is apparently due to inwardly-directed proton translocation. In support of this view, inhibition of the Mg++ dependent ATPase of the membrane by the addition of 2 mM EDTA leads to a corresponding inhibition (>70%) of ATP-induced swelling (see below). Slow swelling of the ghosts in the presence of ATP continued for long periods (>30 min) in the absence of other additions. However, if nigericin (1 µg/ml) was added after ATP, an immediate increase in the swelling rate occurred which lasted for several minutes. The swelling then returned to the slower rate obtained prior to nigericin addition (Figure 13a). This effect of nigericin was absolutely dependent on the presence of ATP. As shown in Figure 13b, if nigericin was added first, there was no effect on the scattering of the ghost suspension. Following ATP addition,

Figure 13. Swelling of ghosts induced by ATP and nigericin.

Ghosts were added to a solution (final protein concentration 100 µg/ml) containing 0.25M sucrose, 10 mM HEPES, 1 mM Mg SO_4, (neutralized to pH 7.0 with 5N NaOH (A,B and D) or 1 M TRIS (C)) in a final volume of 2.5 ml. The immediate increase in light scattering upon addition of ghosts is not shown. The suspensions were equilibrated at 37°C. Reagents were added (arrows) at the following final concentrations: Na_2ATP, 3.0 mM; nigericin (Nig), 1 µg/ml; TRIS-ATP, 2.0 mM; NaCl, 20 mM. Molarities at left in Panel D indicate final concentrations of SITS added. The response shown following SITS addition represents that obtained following addition of 400 µM SITS. An immediate apparent decrease in scattering caused by light absorbance by SITS has been corrected for in Panel D. Top scale represents calibration for Panels A and B. Bottom scale represents calibration for Panels C and D. ΔS—change in scattering.

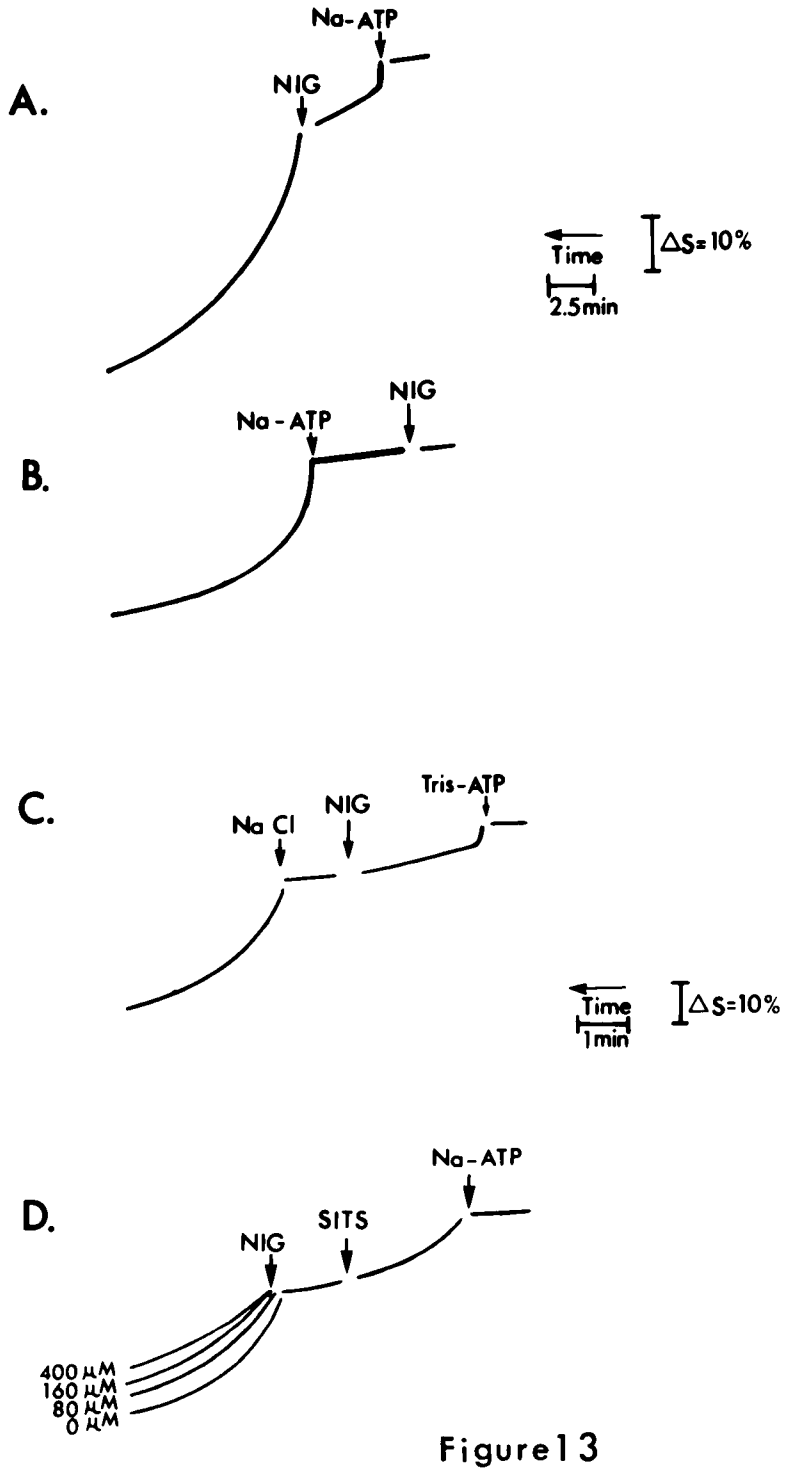
A.
Na-ATP
NIG
Time
2.5min
ΔS=10%
B.
Na-ATP
NIG
C.
Na Cl
NIG
Tris-ATP
Time
1min
ΔS=10%
D.
Na-ATP
SITS
NIG
400 μM
160 μM
80 μM
0 μM

Figure 13

however, a rapid burst of swelling ensued. Since nigericin catalyzes electroneutral exchange between H+ and K+ or Na+, it was reasoned that Na+ of the buffering medium rapidly exchanged for protons which had been pumped into the ghosts. This possibility was tested by replacing Na-HEPES with TRIS-HEPES in the buffering medium. Ghosts were added to the latter medium and the recorder trace was allowed to stabilize as described above (Figure 13c). Addition of TRIS-ATP to a final concentration of 2 mM caused an immediate swelling similar to the one seen with Na-ATP followed by a slower phase of swelling. However, the subsequent addition of nigericin did not elicit an increase in the rate of swelling. If nigericin addition was then followed by the addition of 20 mM NaCl, a rapid phase of swelling was seen. This pheonmenon was also seen when KCl was substituted for NaCl under these circumstances. This result demonstrates that rapid swelling induced by nigericin in the presence of ATP is due in part to an electroneutral exchange of Na+ for H+ catalyzed by nigericin. However, this exchange must also lead to increased influx of anions since a net influx of water takes place under these conditions resulting in swelling of the ghosts (see "Discussion"). In order to test whether the anion movements seen in the presence of ATP and nigericin could be manipulated in a predictable way, different concentrations of the anion transport blocker, SITS (4-acetamido-4′-isothiocyanostilbene-2,2′disulfonic acid) were added to ghosts during Na-ATP stimulated swelling (Figure 13d). The addition of SITS at increasing concentrations had no measurable effect

on ATP-induced swelling. However, the nigericin-induced swelling burst was sensitive to such additions of SITS, suggesting that different mechanisms for anion transport may be involved in the two processes. This result is discussed in detail below.

Another aspect of ion movements in the presence of ATP is shown in Figure 14. Trace A shows that addition of CCCP, an ionophore which has been shown to allow electrogenic H+ movement across biological membranes has little effect on the rate of ATP-induced swelling. Establishment of a K+ diffusion potential (inside positive) by valinomycin and potassium phosphate addition before ATP addition also caused no discernable change in ATP-induced swelling (Figure 14b). In Trace C, a combination of valinomycin and CCCP in the presence of K+ causes rapid swelling in the presence of ATP. This effect is analogous to adding nigericin in the presence of K+ since the combination of valinomycin and CCCP allows exchange between K+ and H+. The above results suggest that ATP-induced H+ movement is at least partially nonelectrogenic in nature. It is unknown at present whether accompanying anion movements are diffusion or carrier mediated, however.

Ghost ATPase activity. The occurrence of an ATP-stimulated, nigericin sensitive 5-HT transport and of ATP dependent ghost swelling suggested the presence of a proton translocating ATPase activity in the dense granule ghost membranes. In order to further characterize this activity, the ghost ATPase was assayed as described in "Experimantal Procedures." The time course of ATPase activity is shown in Figure 15.

Figure 14. Effect of valinomycin and CCCP on ATP-induced swelling.

Ghosts were equilibrated at 37°C exactly as described in Figure 13, page 78. Reagents were then added (arrows) at the following final concentrations: Na_2ATP, 3 mM; CCCP, 2 μM; valinomycin (VAL), 10 μM; potassium phosphate (pH 7.0), 10 mM. Top scale represents calibration for Panels A and B. Bottom scale represents calibration for Panel C.

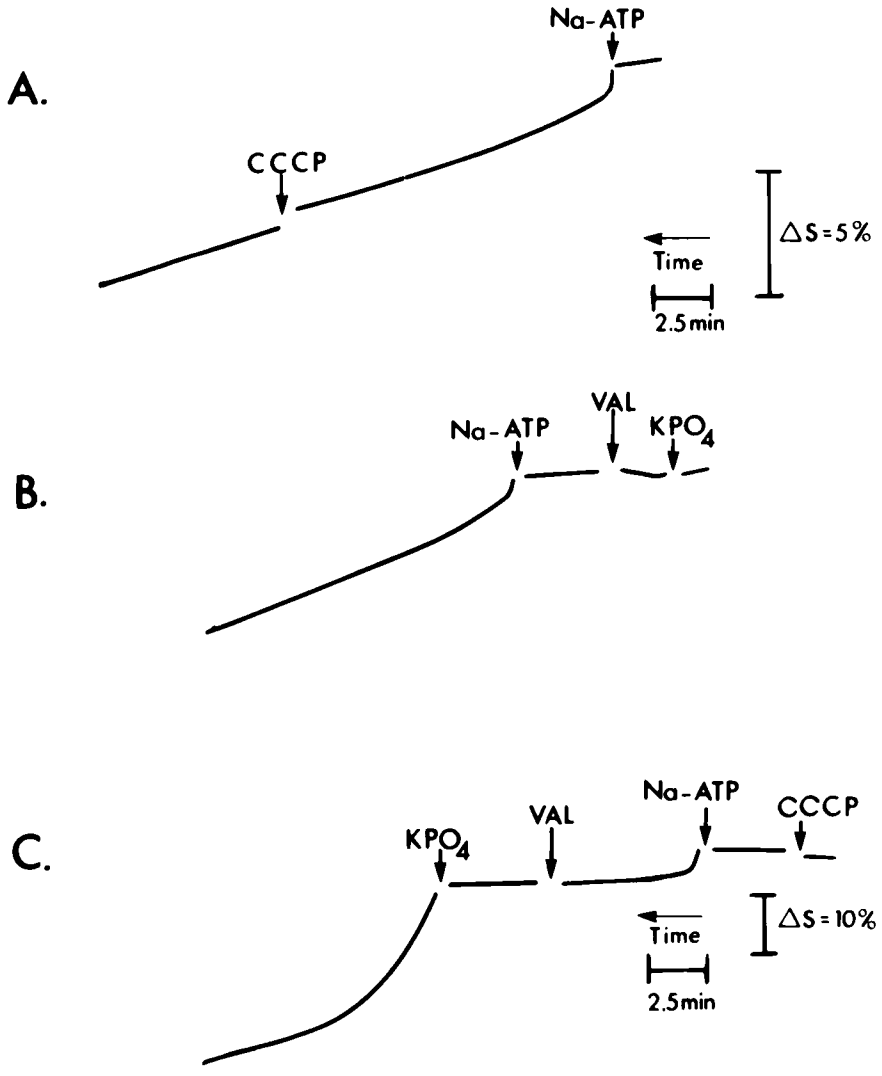
A.
Na-ATP
CCCP
ΔS=5%
Time
2.5min
B.
Na-ATP
VAL
KPO4
C.
KPO4
VAL
Na-ATP
CCCP
ΔS=10%
Time
2.5min

Figure 14

Figure 15. ATPase activity of ghosts.

ATPase activity was monitored at 37°C as described in "Experimental Procedures." Results shown represent data from two separate experiments.

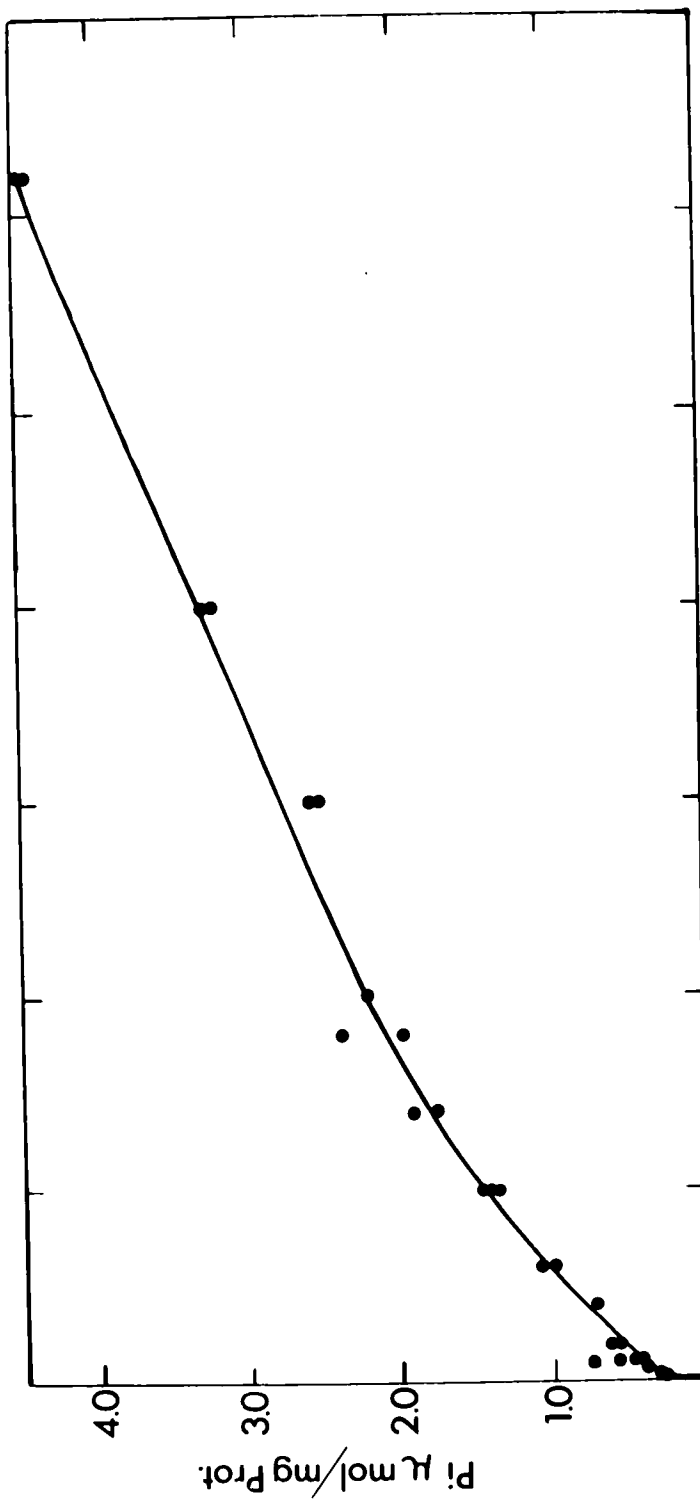

Figure15

An initial burst of ATP hydrolysis in the first minute was followed by a slower phase of activity during the rest of the 30 min assay period. This slower phase was significantly inhibited in the presence of either EDTA (2 mM) or DCCD (0.5 mM) both of which are known to inhibit ATPase activities of other amine storage organelles (e.g., 74, 105) (Table VI). The inhibition of the ATPase by EDTA correlates well with its effect on ATP-induced swelling. Interestingly, Ca++ (2.5 mM) did not substitute for MG++ and caused almost complete inhibition of the ATPase activity. On the other hand, three other agents, namely: (a) reserpine, which inhibits 5-HT transport by dense granules; (b) oligomycin; and (c) ouabain were without significant effect on the ghost ATPase. The lack of inhibition by oligomycin and ouabain indicates that the ATPase activity measured here is not due to contaminating ATPases from mitochondria or plasma membranes respectively.

Also shown in Table VI are the effects of ATPase inhibitors on ATP-stimulated steady state 5-HT transport. Addition of ATPase inhibitors such as DCCD, N-ethylmaleimide or EDTA caused concomitant reduction in 5-HT transport. On the other hand, reserpine, which actually stimulated ATPase activity slightly, strongly inhibited 5-HT transport and ouabain had little effect on either ATPase or 5-HT transport activities.

Generation of a transmembrane ΔpH by ghosts. In order to compare the pH gradients achieved in the ghost preparation following incubation with ATP with those in intact granules directly, the distribution of

TABLE VI

INHIBITION OF STEADY STATE ATPase AND 5-HT TRANSPORT ACTIVITIES

ATPase assays: Dense granule ghosts were princubated for 10 min at 37°C with the desired inhibitor in 50 mM HEPES buffer, pH 7.0 at a final protein concentration of 100 µg/ml. (Control preincubations contained no inhibitors.) All preincubations with the exception of that indicated (*) contained 2.5 mM $MgSO_4$. Following preincubation, Na_2ATP was added to a final concentration of 5 mM and the samples were incubated for another 10 min at 37°C. Following the latter incubation, samples (0.1 ml) were withdrawn and added to 1.0 ml of ice cold 5% TCA. The samples were then treated and assayed for inorganic phosphate by the Malachite green method as described in "Experimental Procedures." Control ATPase activity (mean ± standard error) -3.27 ± 0.2 µmol Pi/10 min/mg protein (3 experiments.)

5-HT transport assays: Dense granule ghosts were preincubated for 10 min at 37°C with the desired inhibitor in 0.25 M sucrose, 50 mM KPO_4, 2.5 mM $MgSO_4$, pH 7.0 at a final protein concentration of 200 µg/ml. (Control preincubations contained no inhibitors.) Following preincubation, Na_2ATP and [^{14}C]5-HT were added simultaneously at final concentrations of 5 mM and 5.7 µM respectively. The samples were allowed to incubate for another 10 min in order to attain a steady state level of 5-HT transport. Aliquots were then withdrawn and filtered as described in "Experimental Procedures." Control 5-HT transport activity (mean ± standard error) -2.5 ± 0.3 nmol 5-HT/10 min./mg protein (3 experiments). ND—not determined. Data shown are the means and standard errors from the numbers of experiments shown in parentheses.

Inhibitor	Concentration	% of Control ATPase Activity	% of Control 5-HT Transport
DCCD	250 µM	50 ± 3(2)	N.D.
DCCD	500 µM	22 ± 3(3)	9 ± 2(3)
N-ethylmaleimide	1 mM	53 ± 9(3)	37 ± 5(3)
EDTA*	2 mM	48 ± 5(3)	42 ± 3(3)
Reserpine	5 µM	115 ± 3(3)	16 ± 2(3)
Oligomycin	60 µg/mg Protein	77 ± 10(3)	N.D.
Ouabain	200 µM	98 ± 7(3)	74 ± 8(3)
$CaCl_2$*	2.5 mM	2 ± 1(2)	N.D.
0 C†		11 ± 3(2)	0

†Preincubated for 10 min at 37°C, then cooled to 0°C and incubated with 5 mM ATP for 10 min.

[^{14}C]methylamine was measured following incubation of ghosts with ATP under various conditions. Experiment A in Table VII shows that a pH gradient of approximately 1.1 units (acid inside) was generated after incubation of ghosts with 5 mM ATP, 2.5 mM $MgSO_4$ for 3 min at 37°C. The ΔpH value declined somewhat upon further incubation. Incubation of ghosts at 0°C with ATP resulted in much lower pH gradients than those measured upon incubation at 37°C (Experiment B). However, these gradients were consistently higher than those found in the presence of nigericin or NH_4^+ ions. Maximal ΔpH values were obtained following a 1 min incubation with ATP at 37°C (Experiment B, Table VII). We found it difficult to measure the initial rate of ΔpH formation using the present methodology since the time required for centrifugation introduced uncertainty into the initial time course measurements. It may be said with certainty, however, that maximal pH gradients are generated within 2 min at 37°C. The magnitude of the pH gradient measured was relatively unaffected either by the presence of various ions (i.e., potassium phosphate or K_2SO_4) (Experiment C) or by the concentration of methylamine used (Experiment D) thus ruling out significant binding of the probe to the ghost membrane.

Stimulation of 5-HT transport by ATP was further studied in the following way. Samples of ghosts were incubated in the standard manner with 5.7 μM 5-HT (Figure 16). After 5 min various concentrations of ATP were added such that the final concentrations shown were attained. Stimulation of 5-HT transport was maximal at 10 mM ATP and was almost negligible at the lowest ATP concentration used (0.5 mM). Addition of

TABLE VII

GENERATION OF ΔpH IN DENSE GRANULE GHOSTS

Dense granule ghosts were incubated at the times and temperatures indicated with 5 mM ATP, 2.5 mM $MgSO_4$ and ^3HOH at a final protein concentration of 0.5 mg/ml. Following the incubation times indicated, [^{14}C]methylamine was added to a final concentration of 8.0 μM and the samples were centrifuged and processed as described in "Experimental Procedures." Samples for determination of trapped volumes were treated exactly as above except that [^{14}C]sucrose was added following incubation. Ionophores and/or salts were added prior to the incubation periods. Results shown for Experiments A, B, C, and D represent data collected from four different preparations. Values shown represent means of duplicate determinations, since values obtained agreed closely.

Medium	Incubation Time and Temperature	[^{14}C]methylamine (in)/ [^{14}C]methylamine (out)	ΔpH = log ((in/out))
Experiment A			
0.25 M Sucrose			
10 mM HEPES pH 7.0	3 min, 37°C	15.7	1.19
"	6 min, 37°C	11.0	1.04
"	10 min, 37°C	11.8	1.07
"	10 min, 0°C	3.9	0.59
Experiment B			
0.25 M Sucrose			
10 mM HEPES pH 7.0	1 min, 37°C	20.4	1.30
"	5 min, 37°C	20.2	1.30
"	10 min, 37°C	17.6	1.25
"	10 min, 0°C	6.0	0.78
0.25 M Sucrose 10 mM HEPES pH 7.0 + 1 μg/ml Nigericin	10 min, 37°C	3.4	0.53
Experiment C			
0.25 M Sucrose 10 mM HEPES pH 7.0 + 20 mM KPO_4	5 min, 37°C	24.6	1.39
0.25 M Sucrose 10 mM HEPES pH 7.0 +20 mM K_2SO_4	5 min, 37°C	29.7	1.47
0.25 M Sucrose 10 mM HEPES 20 mM KPO_4 pH 7.0 +20 mM NH_4Cl	5 min, 37°C	5.1	0.70

TABLE VII (Continued)

Medium	Incubation Time and Temperature	[^{14}C]methylamine (in)/ [^{14}C]methylamine (out)	ΔpH = log ((in/out))
Experiment C (cont'd.)			
0.25 M Sucrose 10 mM HEPES 20 mM KPO_4 pH 7.0 +1 µg/ml Nigericin	5 min, 37°C	5.7	0.76
Experiment D			
0.25 M Sucrose 10 mM HEPES 20 mM K_2SO_4 4 µM methylamine	5 min, 37°C	22.2	1.35
0.25 M Sucrose 10 mM HEPES 20 mM K_2SO_4 4 µM methylamine +1 µg/ml Nigericin	5 min, 37°C	3.5	0.55
0.25 M Sucrose 10 mM HEPES 20 mM K_2SO_4 12µM methylamine	5 min, 37°C	17.1	1.23
0.25 M Sucrose 10 mM HEPES 20 mM K_2SO_4 12 µM methylamine +1 µg/ml Nigericin	5 min, 37°C	3.6	0.56

Figure 16. Stimulation of 5-HT transport by ATP.

Transport of 5-HT was assayed as described under "Experimental Procedures." Samples of ghosts (100 µg protein/ml) were incubated at 37 C with 5.7 µM [^{14}C]5-HT in 0.25 M sucrose 50 mM KPO_4, pH 7.0. At the time shown (arrow) Na_2ATP was added at the final concentrations shown on the right: x, 10 mM ATP; 0, 2 mM ATP; □, 0.5 mM ATP, ● mM ATP; ▲, 5 mM ATP + 1 µg/ml Nigericin.

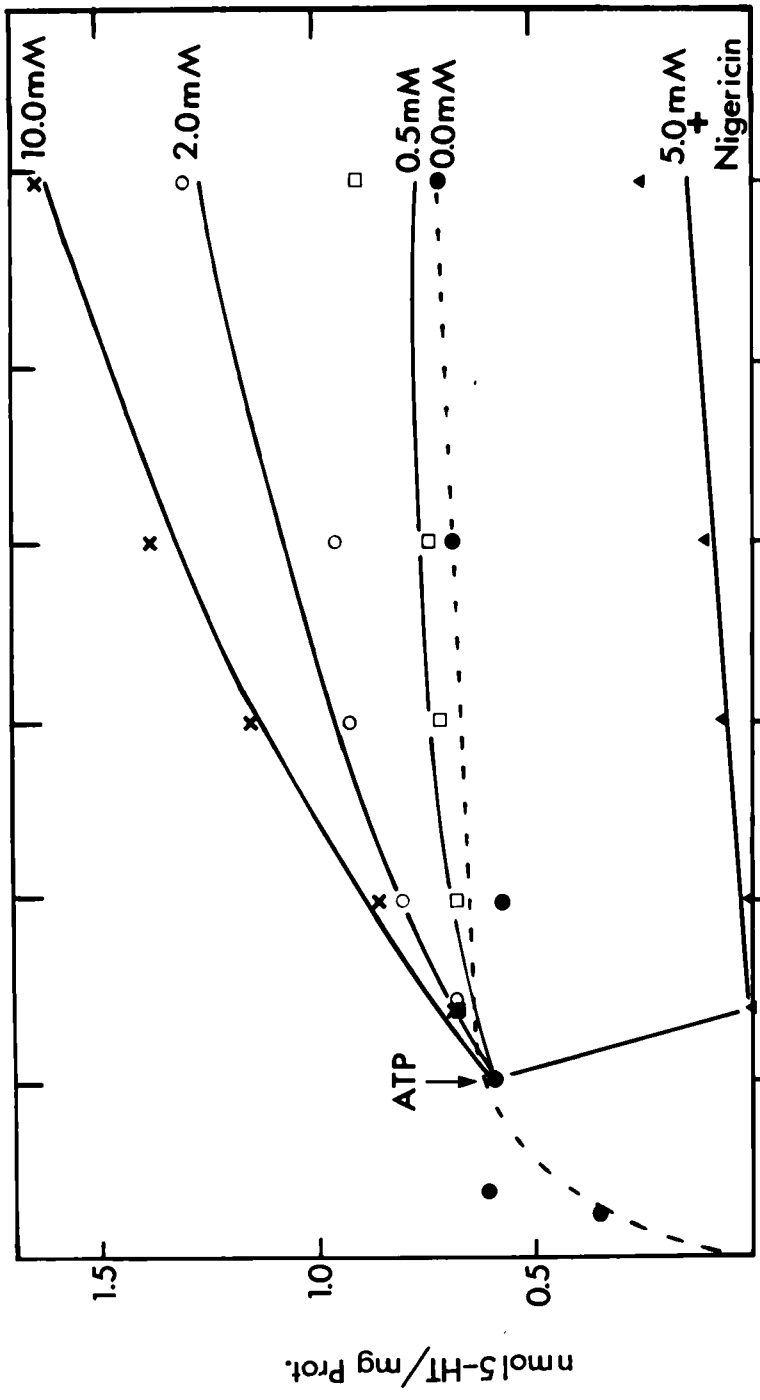
10.0mM
2.0mM
0.5mM
0.0mM
5.0mM
Nigericin
ATP
1.5
1.0
0.5
5
10
15
20
25
30
nmol 5-HT/mg Prot.
Time (min)

Figure 16

nigericin to a final concentration of 1 μg/ml caused almost complete release of accumulated 5-HT in the presence of 5 mM ATP.

Since a pH gradient is generated in the presence of ATP and Mg++ in the ghosts and this ΔpH can apparently supply at lease part of the driving force for 5-HT transport, an artifically imposed ΔpH should also drive increased 5-HT transport in the absence of ATP. The results of such an experiment are shown in Figure 17. Two aliquots of ghosts were incubated separately, each at pH 6.7, and 5-HT transport was assayed at the times indicated as described in "Experimental Procedures." At the time shown (arrow) one assay mixture received 10 μl of 0.5 N NaOH such that its pH was raised to 7.7. The second assay mixture received 10 μl of distilled H_2O and thus remained at pH 6.7. The shift in pH from 6.7 to 7.7 in the first incubation resulted in an immediate increase in 5-HT transport. In contrast, 5-HT transport at a constant pH of 6.7 remained low and reached less than 1/3 of the steady state values attained with the pH jump (Figure 17).

Discussion

It is becoming clear that common mechanisms may exist for the transport of biogenic amines into intracellular storage organelles in cells specialized for biogenic amine storage and release. In particular, it is now well established that a transmembrane ΔpH exists across the membrane of the chromaffin granule (63) and although direct measurements of ΔpH have not yet been performed, the uptake of norepinephrine by synaptic vesicles appears to be greatly reduced by agents which are

Figure 17. Accumulation of 5-hydroxytryptamine in response to an artificially imposed ΔpH.

Ghosts (100 μg protein/ml) were incubated with 5.7 μM [^{14}C]5-HT at 37°C in a buffer containing 0.25 M sucrose, 10 mM HEPES, 10 mM K_2SO_4 and 2.5 mM $MgSO_4$ at pH 6.7. After 5 min of incubation (arrow), 10 μl of 0.5 N NaOH was added such that the pH of the incubation medium was raised to 7.7 (x). Alternatively, 10 μl of deionized H_2O was added (▲). 5-HT transport was assayed as described in "Experimental Procedures."

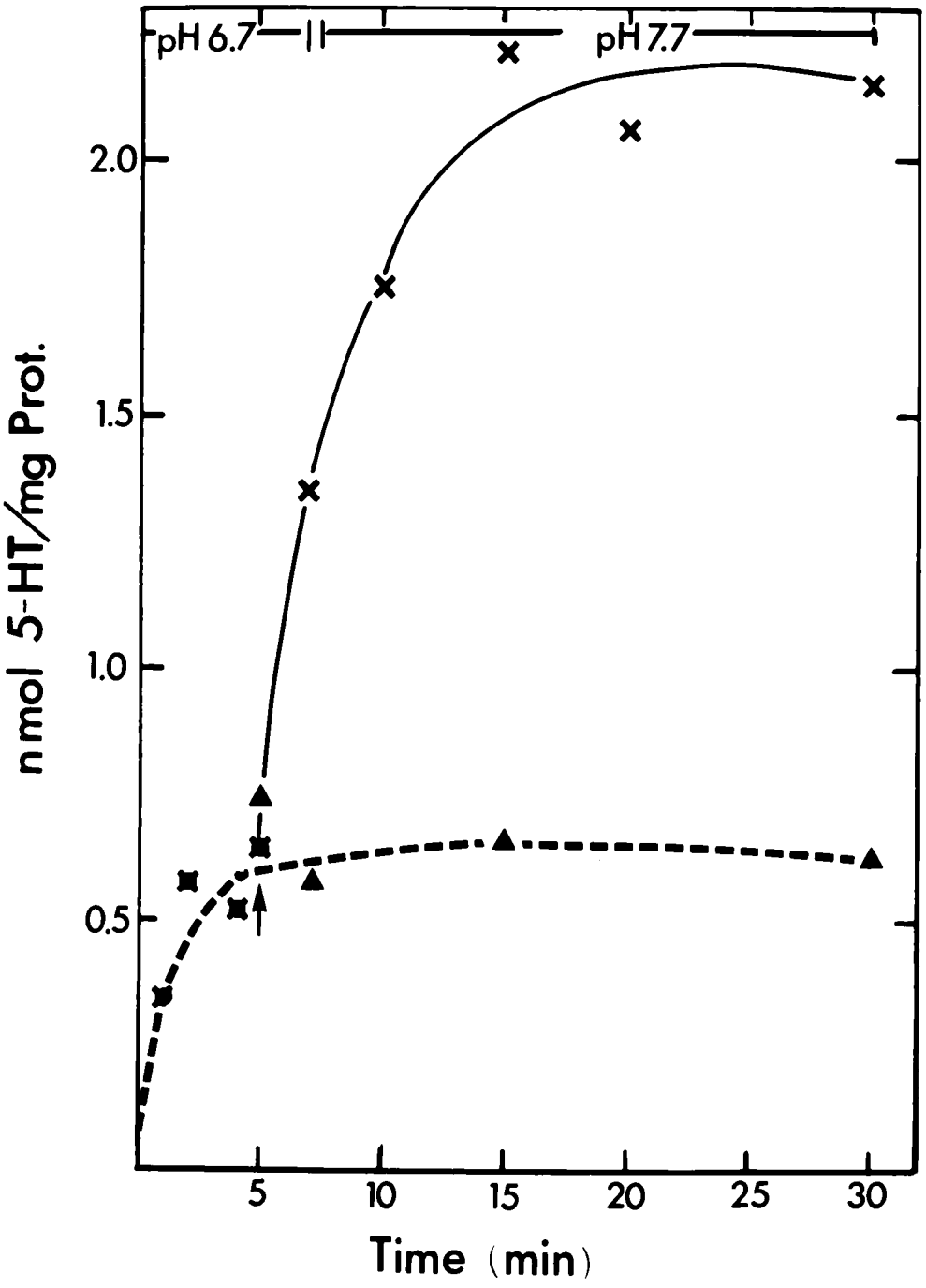

Figure 17

known either to disrupt transmembrane pH gradients by making the membrane permeable to H+ or by inhibiting the function of the membrane bound ATPase, which is thought to act as a proton pump (106). Dense granules of blood platelets have previously been shown to exhibit a transmembrane ΔpH of approximately 1.1 units when isolated from Ficoll gradients (97). In the present study, dense granules isolated from sucrose density gradients have also been shown to exhibit a transmembrane ΔpH (Table IV, page 67). The apparent stability of the dense granule membrane at high sucrose concentrations as shown by a limited permeability to protons is not surprising in view of previous results shown for 5-HT transport in intact dense granules at high medium osmolarities (96). The existence of a transmembrane ΔpH suggests that this may provide at least part of the energy necessary for 5-HT transport. In support of this view, we demonstrate here that the uptake of 5-HT by intact granules is greatly reduced below pH 6.5. Concomitant with the reduced 5-HT transport at low pH values, ΔpH decreases to values near zero at pH 6.0. Increased external pH did not increase 5-HT transport, however, under conditions where an uncoupler was added; thus, ΔpH was destroyed at all external pH values.

Characteristics of ghosts. To study the generation of ΔpH and 5-HT transport by dense granules in more detail, ghosts of dense granules were prepared by hypotonic lysis. Ghost preparations have been useful to clarify solute transport in other systems such as red blood cells (107) and platelets (51, 108). In particular, Phillips

has used ghosts to study transport and energy utilization in chromaffin granules. The advantages in using a ghost preparation are the following: (1) intragranular constituents (e.g., 5-HT) are removed thus greatly reducing the possibility of their interference in transport and other assays, and (2) it is possible to study in a clearer way the generation of ΔpH in the presence of ATP. Ghosts prepared by our method lost 95% of their endogenous 5-HT and preliminary results show that they lost approximately 80% of their ATP. The remaining amine and nucleotide in the ghosts was apprently tightly bound, perhaps in so-called "storage complexes" (40). Attempts to dissociate the storage complexes by addition of EDTA during lysis resulted in a condition under which the ghosts did not reseal.

The ghost preparation transports 5-HT as measured by Millipore filtration assay (Figure 11, page 73). This transport was stimulated maximally by 5 mM ATP (data not shown). 5-HT accumulation occurred in the absence of ATP but reached only 1/3 of the steady state levels found in its presence. This transport was partially inhibited by 20 mM NH_4Cl which has been shown to destroy ΔpH (data not shown). In consequence, the ATP sensitive transport may be due in part to a small ΔpH present in the absence of ATP. This idea is supported by the fact that a small ΔpH is measured in the presence of ATP at 0°C (Table VII, page 89), a condition under which ATPase activity is negligible and thus no ΔpH would be expected. However, small ΔpH values are difficult to interpret as will be discussed below. The possible role of the remaining internal nucleotide in these phenomena cannot be clarified at present. Also explored was the effect of nigericin addition on 5-HT accumulated to

steady state levels. Phillips (73) and Johnson et al. (65) have studied the effect of uncouplers on amine transport in chromaffin granule ghosts and intact chromaffin granules respectively with different results. Phillips showed that the addition of the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) caused release of 5-HT accumulated by ghosts; however, Johnson et al. were unable to demonstrate uncoupler-stimulated release of amines accumulated by intact chromaffin granules. This may indicate that the presence of the intragranular storage complex allows amines accumulated in response to the transmembrane ΔpH to be sequestered in such a way that they are resistant to release by uncouplers. In the present study, nigericin released 5-HT accumulated by dense granule ghosts in response to ATP addition, thus supporting the idea that amine transport and retention in ghosts are dependent on the presence of a transmembrane ΔpH.

Light scattering measurements. Light scattering experiments allow a different insight into the behavior of the ghost preparation. The ghosts are osmotically active over a range of medium osmotic pressures. Swelling in the presence of ATP is apparently due to entry of protons into the ghosts via a proton translocating ATPase. This idea is supported by the fact that agents which inhibit the ATPase (e.g., EDTA) also substantially inhibit ATP-dependent swelling. ATP-dependent swelling was found to take place even in the presence of the proton ionophore CCCP, suggesting that this response may be due in part to nonelectrogenic proton entry. This idea is further supported by the

fact that the establishment of a diffusion potential of K+ (positive inside) induced by adding valinomycin + K+ does not inhibit ATP-induced swelling. These results are similar to those found by Phillips in chromaffin granule ghosts although he found only limited ATP-induced swelling in the absence of added salts. The possible identity of the anion which accompanies proton translocation into dense granule ghosts is discussed below.

Of interest is the nature of the swelling induced by uncouplers in the presence of ATP. It can be seen experimentally that although the ΔpH is collapsed, the ATP is utilized to do osmotic work involved in the swelling. The fact that SITS is shown to inhibit this swelling in a concentration dependent fashion suggests the presence of anion transport sites in the dense granule membrane. SITS has been shown to inhibit anion movements in erythrocytes (109,110). Pollard et al. (111) have postulated that OH^- ion transport leads to osmotic rupture of the platelet dense granule during the platelet release reaction and similar events have been suggested to occur in lysis of parathyroid cell granules (112). The present methodology does not allow the differentiation between OH^- and $SO_4^=$ ions (the only small anions present in the media used) as being responsible for the rapid nigericin-induced swelling. However, transport of OH^- before nigericin addition may be excluded since a pH gradient is formed under these circumstances. The ATP-dependent swelling is not sensitive to SITS addition. The above findings raise the possibility that two components are involved in anion transport: one which operates during proton translocation and is insensitive to anion transport blockers (possibly a nonmediated

diffusion process); and another which is "unmasked" by the addition of uncouplers and is sensitive to anion transport blockers. Recently, Pazoles et al. (113) have provided evidence that the chromaffin granule membrane contains transport sites for Cl^-. As stated above, based on the present results, it seems likely that the dense granule membrane also contains specific anion transport sites. Further characterization of these sites merits consideration.

ATPase activity. The ATPase activity which is presumably responsible for H+ movements into the ghosts was assayed and its activity was found to be correlated with increased 5-HT transport by the ghosts. Thus, agents which inhibited the ATPase activity (DCCD, EDTA, N-ethylmaleimide), also inhibited stimulation of 5-HT transport by ATP. In addition, measurements of ΔpH showed that pH gradients were reduced substantially when ghosts were incubated at 0°C with ATP. ATPase activity was shown to be quite low under the latter conditions. This evidence, again, lends strong support to the idea that the ATPase of the dense granule acts as a proton pump and stimulated 5-HT transport via generation of a transmembrane ΔpH. Heinrich et al. (39) have also found a Mg++-dependent ATPase activity in rabbit platelet dense granules. It is likely that this ATPase has a similar function to the one described here.

An initial burst in ATPase activity was found which was apparently due to rapid hydrolysis of ATP during the first minute of the assay. In order to substantiate the results obtained by measuring inorganic phosphate production using the Malachite green assay, ATPase activity

has also been monitored by following the production of ADP without a need for sampling. The coupled assay system described by Schwartz et al. was used (114). The oxidation of NADH was followed fluorometrically at 450 nm. A burst in ADP production similar to that of inorganic phosphate was seen using this assay (Wilkins and Salganicoff, unpublished results). Such a rapid burst in activity has been noted for other hydrolytic enzymes (115); however, an initial burst of activity is not seen in the chromaffin granule ATPase (e.g., 116). This rapid ATP hydrolysis may be correlated with the rapid initial rate of swelling seen upon addition of ATP to ghosts and also with the apparently rapid formation of the transmembrane ΔpH. This question is currently under investigation.

pH gradients in ghosts. In order to determine the magnitude of the pH gradients generated in the presence of ATP, direct measurements were made using [^{14}C]methylamine as had been performed on intact granules. We found it somewhat more difficult to measure ΔpH in ghosts due to the fact that a slightly greater amount of methylamine binding occurred as opposed to binding in intact granules (compare ΔpH values with nigericin added, Tables IV and VII, pages 67 and 89). The reason for this difference is unclear at present; however, it may relate to the slightly different methods of preparation used. Thus, under conditions where ΔpH would be expected to be completely disrupted (e.g., in the presence of nigericin and K+) a small ΔpH was found. Since the relationship between ΔpH and the H+ concentration gradient is a

logarithmic one, ΔpH values at low H+ concentration gradients are magnified and introduce a small error into all measurements. This problem has been considered previously (70). An attempt to minimize methylamine binding was made by addition of salts (i.e., 20 mM K_2SO_4 or 20 mM potassium phosphate). However, a small methylamine space was always found in the presence of nigericin or NH_4+ ions. A somewhat larger ΔpH was measured at 0°C (Experiment B, Table VII, page 89) and this probably represents a real ΔpH as discussed earlier. In 0.25 M sucrose in the presence of 5 mM ATP and 2.5 mM $MgSO_4$, ghosts rapidly generate a ΔpH of 1.1-1.3 units (at an external pH of 7.0) which declines somewhat upon further incubation. This ΔpH was generated within 2 min at 37°C which is the earliest time point one may measure with confidence using the present methodology. Variation of the methylamine concentration between 4 and 12 μM had virtually no effect on the ΔpH measured, thus ruling out a significant contribution of binding to these results.

Stimulation of 5-HT transport by ATP and artificially imposed ΔpH. In support of the idea that a transmembrane ΔpH can drive 5-HT transport, ATP addition caused a significant stimulation of amine transport into ghosts. This stimulation was reversed by the addition of nigericin (Figure 16, page 91) or NH_4+. These two agents have been shown by us and others to dissipate ΔpH in both intact granules and granule ghosts. Accumulated 5-HT was also released by the addition of nigericin. This finding provides strong evidence that 5-HT transport in platelet dense

granules is intimately related to the presence of a transmembrane ΔpH. Another line of evidence in support of this hypothesis is the experiment in which an aritficial ΔpH was imposed across the ghost membrane. Again, 5-HT transport may be enhanced by the establishment of a gradient of protons (acid inside), this time in the absence of ATP. The latter result supports the idea that ATP hydrolysis drives an inward flux of protons such that the interior of the ghosts becomes more acidic, thus leading to increased 5-HT transport. It also argues against any direct involvement of ATP in 5-HT transport per se (i.e., direct coupling of ATP hydrolysis to 5-HT transport, etc.).

In summary, the present work has examined the role of the transmembrane ΔpH in amine accumulation by the subcellular storage organelles for 5-HT in the blood platelet, the dense granules. These findings support the idea that 5-HT transport into platelet dense granules is driven by a gradient of protons across the membrane. This ΔpH probably generated in vivo by an inwardly directed proton translocating ATPase in the granule membrane and the results presented here strongly suggest its participation in the formation of ΔpH and thus in 5-HT transport. The present results also correlate well with earlier findings in chromaffin granules (65) and synaptic vesicles (106) and, as stated earlier, support the idea that common mechanisms for biogenic amine transport into subcellular storage organelles exist.

CHAPTER IV

GENERAL DISCUSSION

The present work has examined the transport of 5-hydroxytryptamine into platelet dense granules from several standpoints. Chapter II was an investigation of the characteristics of 5-HT transport into intact dense granules with regard to kinetic and structural aspects as well as its behavior under different osmotic conditions. In Chapter III, the energy requirements for 5-HT transport were examined in both intact granules and in ghosts prepared from dense granules. Also investigated in Chapter III were ionic movements in ghosts and the function of the membrane bound ATPase. In this section, it is intended to give a general overview of the present work and to suggest areas for further study. For a more detailed discussion of the results, the reader is referred to the discussion sections in Chapter II (page 25) and in Chapter III (page 55).

The isolation of a homogenous population of dense granules from blood platelets allows the study of 5-hydroxytryptamine transport at the subcellular level. As discussed in Chapter II (page 25), platelet dense granules offer an advantage for the study of 5-HT transport in that they may be isolated free from other biogenic amine-containing organelles. This is not true at present of synaptic vesicles from brain. Therefore, the dense granule serves as a good model for the

study of 5-HT transport and storage in a storage organelle specialized for this purpose.

The results presented here show that 5-HT transport may be studied using a very sensitive radiochemical assay employing the Millipore filtration technique. This technique has been used extensively to measure transport of solutes in other systems such as bacterial membrane vesicles (117) and renal brush border membrane vesicles (118) and also in chromaffin granule ghosts (62). Transport of 5-HT into intact granules in the absence of ATP (Figure 4, page 36) and into ghosts in the presence of ATP (Figure 11, page 73) exhibit similar behavior when studied as a function of time, suggesting that the ghosts prepared by hypotonic lysis are functional membrane structures derived from dense granules. The effects of external pH and of nigericin on 5-HT transport in both intact granules and ghosts support the above conclusion. A study of the kinetics of 5-HT transport into granules (page 38) showed that two mechanisms for 5-HT transport operate: (a) a carrier-linked transport and (b) a passive diffusion. The relatively low K_m for the carrier mediated transport (3.3 μM) suggests that this mechanism may be important <u>in vivo</u> for the accumulation of 5-HT by dense granules. In other words, 5-HT should be rapidly accumulated by the dense granules once it is transported inside the circulating platelet. Thus, the platelet possesses a very efficient mechanism for the storage of 5-HT.

The osmotic behavior of intact granules was examined with respect to 5-HT transport and retention of endogenous 5-HT in Chapter II

(page 25). The intact granules exhibit an extremely high sensitivity to low osmotic pressure in the medium (i.e., to osmotic pressures below 300mOsM) in both of the above aspects. This sensitivity to low osmotic pressure was exploited in order to prepare "ghosts" of dense granules which, as stated above, transport 5-HT efficiently in the presence of ATP and Mg++ (Chapter III, page 73). It is of particular interest that ghosts prepared from dense granules are able to transport 5-HT as efficiently below 300mOsM as above in the presence of ATP (data not shown). This finding suggests that the osmotic behavior of ghosts (as prepared in Chapter III) is different from that of intact granules which transport 5-HT much less efficiently below 300mOsM than above (Figure 8b, page 46). This differential sensitivity to osmotic pressure implies that the "storage complex" in intact granules may play a role in osmotic regulation. This is an area which will require further study for clarification.

Another area which was studied using intact granules was the effect of inhibitors on 5-HT transport. The transport of 5-HT by dense granules is inhibited by several other biogenic amines as well as the 5-HT metabolite, 5-hydroxyindoleacetic acid (Table III, page 50). The lack of structural specificity implied by this inhibitor study suggests that the plasma membrane of the platelet may act as a much more selective permeability barrier than the membrane of the dense granule. As pointed out in Chapter I (page 19), the chromaffin granule transports biogenic amines other than the ones which are found endogenously. It is possible that the inhibitors tested in Chapter II are also transported

by the dense granule amine carrier and thus compete with 5-HT at this level.

Following initial characterization of dense granule 5-HT transport, it was important to determine the source of energy for 5-HT transport in these organelles. The finding that intact granules exhibited increased 5-HT transport with increasing external pH (page 65) supported the idea forwarded by Johnson and Scarpa (97) that a transmembrane ΔpH (acid inside) could drive the transport. In the present study, a transmembrane ΔpH was found in intact granules and it was found that increasing external pH increased ΔpH (Figure 9, page 65). The role of ΔpH in amine accumulation by other organelles such as chromaffin granules and synaptic vesicles appears to be the same as that in dense granules, namely as an energy source for this process.

The advantages in using a ghost preparation to study 5-HT transport have been pointed out in Chapter III (page 55). The ghost preparation studied here transports 5-HT in response to a transmembrane ΔpH which may be imposed artifically or by incubation of the ghosts with ATP and Mg++. Inwardly-directed proton translocation in response to ATP addition during the establishment of ΔpH is accompanied by swelling of the ghosts. This swelling is insensitive to addition of an uncoupler such as CCCP, which allows electrogenic proton movements across membranes. ATP-induced swelling is also insensitive under conditions where a diffusion potential is imposed across the membrane (positive inside the ghosts) by the addition of valinomycin and K+. As stated earlier, both of these conditions would be expected to inhibit

electrogenic H+ movements and thus to inhibit ATP-induced swelling if proton movement were electrogenic. Since neither is inhibitory to ATP-induced swelling, it may be concluded that H+ movement driven by ATP is at least partially nonelectrogenic (see Chapter III, page 55). Quantitation of the electrical potential ($\Delta\psi$) formed during proton translocation must await further study. As pointed out in Chapter I (page 20), results obtained by Phillips (70) indicate that proton translocation in chromaffin granule ghosts is largely nonelectrogenic.

The results presented here fit well within the theoretical framework of the model proposed by Mitchell (119) for energy-dependent transport of solutes across biological membranes. As proposed by the latter author, transport of solutes against their concentration gradient may be driven by an electrochemical gradient of protons across the membrane ($\Delta\mu H+$). This gradient may consist of two components: (a) ΔpH, a transmembrane gradient of protons and (b) $\Delta\psi$, a gradient of electrical potential across the same membrane. Transport of many solutes across biological membranes such as the bacterial membrane (120) or the mitochondrial inner membrane (121) has been shown to be linked to the presence of an electrochemical gradient of protons. Thus Ramos et al. (122) have shown that bacterial membrane vesicles form a transmembrane ΔpH and a transmembrane $\Delta\psi$ in response to addition of appropriate substrates such as ascorbate plus phenazine methosulfate. The same authors have also shown that the transport of solutes such as lactose into bacterial membrane vesicles is dependent upon the presence of a transmembrane electrochemical gradient of protons ($\Delta\mu H+$) (123).

Manipulation of ΔpH or $\Delta\psi$ using appropriate ionophores was shown by the latter authors to affect the transport of solutes in a predictable way. These authors were able to show that the transport of some solutes was dependent upon ΔpH and that of others was dependent upon $\Delta\psi$. Thus the presence of $\Delta\mu H^+$ across biological membranes may drive solute transport via utilization of ΔpH or $\Delta\psi$ in this system. Further study in other systems will undoubtedly show similarities to the bacterial membrane system. Thus experimental evidence from many lines of work using both prokaryotic and eukaryotic models supports the idea of Mitchell that such a transmembrane $\Delta\mu H^+$ may serve as an energy source for solute transport.

Based on the present results, it is possible to propose alternative models for 5-HT transport into the dense granule (Figure 18). (Mechanism D here is similar in some respects to that presented by Johnson et al. (65) for amine transport by chromaffin granules.) The dense granule is pictured on the left of the figure (A). The hydrolysis of ATP by the membrane-bound ATPase leads to a concomitant transport of protons inside the granule. At the same time, anions (A^-) enter via an as yet unknown mechanism. Proton translocation leads to the formation of a transmembrane ΔpH (B) such that the concentration of protons is greater inside the granule than the outside. Following the formation of the pH gradient, 5-HT transport may occur. Two possible mechanisms for this transport are given here. In C, the charged form of 5-HT (5-HT+) is seen crossing the dense granule membrane in exchange for a proton (H^+). Once inside the granule, free 5-HT+ would be in equilibrium with

Figure 18. Proposed mechanisms for 5-HT transport by dense granules.

See text (page 109) for details.

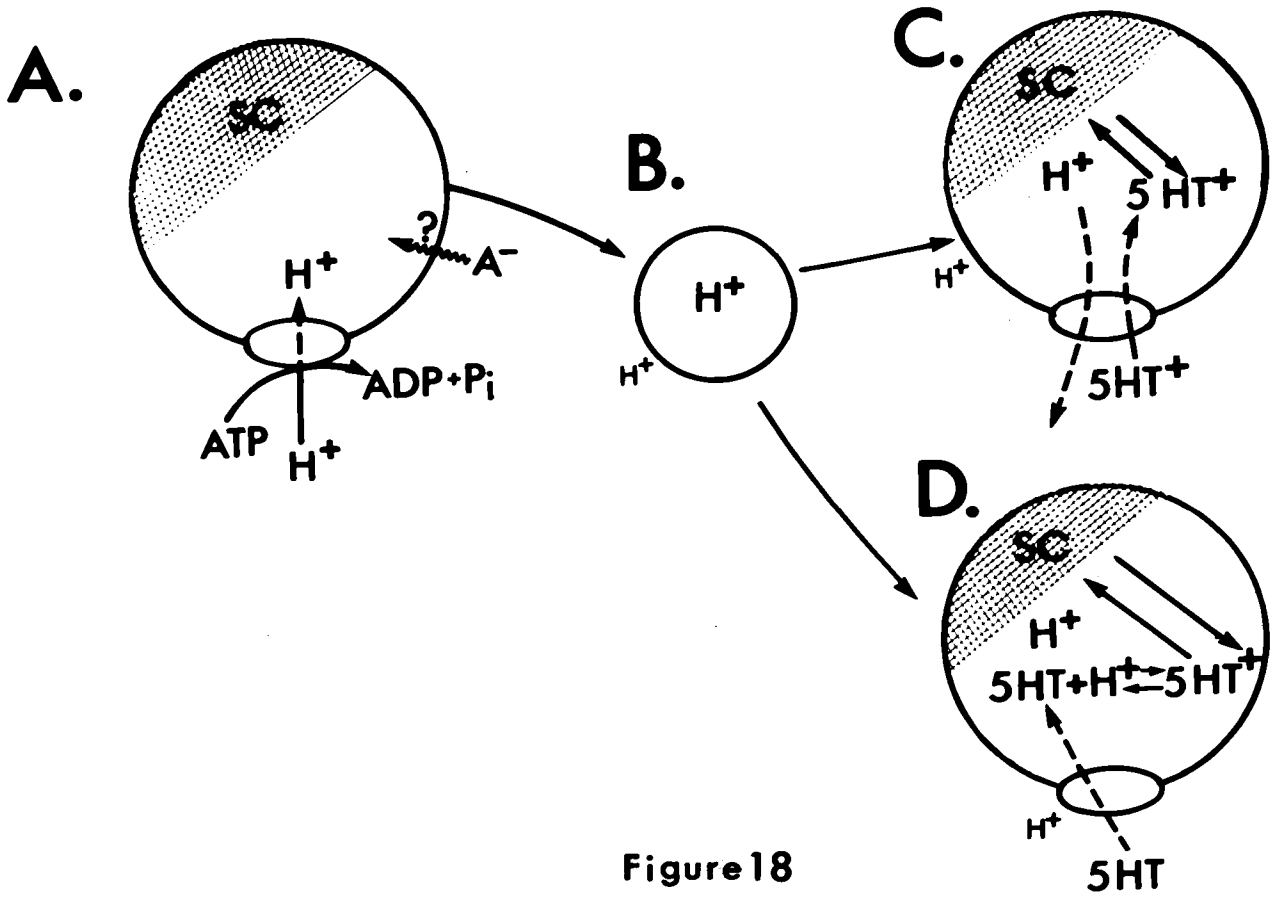

Figure 18

the "bound" form, i.e., bound to the storage complex (SC). Mechanism D shows 5-HT entering the dense granule in the uncharged form (present in very low concentrations at neutral pH). Once inside the granule, protonation of the amine is favored in the acidic environment. The charged form of the amine (5-HT+) crosses the membrane much less readily than its uncharged form. Mechanism D is strictly analogous to the entrance of methylamine in response to ΔpH, although entrance of methylamine does not require carrier-mediation. Once inside the granule, 5-HT+ is again free to bind to the storage complex (SC) (Mechanism D). The results presented here do not distinguish between Mechanisms C and D; however, the fact that 5-HT transport is coupled to ΔpH is unquestionable. Further study is therefore needed to clarify the exact mechanism whereby the pH gradient supplies energy for 5-HT transport in the dense granule. Due to the many similarities between the dense granule, the chromaffin granule and the synaptic vesicle, elucidation of the exact mechanism for amine accumulation in one of these organelles will undoubtedly lend insight into the behavior of the others.

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