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Phosphorus-31 Nuclear Magnetic Resonance Studies of Active Proton Translocation in Chromaffin Granules†

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ABSTRACT: ATP hydrolysis and proton translocation in chromaffin granules were followed using ^{31}P nuclear magnetic resonance. The intragranular pH affects the resonance frequency of the γ-phosphate of granular ATP. By measuring frequency vs. pH in solutions which simulate the intragranular matrix, this may be calibrated to give quantitative pH measurements. The pH in the resting granule is 5.65 ± 0.15. This drops by 0.4 to 0.5 pH unit when ATP is added externally and protons are actively pumped into the granules. Because of differences in the composition and pH of the internal and external solutions, the resonances of internal and external nucleotides and P_i can be distinguished. Consequently, ATP hydrolysis and changes in internal pH may be observed simultaneously and continuously in a single sample of chromaffin granules. From the measured buffering capacity of a reconstituted intragranular solution, pH changes were converted into an absolute number of protons translocated. The net proton flux (protons translocated/ATP hydrolyzed) was about 1.0 immediately after external ATP addition but fell toward zero as the pH gradient increased to a new steady state. These ^{31}P NMR results agree with intragranular pH measurements determined from methylamine distribution and with H$^+$/ATP stoichiometries calculated from pH changes observed in the external medium.

Phosphorus nuclear magnetic resonance has proved to be a powerful method for studying metabolism in working systems, because pH and concentrations of such important compounds as ATP, ADP, AMP, and P_i can be monitored continuously in a single sample. ^{31}P NMR[1] has been exploited to follow pH and metabolite levels in perfused and ischaemic tissues such as muscle (Dawson et al., 1977), heart (Garlick et al., 1977), and kidney (Sehr et al., 1977). At the cellular level, intracellular pH has been measured in respiring and anaerobic cells of *Escherichia coli* (Navon et al., 1977). At the level of the organelle, ^{31}P NMR is a potentially valuable technique for bioenergeticists investigating the role of pH gradients in energy coupling. ^{31}P NMR can simultaneously monitor concentrations of phosphate compounds and the internal pH in organelles which contain or can be loaded with a high concentration of an appropriate phosphate compound.

Chromaffin granules, the catecholamine storage vesicles of the adrenal medulla (for a review, see Winkler, 1976), contain high concentrations of ATP (0.1 M), adrenaline (0.55 M), and chromogranin protein (120 mg/mL). The ATP, while its function in vivo is unknown, provides a convenient indicator of internal pH. The pK_a of the γ-phosphate is close to the intragranular pH, and the resonance frequency shifts markedly with the degree of protonation. Moreover, the granules possess an electrogenic, inwardly directed, proton-translocating ATPase, which hydrolyzes extragranular but not intragranular ATP. We have shown that this proton pump can change the intragranular pH and shift the resonance frequency of the γ-phosphate of intragranular ATP (Casey et al., 1977; Njus and Radda, 1978). We report here the use of ^{31}P NMR to follow simultaneously proton translocation and ATP hydrolysis in chromaffin granules.

Materials and Methods

Chromaffin granules were isolated from bovine adrenal

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[1] Abbreviations used are: ATPase, adenosine triphosphatase; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; ppm, parts per million.

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TABLE I: Net Proton Flux per ATP Hydrolyzed.[a]

time interval (min after ATP addit)	H^+ translocated/ATP hydrolyzed	
	NMR method	external pH method
0–4	1.03	1.14 ± 0.11
4–11	0.73	0.59 ± 0.13
11–19	0.65	0.07 ± 0.14
19–25	0.34	−0.18 ± 0.13

[a] For each time interval, the number of protons translocated was determined and divided by ATP hydrolyzed. Ratios derived from extragranular pH changes (see Materials and Methods) are averages of two experiments. Ratios determined by NMR (see Results) were calculated from pH changes and ATP hydrolysis occurring between successive points in Figure 4.

medullas as described by Kirshner (1962), except that 0.3 M sucrose, 10 mM Hepes (pH 7.0) was used as the isolation medium. Granules were sedimented and washed three times and used within 14 h of the cattle being slaughtered. The reconstituted intragranular solution was obtained from chromaffin granules prepared from 50 adrenal glands. The final granule pellet was immediately dispersed in 200 mL of distilled water, and membranes of the lysed granules were removed by centrifugation for 20 min at 39 000*g* and 4 °C. Centrifugation was repeated to make sure that the supernatant was free of membranes. After lyophilization for 24 h, the resulting product (815 mg) was stored at −20 °C. An aliquot of the final supernatant was assayed for protein and adrenaline to determine the original intragranular volume. Assuming intragranular concentrations of 550 mM adrenaline and 120 mg of protein/mL, the adrenaline and protein assays gave internal volumes of 2.50 and 2.80 mL, respectively. Sorbitol-excluding volume, determined on an aliquot of the starting granules, was 2.09 mL. The internal volume was taken as 2.50 mL/815 mg of residue. Portions of the residue were weighed and reconstituted with the appropriate amount of water shortly before use.

^{31}P NMR spectra of defined ATP solutions (Figure 1) were recorded at 20 °C on a Bruker WH-90 spectrometer (2.1T) using proton decoupling and an external D_2O lock for field stabilization. Chromogranin proteins were prepared by lysing granules and removing membranes as above. They were then purified by dialysis. Sodium-free ATP was prepared by passing Na_2ATP through a Dowex Type 50HW ion-exchange column. The effluent was collected and lyophilized.

All other ^{31}P NMR spectra were recorded at 129.2 MHz on a spectrometer built in Oxford (Hoult and Richards, 1975). The spectrometer, equipped with temperature control (±0.5 °C) and field stabilization via a deuterium lock, was operated in the Fourier transform mode employing 70° pulses at 1-s repetition time. A capillary containing D_2O and methyltriethylphosphonium iodide was inserted into the sample tube to provide the field lock and a reference signal. To permit comparison with other data, chemical shifts are expressed as parts per million with respect to 88% phosphoric acid, which has a resonance frequency 37.79 ppm upfrequency from that of methyltriethylphosphonium iodide.

The reconstituted intragranular solution had a pH of 5.77. To obtain the frequency vs. pH curve (Figure 2), 2 N HCl was added in steps to 1.15 mL of solution. The pH was measured immediately after HCl addition, three spectra were recorded, and the pH was measured again. When the titration reached pH 4.4, the pH was adjusted to 5.7 with 10 N NaOH and the sample was titrated to more alkaline pH with 2 N NaOH. Spectra were recorded at 20 °C. Free-induction decays were accumulated from 130 transients.

To follow intragranular pH changes and extragranular ATP hydrolysis (Figure 4), granules were suspended in 10 mM Hepes, 0.3 M sucrose (pH 7.0); 0.3 mL of the granule suspension (27 mg of protein) was mixed with 0.9 mL of 160 mM KCl, 150 mM sucrose, 53 mM Mes (pH 6.40). Sorbitol-excluding volume (intragranular volume) of 1 mL of this suspension was 70 μL. Four micrograms of oligomycin, which does not affect granular ATPase activity (Bashford et al., 1976), was added to inhibit any contaminating mitochondrial ATPase activity. At $t = 0$, 130 μL of 100 mM ATP, 100 mM $MgSO_4$ (pH 7) was added. Spectra were recorded periodically at 25 °C. After 120 min, the sample was removed and the pH measured as 6.35. For each spectrum, 256 free-induction decays were accumulated (total accumulation time = 4.27 min). In each spectrum, the position of the γ-phosphate signal of the intragranular ATP was noted and used to determine the internal pH. The integrals of the various extragranular phosphate signals were compared to the integral of the reference signal (methyltriethylphosphonium iodide) to determine concentrations. The phosphate signal from the reference capillary was calibrated by taking the intensity of the ATP β-phosphate signal observed immediately after ATP addition as 9.8 mM.

H^+/ATP ratios were determined from pH changes in the medium (Table I) by a modification of the method of Thayer and Hinkle (1973). Granules (30–35 mg of protein) in 2.5 mL of 1 mM Mes, 350 mM sucrose (pH 6.15) were mixed with 2.5 mL of 1 mM Mes, 150 mM sucrose, 240 mM KCl (pH 6.15) and 50 μL of 0.4 mg/mL oligomycin, and the pH of the mixture was adjusted to 6.15. A 4.0-mL aliquot was added to the electrode chamber thermostated at 25 °C. pH was recorded using an EIL Vibron electrometer. After the pH had stabilized, 100 μL of 100 mM ATP, 50 mM $MgSO_4$ (pH 6.15) was added. The pH was recorded and 100-μL samples were periodically withdrawn and assayed for inorganic phosphate. pH changes caused by known quantities of HCl were used to convert the ATP-dependent pH changes into numbers of protons translocated. The rate of ATP hydrolysis was assumed to be equal to the rate of P_i generation.

Phosphate was assayed by adding a sample in 0.5 mL of 5% trichloroacetic acid to 0.5 mL of phosphate reagent (4 g of $FeSO_4 \cdot 7H_2O$ and 1 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 100 mL of 1.26 N H_2SO_4). After 10 min at 20 °C, absorbance at 700 nm was measured.

Protein was assayed using Biuret reagent as described (Casey et al., 1976), and adrenaline was assayed by the method of Anton and Sayre (1962). The determination of sorbitol-excluding volume is given by Casey et al. (1977). pH measurements (except those used to determine H^+/ATP ratios) were made with a Findip digital pH meter equipped with an Activion glass electrode. [^{14}C]Sorbitol and ^3H_2O were obtained from the Radiochemical Centre, Amersham, Bucks. Na_2ATP, oligomycin, adrenaline, and noradrenaline were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey.

Results

When the terminal phosphate group of ATP is titrated through its pK, its ^{31}P NMR resonance shifts by about 5 ppm (Cohn and Hughes, 1960), making it a sensitive pH indicator. ATP contained in chromaffin granules is clearly observable by ^{31}P NMR, and the resonance frequency of the γ-phosphate of intragranular ATP shifts with intragranular pH changes caused by proton pumping (Casey et al., 1977). To quantify intragranular pH measurements, it is necessary to establish

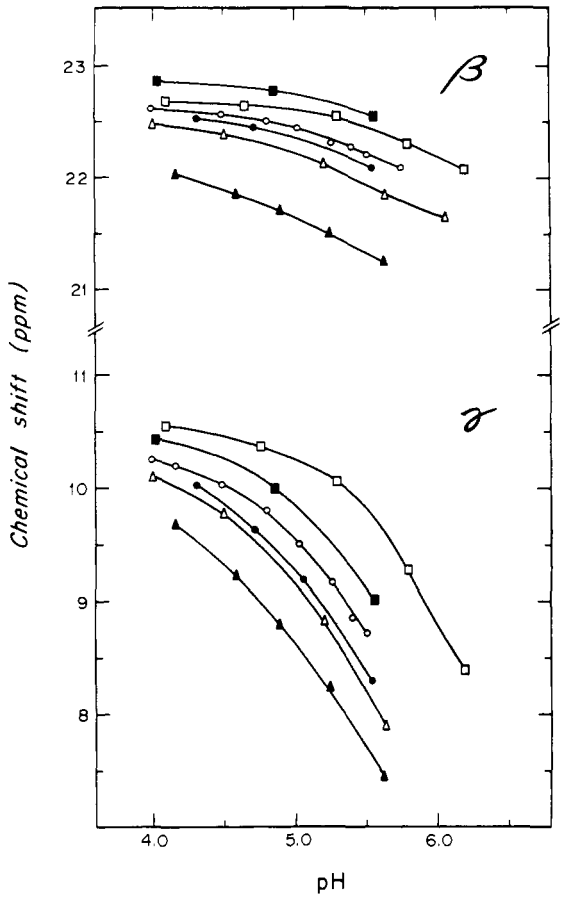

FIGURE 1: pH dependencies of the frequencies of the β- and γ-phosphate resonances of ATP in the presence of various chromaffin granule components. Curves represent the following mixtures: 122 mM Na_2ATP (□); 122 mM sodium-free ATP, 550 mM adrenaline (■); 122 mM Na_2ATP, 550 mM adrenaline (○), 122 mM Na_2ATP, 550 mM adrenaline, 180 mg/mL chromogranin protein (●); 122 mM Na_2ATP, 550 mM noradrenaline (△); 122 mM Na_2ATP, 550 mM adrenaline, 20 mM $CaCl_2$, 8 mM $MgCl_2$, 180 mg/mL chromogranin protein (▲). Frequencies are expressed as chemical shifts in parts per million with respect to external 88% phosphoric acid.

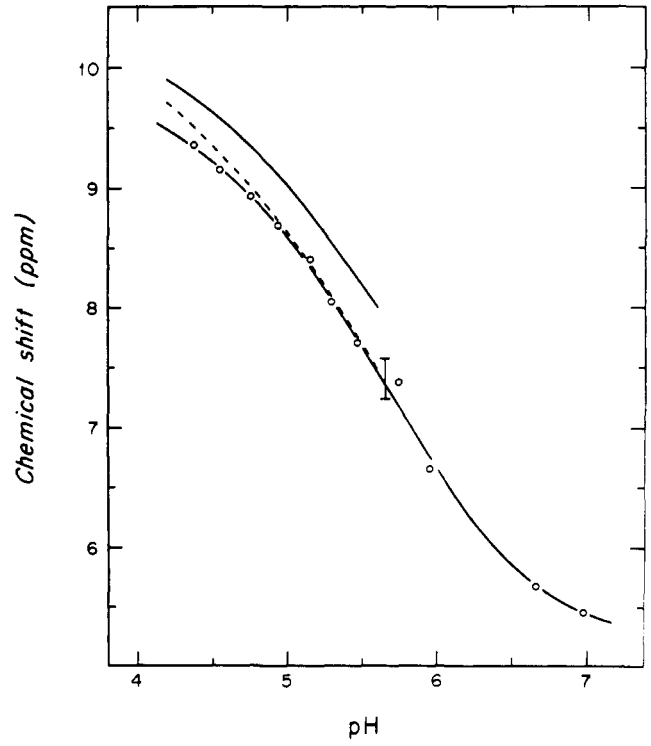

FIGURE 2: pH dependence of the resonance frequency of the γ-phosphate of ATP in the chromaffin granule matrix. Curves for adrenaline granules (—) and noradrenaline granules (- - -) were calculated from the curves for defined solutions shown in Figure 1. Points (○) were obtained by titrating the reconstituted solution. The error bar shows the variation in the frequency of the γ-phosphate signal observed in 15 granule preparations. Frequencies are expressed as chemical shifts in parts per million with respect to 88% phosphoric acid.

a calibration curve giving resonance frequency vs. pH. However, because the ^{31}P NMR resonance positions of the ATP phosphates are greatly influenced by other molecules and metal ions present in the granular matrix, the calibration curve must be obtained in a solution matching the intragranular solution as closely as possible. We have studied both defined solutions made up to match the granule composition and a reconstituted solution extracted from freshly prepared granules.

Studies on Defined Solutions. In Figure 1, ATP resonance frequency vs. pH is shown in the presence of other granular components. In these defined solutions, the effects of individual granule components on the ATP resonances can be seen. However, in solutions containing high concentrations of catecholamines, precipitation occurs above pH 6, limiting the range of the titrations. Adrenaline and noradrenaline both cause upfrequency shifts but that caused by noradrenaline is larger. The adrenaline-dependent shift increases with concentration up to 550 mM, but catecholamine in excess of 550 mM does not cause a further shift in the ATP resonances (Ritchie, 1975). Large amounts of monovalent ions are introduced by using the commercially available disodium salt of ATP. However, while the shifts produced by salts in the absence of adrenaline are fairly large (1 M KCl = 1.3 ppm), the shifts in the presence of 550 mM adrenaline are much smaller (1 M KCl = 0.6 ppm) (Ritchie, 1975). To correct for this salt effect, some titrations were done with sodium-free ATP. As noted by others (Cohn and Hughes, 1962), divalent metal ions cause large upfrequency shifts in the resonance frequencies of the ATP β- and γ-phosphates. The acidic proteins, chromogranins, cause only small shifts in the ATP resonances.

Final calibration curves are shown for solutions containing either 550 mM adrenaline or 550 mM noradrenaline and 122 mM ATP, 120 mg/mL chromogranins, 20 mM $CaCl_2$, and 8 mM $MgCl_2$ (Figure 2). These solutions mimic the compositions of the two major granule populations in the adrenal medulla, the adrenaline- and the noradrenaline-containing granules (Winkler, 1976). There is some disagreement over the precise metal-ion content of the granules; we have used the original values determined by Borowitz et al. (1965).

Studies on Reconstituted Intragranular Solution. Because of the uncertainty about the exact composition of the internal matrix, particularly about the concentration of divalent cations, we also generated an ATP resonance vs. pH curve with a reconstituted intragranular solution. Granules were lysed in distilled water, the membranes were removed by centrifugation, and the lysate was concentrated by lyophilization as described under Materials and Methods. Little degradation of ATP occurs during this preparation, and the ^{31}P NMR spectrum of the reconstituted solution is virtually identical to a spectrum of intact granules.

This solution has a high concentration of sucrose (about 0.3 M) and some Hepes buffer (about 10 mM) introduced from buffer trapped in the granule pellet. However, control experiments show that neither affects the frequency of the ATP signals. This reconstituted solution does not precipitate above

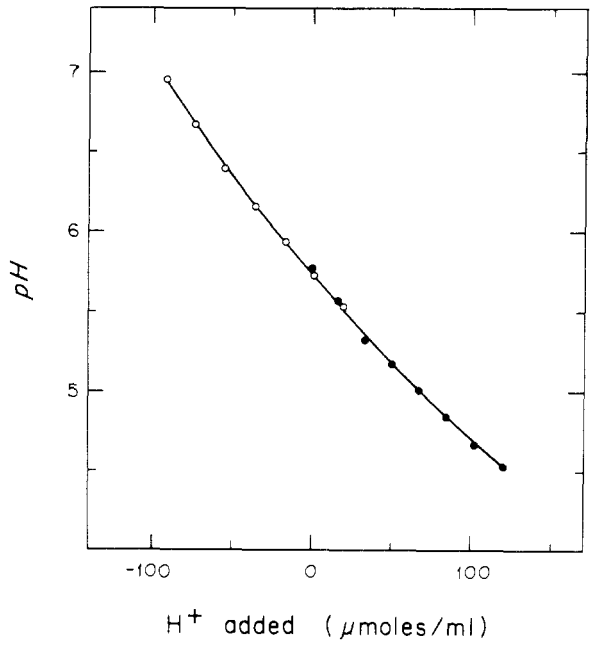

FIGURE 3: Titration of the reconstituted intragranular solution. 1.15 mL of reconstituted solution was titrated to pH 4.5 with 2 N HCl (●), adjusted to pH 5.5 with 10 N NaOH, and then titrated to pH 7 with 2 N NaOH (○). The amount of acid or base added is normalized to 1 mL of the original solution.

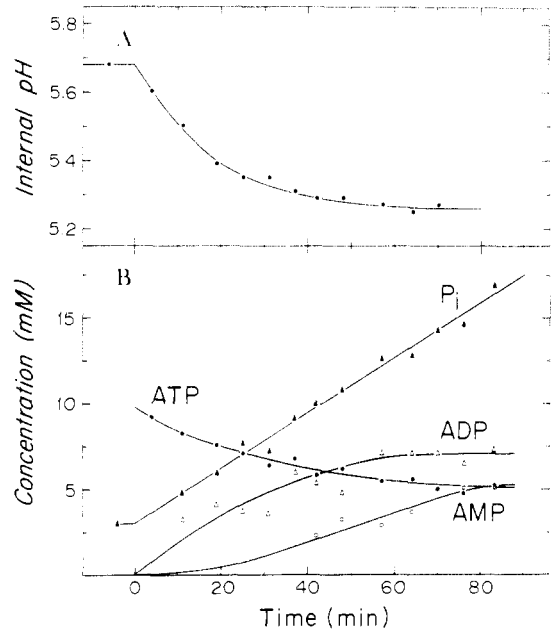

FIGURE 4: Changes in intragranular pH and concentrations of extragranular phosphate compounds after ATP addition. ^{31}P NMR spectra were taken at intervals after the addition of 10 mM MgATP. From each spectrum, the following information was calculated: (A) Intragranular pH was determined from the resonance frequency of the internal ATP γ-phosphate signal. (B) Concentrations of extragranular phosphorus nuclei were determined from integrals of the signals: ATP β-phosphate (●), ADP β-phosphate (△), AMP (○), P_i (▲). Points are plotted at the middle of the accumulation period.

pH 6, and a complete titration curve could be obtained (Figure 2). While many minor constituents (ascorbate, dopamine, ADP, AMP, and various trace metals) have been omitted from the defined solutions and additional sucrose is present in the reconstituted solution, it is evident that the curves obtained from these solutions agree quite closely. From the ATP resonance frequency, pH may be determined within 0.15 unit. Moreover, because the curves all have identical slopes in the pH range 5.0–5.5, pH changes can be determined precisely (±0.02 unit).

A titration of the reconstituted intragranular solution (Figure 3) gives a measure of the buffering capacity of the internal solution. From this curve, we may estimate the number of protons which must be translocated to cause a given pH change in a given volume of intragranular space. Buffering capacity of the membrane was found to be about 12% of the buffering capacity of the granule interior in the pH range 5.0–5.5. Consequently, buffering contributed by the inner surface of the membrane was neglected.

Studies on Intact Chromaffin Granules. The γ-phosphate resonance frequencies in 15 different preparations averaged 7.41 ± 0.17 ppm with respect to 88% phosphoric acid. Consequently, the average internal pH in "resting" chromaffin granules is about 5.65 (Figure 2). The variation in intragranular pH is about 0.08 pH unit. When granules are suspended in a medium containing a permeant anion (Casey et al., 1976), addition of external ATP causes the γ-phosphate resonance of intragranular ATP to shift a downfrequency by 0.8 to 1.0 ppm (Casey et al., 1977), corresponding to a decrease in internal pH of 0.4 to 0.5 pH unit. Figure 4A shows the change in intragranular pH observed in spectra taken at intervals after ATP addition. The number of protons translocated between successive spectra can be calculated from the pH change if the buffering capacity (Figure 3) and the volume of the intragranular space are known. Since ATP addition does not affect intragranular volume (Casey et al., 1977), the volume of the starting sample can be used in these calculations.

Because the internal and external solutions differ in composition and pH, the β and γ signals of intragranular ATP and extragranular ATP can be distinguished. Intragranular ADP, AMP, and P_i are present in negligible concentrations, so the external β-ADP, AMP, and P_i signals are also well resolved (for typical spectra, see Casey et al., 1977). By comparing the integral of each resonance peak to a calibrated reference peak, the concentrations of metabolites in the sample may be determined.

Figure 4B shows the changes in concentration of extragranular ATP, ADP, AMP, and P_i calculated from spectra taken at intervals after ATP addition. ATP hydrolysis and generation of inorganic phosphate and ADP are apparent. The slow appearance of AMP indicates that an adenylate kinase is converting ADP to AMP and ATP. [Although no adenylate kinase activity is present in the chromaffin granule, preparations contain some activity because of mitochondrial contamination (Hagen and D'Iorio, 1965; Lagercrantz et al., 1970).] Since the observed decrease in ATP concentration is the difference between hydrolysis by the ATPase and regeneration by the adenylate kinase, the amount of ATP hydrolyzed is given by $\Delta AMP - \Delta ATP$. The ATPase activity observed in Figure 4B is 6.02 nmol min^{-1} (mg of protein)$^{-1}$.

Because we know the number of protons translocated and the amount of ATP hydrolyzed between successive spectra in Figure 4, we may calculate the ratio of protons translocated to ATP molecules hydrolyzed. As shown in Table I, this ratio is initially about 1.0 but falls rapidly as the pH gradient increases.

H^+ translocation can also be monitored by measuring pH changes in the external solution. If the experiment is done at pH 6.15, neither ATP hydrolysis nor the adenylate kinase reaction affects the pH; any pH change in the medium must be caused by proton translocation (Thayer and Hinkle, 1973). Since the adenylate kinase reaction does not release inorganic phosphate, the ATPase activity is equal to ΔP_i. ATPase ac-

tivity, determined by periodically assaying samples for phosphate, was 7.1 nmol min^{-1} (mg of protein)$^{-1}$. H^+/ATP ratios determined by this method agree quite well with those obtained by ^{31}P NMR (Table I). Reserpine (0.1 mM) and carboxyatractyloside (0.2 mM), added to inhibit catecholamine and ATP translocation (Kirshner, 1962; Kostron et al., 1977), did not affect the H^+/ATP stoichiometry.

Discussion

Since Mitchell (1961) proposed that a proton gradient mediates energy transduction in mitochondria, the problem of measuring pH inside organelles has received wide attention. By far the most popular technique has been to follow the distribution of weak acids and bases. When using this method, it is assumed that the weak acid or base equilibrates across the membrane only as the neutral species (Rottenberg et al., 1972), but it is difficult to be certain that this assumption holds. Moreover, the tracer must not interact with organellar components and the ionic composition of the internal solution must not affect its pK_a. Finally, the method requires either centrifugation of the sample or dialysis and stirring, either of which may damage the membrane. ^{31}P NMR provides a valuable check on this method because it does not require any disturbance of the sample and is based on completely different assumptions. In chromaffin granules, methylamine distribution measures an internal pH of 5.5 (Johnson and Scarpa, 1976; Casey et al., 1977). It also detects a pH drop of 0.3 to 0.4 unit when ATP is added to the granules (Casey et al., 1977). Both of these are in good agreement with results obtained with ^{31}P NMR.

When ATP is added to a chromaffin granule suspension in the presence of a permeant anion, the electrogenic proton pump translocates H^+ and the permeant anion follows to neutralize the charge (Casey et al., 1977). In the absence of a permeant anion, the ATP-dependent pH drop is not seen. Because we normally use Cl^- as the permeant anion, the pH changes are effectively caused by an HCl influx. For that reason, HCl was used for the titrations shown in Figures 2 and 3.

The ATP-dependent drop in the intragranular pH seems to be limited by an increase in the H^+ leakage rather than by a decrease in proton pumping. The ATPase activity does not decrease appreciably as the limit is reached. Furthermore, the energy theoretically obtainable from ATP hydrolysis is more than enough to drive protons across the increased pH gradient. When the intragranular pH reaches its lower limit, the free energy of hydrolysis $\Delta G^{\circ\prime}_{ATP} + 1.36 \log ([ADP][P_i]/[ATP])$ is about 9 kcal/mol. This is calculated from the data in Figure 4, assuming a $\Delta G^{\circ\prime}_{ATP}$ of -6.4 kcal/mol (Rosing and Slater, 1972). Assuming a stoichiometry of two protons per ATP, this provides enough energy to drive protons across the observed pH gradient (1.2 units) plus an electrical potential of $\sim$120 mV. It is unlikely that the potential is this large, since the permeant Cl^- ion should tend to dissipate the electrical potential, and, even in the absence of Cl^-, potentials greater than 70 mV have not been observed (Pollard et al., 1976; Drake, Njus and Radda, unpublished results).

Both ^{31}P NMR and the external pH method show a value of about 1.0 for the initial ratio of protons translocated to ATP hydrolyzed. These measurements, of course, give the net proton flux per ATP. To determine the H^+/ATP stoichiometry of the proton pump, corrections must be made for the H^+ leak.

The H^+/ATP ratio is time dependent because ATP hydrolysis progresses at a nearly constant rate, whereas the internal pH falls at a decreasing rate. The stoichiometry determined by measuring the pH in the external medium falls artifactually quickly, however, because gradual lysis of the acidic granules contributes protons to the external medium and counteracts the effect of inward proton translocation. Since equilibration of the electrode requires stirring, granule disintegration is quite noticeable, especially at later times (Table I). Consequently, ^{31}P NMR provides a better method of following the stoichiometry for this system.

In mitochondria, ATP is hydrolyzed rapidly. Therefore, by adding a pulse of ATP and following the rise of the pH gradient as ATP is hydrolyzed and the subsequent decay as the ATP is exhausted, one can estimate the H^+ leak and thus correct the H^+/ATP stoichiometry (Thayer and Hinkle, 1973). Because chromaffin granules hydrolyze ATP much more slowly, we must estimate the leak by a different method. The observed proton flux is the difference between the rates of H^+ pumping and leakage, so

$$([H^+]/[ATP])_{obsd} = ([H^+]/[ATP])_{pump} - [H^+]_{leak}/[\text{ATPase activity}] \quad (1)$$

The inward and outward components of the H^+ leak (I_{in} and I_{out}) have the ratio

$$RT \ln \left(\frac{I_{in}}{I_{out}}\right) = \mu^{H+}{}_{out} - \mu^{H+}{}_{in} \quad (2)$$

where $\mu^{H+}{}_{in}$ and $\mu^{H+}{}_{out}$ are the electrochemical potentials of H^+ on the inside and outside of the granule (Ussing, 1954). Therefore, the net passive H^+ flux is given by

$$[H^+]_{leak} = (I_{out} - I_{in}) = g_{H+}\{[H^+{}_{in}]e^{(F\Delta\psi/RT)} - [H^+{}_{out}]\} \quad (3)$$

where g_{H+} is a constant, $\Delta\psi$ is the membrane potential, and $[H^+{}_{in}]$ and $[H^+{}_{out}]$ are the internal and external proton concentrations. Because $[H^+{}_{in}] \gg [H^+{}_{out}]$ and $\Delta\psi$ is small and fairly constant, this can be simplified to

$$[H^+]_{leak} = C[H^+{}_{in}] \quad (4)$$

where C is a constant. When eq 4 is substituted into eq 1, it is clear that $[H^+{}_{in}]$ and $([H^+]/[ATP])_{obsd}$ are linearly related and the intercept ($[H^+{}_{in}] = 0$) will be $([H^+]/[ATP])_{pump}$. The data shown in Table I, when analyzed in this way, indicate a stoichiometry of about 2.0 (Figure 5).

According to this view, the drop in the internal pH is limited not by the size of the pH gradient or membrane potential but by the internal pH. The internal proton concentration simply becomes so high that the passive leak equals the pumping rate. This is supported by a number of other observations. First, the internal pH reached by proton pumping is only slightly dependent on the external pH or pH gradient (Casey et al., 1977). Second, Flatmark and Ingebretsen (1977), using Bromothymol blue as an indicator of internal pH, have reported a higher initial stoichiometry (1.58) in chromaffin granule ghosts. In ghosts, which have a higher internal pH, the H^+ leak should be smaller and hence the observed stoichiometry should be higher.

The estimation of H^+/ATP ratios by the NMR method depends critically on the value chosen for the buffering capacity. If we are underestimating the buffering capacity, we will be underestimating the stoichiometry in the same proportion. Because the ratios determined by the NMR method are similar to those obtained by the external pH method, we think that our buffering capacity must be fairly accurate.

We found the buffering capacity to be nearly constant [92 μmol of H^+ (pH unit)$^{-1}$ (mL of intragranular volume)$^{-1}$] over the pH range 4.5 to 7.0. Since granular protein is about 40% of the dry weight (Winkler, 1976) and the internal volume is about 3.5 μL/mg of granule protein (Njus and Radda, un-

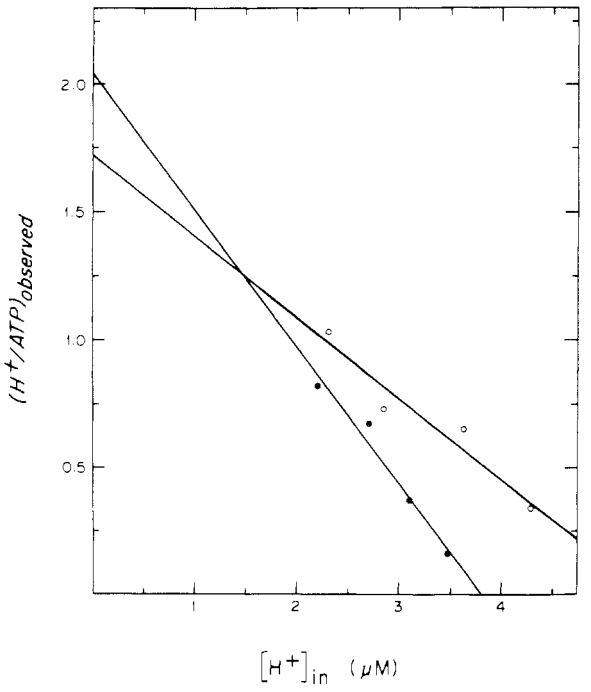

FIGURE 5: Stoichiometry of ATP-dependent proton translocation. For two experiments of the type shown in Figure 4, $([H^+]/[ATP])_{obsd}$ was determined for intervals between successive ^{31}P NMR spectra as described in Table I. These values were plotted against $[H^+]_{in}$, calculated as the average of values measured in spectra taken at the beginning and end of the interval. Straight lines, fit to the data points by the least-squares method, indicate proton pump stoichiometries ($[H^+]/[ATP]$) of 1.72 (○) and 2.04 (●).

published results), our buffering capacity estimate is 130 μmol of H^+ (pH unit)$^{-1}$ (g of dry weight)$^{-1}$. By contrast, Johnson et al. (1978), using different methods, obtained a value of about 100 μmol of H^+ (pH unit)$^{-1}$ (g of dry weight)$^{-1}$ at pH 6.25, but this rose to about 300 at pH 5.75.

From the ^{31}P NMR spectra of defined solutions (Figure 1), it is possible to see how each constituent adds to the upfrequency shift of the intragranular ATP resonances. Unfortunately, since the entire titration curve cannot be obtained, it is difficult to tell whether each compound affects the pK of the phosphate group or the chemical shifts of the phosphates. For this reason, we have not attempted to deduce the nature of the interactions of each of these compounds with ATP. NMR studies of the structure of the intragranular complex have been made by others (Sharp and Richards, 1977a,b; Daniels et al., 1974).

The calibration curve (Figure 2) shows that, in the intragranular solution, the pK of the γ-phosphate of ATP is depressed from about 6.5 to about 5.6, demonstrating the utility of ^{31}P NMR in obtaining the pK of compounds in complex, buffered solutions in which it would be difficult to obtain these values by standard titrimetric methods.

Table II shows the line width at half-height of the three resonances of ATP in intact granules and in the reconstituted intragranular solution. There is considerable broadening of the β and γ signals from the intact granules. Daniels et al. (1974) attributed this to decreased mobility of the phosphate groups. However, the presence of 300 mM sucrose in the reconstituted solution should increase the viscosity over that in the granules rather than decrease it. We suggest instead that the broadening is caused by heterogeneity in the granules. The preparation consists of a mixed population of adrenaline- and noradrenaline-containing vesicles. Either this difference or heterogeneity

TABLE II: Comparison of Half-Height Widths of the Phosphate Resonances of ATP in Intact Granules and in the Reconstituted Matrix.[a]

	half-height widths (Hz)			chemical shifts (ppm)		
phosphate	granules	reconst matrix	diff	upon protonat	upon replac adren with noradren	upon Mg^{2+} binding
α	95	87	8	0.8		0.4
β	129	110	19	1.7	0.3	3.4
γ	193	128	65	5.0	0.5	3.6

[a] The data on line widths at half-height do not contain contributions introduced by the convolution of the free-induction decay. Chemical shifts upon protonation were determined by titration of the reconstituted intragranular solution (Figure 2). Shifts upon replacing adrenaline with noradrenaline are taken from Figure 1 (pH 5.5). Data for Mg^{2+} binding (obtained at pH 6.3 in the presence and absence of 0.1 M Mg^{2+}) is from Cohn and Hughes (1962).

in internal pH could cause the observed line broadening. The broadening of the three ATP resonances correlates with their sensitivity to pH and with the shift seen on replacing adrenaline with noradrenaline. The dependence of the signals on Mg^{2+} concentration does not correlate with this broadening, suggesting that a variation in divalent cation content does not provide an alternative explanation.

Finally, it is worth noting that the ^{31}P NMR spectrum of freshly excised adrenal glands is dominated by the signals from ATP in the chromaffin granules. These signals are nearly identical to those in isolated granules, indicating that the preparation does not greatly perturb characteristics of the granular matrix. Because of the prominence of the granule signals, ^{31}P NMR is an attractive technique for observing granular changes in perfused and functioning adrenal glands.

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Mobile and Immobile Proteins of Synaptosomal Plasma Membrane†

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ABSTRACT: The effects of several cross-linking reagents on mouse brain synaptosomal plasma membrane and synaptic junction fractions were examined and compared. The rate of cross-linking by dimethyl suberimidate of many synaptosomal plasma membrane polypeptides was inversely correlated with their size, and these species were found together in cross-linked complexes fractionated on Sepharose 2B; several low molecular weight species, however, believed to include tubulin and actin, were cross-linked relatively slowly and were preferentially found in fractionated complexes of highest molecular weight. In contrast to those of synaptosomal plasma membrane, most of the polypeptides of synaptic junctions were cross-linked with the shorter molecule dimethyl adipimidate, and the rate of this process did not bear any marked relationship with molecular weight. Many of these species were found to be cross-linked in situ with disulfide bonds, as previously reported by others; the molecular weight of these in situ complexes was greater than 40×10^6. Most of the in situ cross-links were cleaved by treating intact synaptic junctions with B-mercaptoethanol, but species of such reduced preparations were still cross-linked with adipimidate or *o*-phenanthroline-Cu^{2+}. These results suggest that many polypeptides in synaptosomal plasma membrane are freely mobile and probably randomly distributed in the lipid bilayer, while several structural proteins may exist in ordered arrays. All of the species in the synaptic junction, in contrast, appear to be immobilized; some of these polypeptides are linked by disulfide bonds, while others are joined by noncovalent bonds and may form bridges between disulfide-linked species. The synaptic junction complexes may form one continuous network in this specialized membrane.

The lateral disposition of proteins in biological membranes is information essential to understanding the latter's functions, but is difficult to obtain (Peters & Richards, 1977). Examination of freeze-fractured membranes by electron microscopy reveals numerous electron-dense particles generally believed to be proteins (Branton, 1969), but the identification of these particles with specific species is generally not possible except in the simplest systems. Examination of cells labeled with such reagents as fluorescent antibodies or radioactive lectins has demonstrated that many species are freely mobile in the lipid bilayer (Frye & Edidin, 1970; Taylor et al., 1971; Kelly et al., 1976), while others are restricted to certain areas (Kelly et al., 1976); however, such methods necessarily can give information about only a few species.

In an earlier study (Smith & Loh, 1978), we demonstrated that most of the proteins of synaptosomal plasma membrane (SPM),[1] a complex system of crucial importance to nervous transmission, can be cross-linked with dimethyl suberimidate (DMS). Our observations led us to conclude that some, but not necessarily all, of these species were freely mobile in the lipid bilayer. In the present study, we have examined this question more thoroughly, by compositional analysis of the cross-linked material, and by comparing the cross-linking characteristics of SPM proteins with those of the synaptic junction (SJ); the latter is a small specialized area isolated from SPM which contains many polypeptides known to be immobilized in situ (Kelly & Cotman, 1976).

Materials and Methods

Preparation of SPM and SJ's. SPM was prepared as pre-

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[1] Abbreviations used: SPM, synaptosomal plasma membrane; SJ, synaptic junction; DMM, dimethyl malonimidate; DMA, dimethyl adipimidate; DMS, dimethyl suberimidate; $NaDodSO_4$, sodium dodecyl sulfate; TEA, triethanolamine.

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