Active Proton Uptake by Chromaffin Granules: Observation by Amine Distribution and Phosphorus-31 Nuclear Magnetic Resonance Techniques†

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ABSTRACT: The hydrogen ion activity within isolated chromaffin granules can be estimated from the distribution of the weak base methylamine and from phosphorus-31 nuclear magnetic resonance spectra of ATP contained in the granules. Following the addition of ATP to the external medium, the internal pH drops by 0.2 to 0.5 unit. This change occurs only in medium containing a permeant anion such as chloride and is abolished by an uncoupler of oxidative phosphorylation. These results indicate that the chromaffin granule membrane possesses an electrogenic proton pump directed inward.

Chromaffin granules, the catecholamine-storing vesicles of the adrenal medulla, accumulate adrenaline by a mechanism that appears to be coupled to a proton-translocating ATPase.[1] The uptake of adrenaline into chromaffin granules is driven by ATP hydrolysis (Hasselbach and Taugner, 1970) and is inhibited by uncouplers of oxidative phosphorylation (Bashford et al., 1975b). The existence of a proton-translocating ATPase is further suggested by two other observations. First, the addition of ATP and Mg^{2+} to chromaffin granules enhances the fluorescence of the probe 1-anilinonaphthalene-8-sulfonate (Bashford et al., 1975a) as in other proton-accumulating systems (e.g., Azzi, 1969). Second, ATP-induced lysis of chromaffin granules can be attributed to an electrogenic proton pump (Casey et al., 1976). In chloride-containing media, ATP drives a coupled proton/chloride uptake leading to osmotic lysis of the granules.

Because the chromaffin granule contents have a high buffering capacity, extensive proton translocation must occur before an internal pH change is detectable. However, electrogenic proton translocation generates an opposing electrical potential which limits the amount of proton transport that can occur. This electrical restraint can be relaxed by adding a permeant anion to the medium and permitting a coupled proton/anion influx. However, H^+/anion uptake raises the internal osmolarity and will cause lysis unless the granules are in a hypertonic medium. Consequently, the observation of extensive proton uptake requires that the external medium both contain a permeant anion and be hypertonic. Under these conditions, we have found that ATP addition causes a drop in intragranular pH. The pH drop is abolished by the uncoupler S-13. It occurs in the presence of the permeant chloride ion but not in media containing only sucrose or the impermeant sulfate ion. Two different methods have been used to determine the internal pH. Measuring the distribution of the weak base methylamine is a commonly used technique. A new and nonperturbing method uses phosphorus-31 nuclear magnetic resonance (^{31}P NMR) to monitor the protonation of ATP contained within the granules.

Materials and Methods

Chromaffin granules were isolated from bovine adrenal medullae as described by Bashford et al. (1975b). They were suspended in 0.3 M sucrose–10 mM Hepes (pH 7.0) and kept on ice until required. All experiments were performed within 15 h of the cattle being slaughtered.

The [^{14}C]sorbitol-excluding volume and the [^{14}C]methylamine distribution were determined from the relative activities of these isotopes in the pellet and supernatant by a method similar to that of Johnson and Scarpa (1976). To determine the sorbitol-excluding volume, chromaffin granules (0.5 ml or 2.5–5 mg of protein) were added to a microcentrifuge tube followed by 10 μl of [^{14}C]sorbitol (1.0 μCi/ml, 0.1 mM final concentration) and 10 μl of ^3H_2O (2 μCi/ml final concentration). After allowing 2 min for equilibration, the tubes were centrifuged for 10 min at 27 000g in a Sorvall RC2-B centrifuge at 25 °C and the pellet and supernatant separated. A 50-μl sample was taken from the supernatant and mixed with 200 μl of 14% perchloric acid. The pellet was mixed with 500 μl of 14% perchloric acid. The supernatant and pellets remained in the acid overnight. A 200-μl aliquot from each tube was then taken, mixed with 10 ml of scintillation fluid and 200 μl of 3 M formic acid, and counted. After correcting counts to ^{3}H and ^{14}C, the volume of the pellet (V') was calculated as

$$V' = \frac{500(^3H_{pellet})}{5(^3H_{sup}) - {}^3H_{pellet}}\ \mu l$$

Sorbitol-excluding volume was calculated as

$$V_{int} = V' - \frac{(500 + V')(^{14}C_{pellet})}{5(^{14}C_{sup})}\ \mu l$$

Methylamine distribution was determined by a similar procedure except that 10 μl of [^{14}C]methylamine (1.4 μCi/ml, 24 μM final concentration) was added instead of [^{14}C]sorbitol and the pH of the supernatant solution was measured immediately after centrifugation. The volume of the pellet (V'') was determined as V' above. External methylamine concentration was calculated from the supernatant counts:

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[1] Abbreviations used are: ATPase, adenosine triphosphatase; Hepes, *N*-2-hydroxyethyl-1-piperazine-*N'*-2-ethanesulfonic acid; S-13, *N*-(3-*tert*-butyl-5-chlorosalicilyl)-2-chloro-4-nitroanilide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

TABLE I: ATP-Dependent pH Change in Different Media.[a]

Medium	Additions	V_{int} (μl)	$[MeNH_3^+]_{ext}$ (cpm/μl)	$[MeNH_3^+]_{int}$ (cpm/μl)	ΔpH	External pH	Internal pH
Sucrose		3.64 ± 0.16	2053 ± 11	51 470 ± 2290	1.40 ± 0.02	6.71	5.31 ± 0.02
	ATP	3.00 ± 0.34	2098 ± 25	47 990 ± 5170	1.36 ± 0.05	6.60	5.24 ± 0.05
	ATP + S-13	2.78 ± 0.14	2087 ± 122	37 400 ± 3000	1.25 ± 0.02	6.55	5.30 ± 0.02
KCl		6.00 ± 0.23	2331 ± 7	21 000 ± 720	0.95 ± 0.01	6.41	5.46 ± 0.01
	ATP	6.32 ± 0.18	2074 ± 13	38 930 ± 1370	1.27 ± 0.01	6.42	5.15 ± 0.01
	ATP + S-13	4.97 ± 0.59	2257 ± 10	20 600 ± 2240	0.96 ± 0.05	6.37	5.41 ± 0.05
K_2SO4		5.82 ± 0.20	2470 ± 26	12 520 ± 500	0.70 ± 0.01	6.34	5.64 ± 0.01
	ATP	5.41 ± 0.34	2311 ± 4	14 890 ± 940	0.81 ± 0.03	6.33	5.52 ± 0.03
	ATP + S-13		2403 ± 22	10 510 ± 290	0.64 ± 0.01	6.30	5.66 ± 0.01

[a] For each medium, twelve 0.25-ml aliquots of chromaffin granules (2.5 mg of protein) in 0.3 M sucrose–10 mM Hepes (pH 7.0) were mixed with an equal volume of hypertonic buffer. Five microliters of 150 μM S-13 in ethanol was added to four tubes. At t = 0, 25 μl of 100 mM ATP–100 mM $MgSO_4$ (pH 7) was added to these four tubes and to four others. All samples were incubated at 25 °C for 30 min. Two samples in each group of four were used to determine sorbitol-excluding volume (V_{int}) and two to determine methylamine distribution. V_{int} was not determined in K_2SO_4 medium with ATP and S-13. The average volume in the other K_2SO_4 samples (5.61 μl) was used in calculating methylamine distribution. The media used were: (a) 40 mM Mes–0.6 M sucrose (pH 6.6); (b) 40 mM Mes–300 mM KCl (pH 6.6); (c) 40 mM Mes–200 mM K_2SO_4 (pH 6.6).

$$[MeNH_3^+]_{ext} = \frac{(^{14}C_{sup})}{40} \text{ cpm}/\mu\text{l}$$

Internal methylamine concentration was calculated as

$$[MeNH_3^+]_{int} = \frac{\left(\frac{500 + V''}{5}\right)(^{14}C_{pellet}) - (^{14}C_{sup})(V'' - V_{int})}{40V_{int}} \text{ cpm}/\mu\text{l}$$

^{31}P NMR spectra were recorded at 129 MHz on a spectrometer built in Oxford (Hoult and Richards, 1975). The spectrometer, equipped with temperature control and field stabilization via a deuterium lock, was operated in the Fourier transform mode. Accumulated free induction decays were obtained from 256 transients, employing a 1-s interpulse time (total accumulation time was 4 min, 16 s). The sample contained about 80 mg of protein/ml. A capillary containing D_2O was inserted into the sample tube to provide the deuterium lock. All shifts are reported in parts per million (ppm).

Protein was assayed using biuret reagent as described (Casey et al., 1976). [^{14}C]Methylamine, [^{14}C]sorbitol, and ^3H_2O were obtained from the Radiochemical Centre, Amersham, Bucks. Disodium ATP was obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey. The uncoupler *N*-(3-*tert*-butyl-5-chlorosalicilyl)-2-chloro-4-nitroanilide (S-13) was kindly provided by Dr. Britton Chance.

Results

The distribution of weak acids or bases has been used commonly to measure pH gradients across the membranes of chloroplasts, mitochondria, lysosomes, and cells (Waddell and Bates, 1969; Rottenberg et al., 1971, 1972; Reijngoud and Tager, 1973; Kashket and Wilson, 1973; Goldman and Rottenberg, 1973; Henning, 1975). This method is based on the premise that the neutral form of the compound is in equilibrium across the membrane. It follows that

$$\frac{[MeNH_3^+]_{in}}{[H^+]_{in}} \times 10^{-pK} = [MeNH_2]_{in} = [MeNH_2]_{out} = \frac{[MeNH_3^+]_{out}}{[H^+]_{out}} \times 10^{-pK}$$

The pK of methylamine, 10.6, ensures that less than 0.1% of the methylamine is in the neutral form in the physiological pH range and the distribution we measure is that of the charged species. We have used this method to measure changes in the pH of the interior of the chromaffin granule.

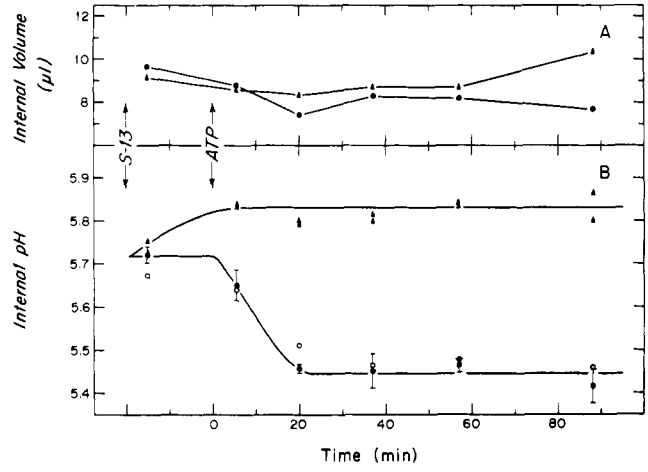

FIGURE 1: Time course of ATP-dependent pH change observed by methylamine distribution. Chromaffin granules in 14 ml of 150 mM KCl–150 mM sucrose–20 mM Hepes (pH 6.6) were incubated at 25 °C. At t = 0, 0.7 ml of 100 mM ATP–100 mM $MgSO_4$ (pH 7) was added. Periodically, four 0.5-ml samples were taken, two to determine sorbitol-excluding volume and two to determine methylamine distribution. The averages and standard deviations of the sorbitol-excluding volumes and the internal pH are plotted vs. the end of the incubation time (●). Concurrently, chromaffin granules in 8 ml of 150 mM KCl–150 mM sucrose–20 mM Hepes (pH 6.6) were incubated at 25 °C. Fifty microliters of 170 μM S-13 was added at t = −20 min and 0.4 ml of 100 mM ATP–100 mM $MgSO_4$ (pH 7) was added at t = 0. Periodically, two 0.5 ml samples were taken, one to determine sorbitol-excluding volume and one to determine methylamine distribution. These values are also plotted vs. the end of the incubation time (▲). "ATP" and "S-13" samples gave average sorbitol-excluding volumes of 8.5 ± 0.9 μl. Internal pH's calculated using this average volume are shown by open symbols [(○) +ATP; (△) +ATP and S-13].

As shown in Table I, ATP induces a pH change in KCl-containing medium but not in sucrose or K_2SO_4. The ATP-dependent change appears to be complete after about 30 min incubation (Figure 1). The time course as determined by methylamine distribution is only approximate since the sample is centrifuged for 10 min after incubation and a further 5 or 10 min is required before the supernatant and pellet can be

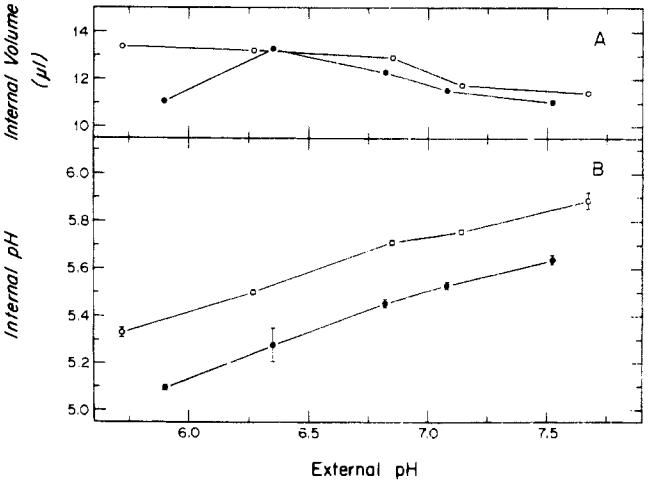

FIGURE 2: ATP-dependent pH change as a function of external pH. At each pH, control points (○) were obtained by mixing four 0.25-ml aliquots of chromaffin granules (4.6 mg of protein) in 0.3 M sucrose–2 mM Hepes (pH 7.0) with equal volumes of 300 mM KCl–40 mM buffer. After incubation for 30 min at 25 °C, two samples were used to determine the sorbitol-excluding volume and two were used to determine the internal pH by methylamine distribution. Points with added ATP (●) were obtained in the same way, except that 25 μl of 100 mM ATP–100 mM $MgSO_4$ (pH 7) was added to all four samples at the beginning of the 30-min incubation period. Mes was used as the buffer for points below pH 7; Hepes was used for points above pH 7.

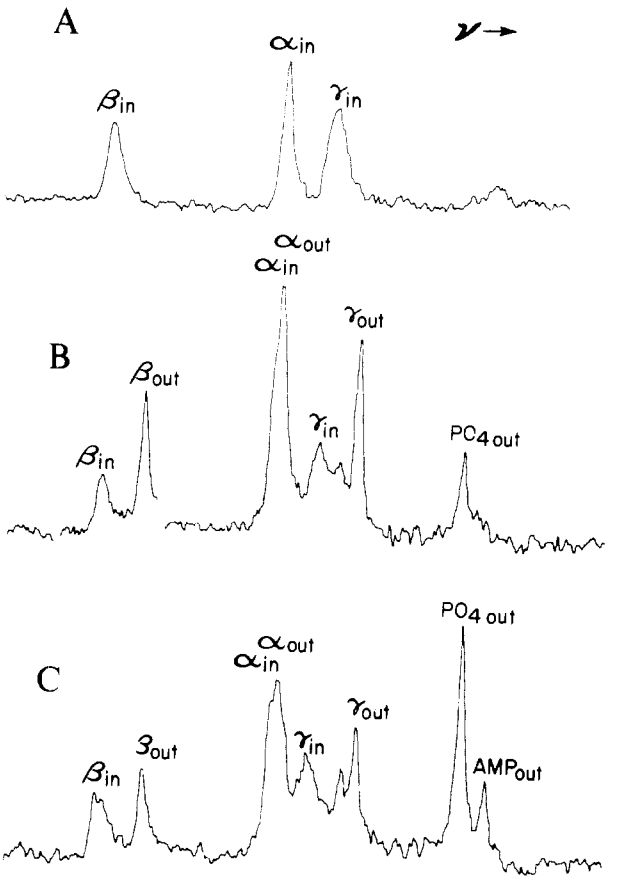

FIGURE 3: ^{31}P NMR spectra of chromaffin granule samples. Spectra were obtained as described in Materials and Methods. Chromaffin granules in 0.8 ml of 120 mM KCl–40 mM Mes–185 sucrose (pH 6.44) were incubated at 25 °C (A). Chromaffin granule suspension (0.8 ml). (B) Chromaffin granule suspension 1–5 min after the addition of 0.1 ml of 100 mM ATP–100 mM $MgSO_4$ (pH 7). (C) Chromaffin granule suspension 34–38 min after the addition of Mg–ATP.

separated. Since some reequilibration of methylamine may occur during this time, it is not possible to associate a methylamine distribution measurement with a precise instant. The pH change is abolished by the mitochondrial uncoupler S-13 (Figure 1). The uncoupler was added as an ethanolic solution, but ethanol alone had no effect. There may have been some systematic variations in internal volume during the incubation time, but these were too small to cause the observed variation in pH since the pH values calculated on the basis of the average internal volume are nearly the same as those calculated on the basis of internal volumes measured at each time point. As reported by Johnson and Scarpa (1976), the internal pH is only slightly dependent on the external pH (Figure 2). Moreover, the ATP-induced pH change does not seem to vary significantly with the external pH.

Chromaffin granules naturally contain a high concentration of ATP whose γ-phosphate has a pK around 6. This is close to the intragranular pH (Johnson and Scarpa, 1976). Since the ^{31}P NMR resonance peak shifts with the degree of protonation, intragranular ATP may serve as a particularly useful indicator of internal pH changes. A drop in pH causes a down frequency shift in the γ-phosphate resonance peak (Cohn and Hughes, 1960; Ritchie, 1975).

Figure 3A shows a typical ^{31}P NMR spectrum of isolated chromaffin granules with the internal ATP resonance peaks. When external ATP is added, the internal and external resonance peaks can be distinguished because of differences in pH and divalent cation concentrations in their respective media (Figure 3B). During the 30 min following its addition, the external ATP is hydrolyzed by the granular ATPase. The extent of this hydrolysis can be seen as an increase in the external inorganic phosphate peak and as a decrease in the external ATP signals (Figure 3c). The integrals of the intragranular ATP signals are constant during this time indicating that the granules remain stable during the period of observation. ATP hydrolysis is also accompanied by a down frequency shift in the resonance peak of the γ-phosphate of intragranular ATP (Figure 4), indicating a drop in internal pH. The time course for the shift (Figure 5) is similar to that for increased uptake of methylamine. The timing of the measurements was more accurately defined in the ^{31}P NMR measurements than in the methylamine distribution determinations. The shift was not seen if the mitochondrial uncoupler S-13 was added to the medium before ATP. The shift occurred when the permeant anion Cl^- was present in the external medium but did not occur in a medium containing only buffered sucrose or buffered sucrose and the impermeant sulfate anion (Figure 6). The most convenient temperature for carrying out methylamine-distribution experiments was 25 °C and this temperature was also used in the ^{31}P NMR experiments. However, the ATP-induced shift described above also occurred at the physiologically more meaningful temperature of 37 °C, although the rate was faster.

Discussion

The weak base methylamine has been used widely to estimate the internal pH of cells (Kashket and Wilson, 1973), chloroplasts (Rottenberg et al., 1971, 1972), lysosomes (Reijngoud and Tager, 1973; Goldman and Rottenberg, 1973; Henning, 1975), and chromaffin granules (Johnson and Scarpa, 1976; Pollard et al., 1976). Johnson and Scarpa (1976) reported that methylamine is concentrated by chromaffin granules and, thus, that the intragranular pH is acidic (pH 5.5). Methylamine binding rather than uptake was dismissed

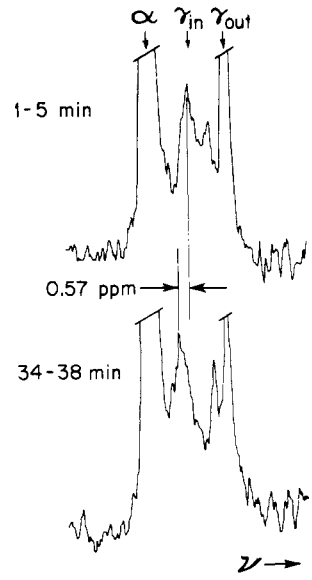

FIGURE 4: ATP-dependent shift in the γ peak of intragranular ATP. The spectra shown in Figures 3B and 3C are reproduced to show the shift in the position of the γ-phosphate peak of ATP contained within the chromaffin granules.

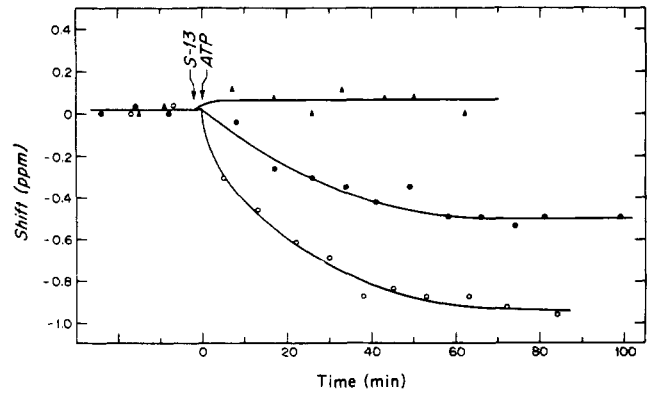

FIGURE 5: Time course of ATP-dependent shift in the γ peak of intragranular ATP. For each series, 0.2 ml of chromaffin granules in 0.3 M sucrose–10 mM Hepes (pH 7.0) were diluted into 0.6 ml of 160 mM KCl–53 mM Mes–150 mM sucrose (pH 6.34) and incubated at 25 °C. At $t = -2$ min, 10 μl of 1.5 mM S-13 was added to one sample (▲). At $t = 0$, 100 mM ATP–100 mM $MgSO_4$ (pH 7) was added to all three samples: 100 μl to one (○) and 60 μl to the other two (● and ▲). Spectra were obtained as described in Materials and Methods. The position of the γ-phosphate peak is plotted vs. the time at which the accumulation of the spectrum was completed.

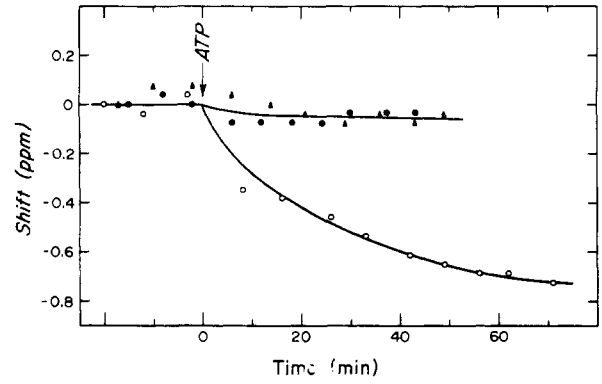

FIGURE 6: Dependence of the ATP-dependent shift in the γ peak of intragranular ATP on medium. Chromaffin granules (0.2 ml) in 0.3 M sucrose–10 mM Hepes (pH 7.0) were diluted into 0.6 ml of hypertonic buffer and were incubated at 25 °C. At $t = 0$, 0.1 ml of 100 mM ATP–100 mM $MgSO_4$ (pH 7) was added, and spectra were taken periodically as in Figure 5. The final solutions were 120 mM KCl–185 mM sucrose–40 mM Mes–2.5 mM Hepes (pH 6.37) (○), 80 mM K_2SO_4–185 mM sucrose–30 mM Mes–2.5 mM Hepes (pH 6.41) (●), and 375 mM sucrose–30 mM Mes–10 mM Hepes (pH 6.38) (△).

since the pH gradient measured was independent of the methylamine concentration and lysed granules showed an apparent pH gradient of only 0.1 pH unit.

While systematic errors in methylamine distribution pH measurements cannot be rigorously excluded, these should not affect the qualitative observation of pH changes. Johnson and Scarpa (1976) showed that the intragranular pH measured by the methylamine distribution technique responds in the predicted direction to K^+–H^+ exchange catalyzed by the ionophore nigericin or by valinomycin and the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Thus, the methylamine distribution method does report changes in the pH of chromaffin granules. The possibility that ATP-dependent methylamine uptake is caused by a change other than a drop in pH must be excluded.

Measuring the internal pH of chromaffin granules is particularly complicated by the fact that the intragranular space contains 110 mM ATP, 500 mM catecholamine, 120 mg of acidic protein/ml, divalent cations, and other components in a poorly defined solution of questionable homogeneity (Kirshner and Kirshner, 1971; Winkler, 1976). ATP-dependent changes in the homogeneity or composition of this internal solution might conceivably account for the observed ATP-dependent changes in methylamine uptake. However, this possibility is ruled out by the fact that chromaffin granule ghosts in which the internal components have been replaced by 0.3 M sucrose–10 mM Hepes (pH 7.0) (Phillips, 1974) also exhibit the ATP-dependent change in methylamine distribution (Casey, Njus, Radda, and Sehr, unpublished data).

The methylamine distribution method requires a fairly accurate estimation of the internal volume of the chromaffin granule sample. However, the ATP-dependent increase in methylamine uptake cannot be attributed to systematic errors in measuring volume. A 70% increase in volume would be required to account for the observed ATP-dependent increase in methylamine uptake. A volume change of this magnitude would certainly appear in measurements of sorbitol-excluding volume. Furthermore, in medium of the osmolarity used here, ATP does not cause any changes in the light scattering of a chromaffin granule suspension, a property that should reflect granular volume (Casey et al., 1976).

It is conceivable that the ATP-induced increase in methylamine uptake might occur by the same mechanism as active catecholamine uptake. However, reserpine, which blocks catecholamine uptake, had no effect on the ATP-induced pH changes observed with methylamine.

^{31}P NMR of phosphate compounds is a new, nonperturbing, and convenient technique for measuring pH. Chromaffin granules, naturally loaded with ATP, are an ideal subject. This technique is somewhat difficult to calibrate since such factors as catecholamine and divalent cation concentrations will change the chemical shift and the p*K* of the ATP γ-phosphate. Ritchie (1975) has studied the pH dependence of the α-, β-, and γ-phosphate resonances of ATP in solutions made up to reproduce the composition of the chromaffin granule matrix. She concluded that the internal pH of the chromaffin granule is 5.5, in agreement with the value obtained from methylamine distribution experiments. Nevertheless, since the internal composition of the chromaffin granule is poorly defined, the

possibility that ATP-dependent shifts are caused by changes in factors other than pH must be examined. Shifts cannot be attributed to changes in the concentrations of intragranular components. Changing these concentrations by increasing the osmolarity of the external medium from 300 to 900 mOsM does not affect the γ-phosphate resonance peak significantly. Similarly, the down frequency shift cannot be caused by uptake of ions from the medium. Increases in ionic strength or divalent cation concentration cause the γ-phosphate resonance to shift to higher frequencies (Cohn and Hughes, 1962; Seeley, 1975).

As the γ-phosphate of ATP is titrated through its pK, the resonance peak shifts by about 5 ppm with a maximum rate of about 2.5 ppm/pH unit (Seeley, 1975). Although various compounds affect the pK and chemical shift of the ATP γ-phosphate, Ritchie (1975) found that the shape of the titration curve is not significantly distorted by adrenaline, divalent cations, or acidic chromaffin granule proteins. This implies that the pH change responsible for a shift of 1 ppm must be at least 0.4 pH unit. This is somewhat larger than the ΔpH observed with methylamine distribution. This could be caused by the different conditions under which the measurements were made. In particular, chromaffin granules and external ATP are present in much higher concentration in the ^{31}P NMR experiments than in the methylamine distribution studies. Moreover, the methylamine distribution method measures a ΔpH averaged over all sorbitol-excluding volume. If some of this volume is contributed by nonpumping contaminants, the measured pH change will be smaller than the actual ΔpH. The ^{31}P NMR method, on the other hand, responds only to ATP-rich chromaffin granules and hence will not be affected by impurities in the preparation, even if lysosomes that may have an ATP linked pump (Mego, 1975) were present.

We clearly observe ATP-dependent changes with both ^{31}P NMR and methylamine distribution methods. The time courses of the changes are similar, the medium requirement is identical, and both are uncoupler sensitive. This suggests that both methods are responding to the same ATP-induced change. We have shown already that ATP-induced lysis of chromaffin granules has the same medium requirement and is also uncoupler sensitive (Casey et al., 1976). It is attributable to a coupled influx of protons and chloride. Both pH measuring techniques indicate a drop in intragranular pH consistent with this proton influx. Consequently, it is reasonable to conclude that these techniques are indeed measuring changes in pH.

These pH changes cannot be attributed to contaminants in the preparation. Chromaffin granules are the only organelle in the adrenal medulla containing enough ATP to contribute to the NMR spectrum. Mitochondria, the only other proton pumping organelles contaminating the preparation, should, if anything, raise their internal pH on addition of ATP. Furthermore, we have observed ATP-dependent pH changes using the methylamine distribution method in chromaffin granule ghosts purified by the method of Phillips (1974).

Pollard et al. (1976) have used the distribution of the permeant anion thiocyanate to measure electrical potential difference across the chromaffin granule membrane. They found that the addition of ATP caused the membrane potential difference to reverse sign from 45 mV inside negative to 30 mV inside positive. They assumed that protons would remain in equilibrium with this potential difference as given by the Nernst equation, so the increase in the potential of the granule interior was interpreted as a decrease in internal proton concentration or an increase in internal pH. However, if a proton pump is activated as we propose, equilibrium conditions as described by the Nernst equation certainly are not applicable. Protons pumped into the granule will raise the potential of the interior but lower the pH. These workers did not use hypertonic media in their studies of ATP-dependent pH and potential changes, and lysis of the chromaffin granules may have complicated the experimental results. Furthermore, their experiments were performed at 0 °C and the properties of the membrane at this temperature could be misleading. Consequently, we consider the conclusion of Pollard et al. (1976) —that ATP causes alkalinization of the chromaffin granule interior—to be incorrect.

Our results are a clear indication by two corroborating techniques that the chromaffin granule membrane possesses an inwardly directed proton pump. It is detectable by measurement of the bulk internal pH only in the presence of a permeant anion indicating that the pump is electrogenic. In this connection we note that the ATPase activity is not significantly dependent on the nature of the anion present (Casey et al., 1976; Bashford et al., 1975a,b) indicating that Cl^- is not exerting some structural effect on the enzyme. Bashford et al. (1976) have implicated a proton translocating ATPase in ATP-dependent catecholamine uptake. The fact that active uptake of catecholamines can take place in sucrose medium implies that transmembrane differences in electrical potential may drive catecholamine uptake.

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Determination of the Microscopic and Macroscopic Acid Dissociation Constants of Glycyl-L-histidyl-L-lysine and Related Histidine Peptides†

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ABSTRACT: Proton magnetic resonance studies of the acid-base chemistry of the glycyl ammonium, histidyl imidazolium, and lysyl ammonium groups of glycyl-L-histidyl-L-lysine and of the glycyl ammonium and histidyl imidazolium groups of glycyl-L-histidine and glycyl-L-histidylglycine are described. Chemical-shift data indicate that, at the molecular level, the glycyl ammonium and the histidyl imidazolium groups are titrated over the same pH range, with the acidity of the imidazolium group some 8 to 10 times that of the glycyl ammonium group, depending on the peptide. The lysyl ammonium group of Gly-His-Lys is much less acidic and is titrated over a higher pH range. Microscopic and macroscopic acid-dissociation constants were determined from chemical-shift data for each of the peptides. It is shown how microscopic formation constants for protonated metal complexes of these ligands, which are being used increasingly as models for the binding of metal ions by proteins, can be calculated from the macroscopic formation constants and the microscopic acid-dissociation constants. The acid-base chemistry of Gly-His-Lys is discussed with respect to its recently discovered biological activity.

In this paper, we characterize the acid-base chemistry of glycyl-L-histidyl-L-lysine and of the related peptides glycyl-L-histidine and glycyl-L-histidylglycine at the molecular level by nuclear magnetic resonance spectroscopy. Both microscopic and macroscopic acid-dissociation constants have been derived from chemical-shift measurements, and the distributions among the various protonated forms have been determined from the microscopic acid-dissociation constants.

The microscopic acid-base chemistry of these peptides is of interest in view of the use of histidine-containing peptides as model systems for the binding of metal ions by the histidyl residues of proteins (Agarwal and Perrin, 1975a,b; Aiba et al., 1974; Yokoyama et al., 1974). For example, recent studies of the copper(II) and cobalt(II) complexes of Gly-His and Gly-His-Gly in aqueous solution have indicated the presence of complexes in which either the amino nitrogen is protonated while the imidazole-3-nitrogen is metal complexed or the reverse (Agarwal and Perrin, 1975a,b). With the microscopic acid-dissociation constants reported in this paper, microscopic formation constants can be derived for the protonated complexes; such constants should provide better models for the binding of metal ions by the histidyl residues of proteins.

The microscopic acid-base chemistry of Gly-His-Lys is of interest in view of the report by Pickart and Thaler that a factor isolated from human serum prolongs the survival of normal rat liver cells and enhances the growth of hepatoma cells in culture (Pickart and Thaler, 1973). An amino acid analysis indicated the active factor to be a tripeptide formed by glycine, histidine, and lysine, and the synthetic tripeptide Gly-His-Lys was found to have biological properties similar to those of the native factor (Pickart et al., 1973). Pickart and Thaler suggested that the mechanism of action of the native factor and the synthetic tripeptide may reside in the high affinity of their polar side chains for DNA. The specific interaction of the imidazole moiety of histidyl residues of histidinamide and of small, synthetic histidine-containing oligopeptides with DNA is thought to be dependent on the protonation state of the interacting groups of the oligopeptide (Fritzsche, 1972; Goufevitch et al., 1974). The microscopic constants and the distribution between the protonation tautomers described in this paper should be of use in interpreting DNA binding experiments with Gly-His-Lys and similar peptides.

Experimental Section

Gly-His-Lys (Terochem Laboratories), Gly-His (Sigma) and Gly-His-Gly (Sigma) were used as received. pH measurements were made with an Orion 801 pH meter equipped with a Fisher microprobe combination electrode, which was calibrated with standard solutions of pH 4.00, 7.00, and 10.00. Acid-dissociation constants were evaluated both as mixed activity-concentration constants (activity of hydrogen ion and concentration of acid and its conjugate base) and as concentration constants. The acid-dissociation constants reported in Table I are mixed constants; factors for conversion to concentration constants are given in the footnote to Table I. pH measurements were converted to hydrogen ion concentration as described previously (Rabenstein and Sayer, 1976).

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