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Proton NMR Studies of Nucleotide and Amine Storage in the Dense Granules of Pig Platelets†

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ABSTRACT: ^{1}H NMR measurements have been conducted at 360 MHz on isolated pig platelet dense granules. Resonances of the H8, H2 protons of the adenine ring, H1′ protons of the ribose moiety, and the aromatic hydrogens of 5-hydroxytryptamine (5HT) have been identified in spectra of intact dense granules. Like the ^{31}P resonances of the nucleotides contained in the dense granules (Uğurbil et al., 1984), the line widths and the intensities of these resonances were sensitive to sample temperature and osmolarity of the suspension medium. Their chemical shifts indicate that 5HT in the granule interior is predominantly bound to the nucleotides through ring-stacking interactions. Association of 5HT with the nucleotides was also confirmed by the presence of intermolecular nuclear Overhauser effect (NOE) between 5HT and nucleotide protons. Large and negative intermolecular NOE's observed among the nucleotide H8, H2 and H1′ protons, together with upfield shifts undergone by these protons within the dense granules, demonstrate that the nucleotides form a complex where they are in close proximity of each other. The formation of this complex apparently does not require the presence of amines since removal of 5HT and histamine did not change the chemical shifts of the nucleotide protons. From T_1 and T_2 data, rotational correlation time of 4 ns was calculated for the nucleotides in the dense granule interior at 35 °C. A resonance tentatively identified as H2 of histamine was found to shift upon manipulation of the intragranular pH; it was used as an indicator of pH changes within the granule interior during 5HT uptake and showed that 5HT accumulation increases the intragranular pH. These results demonstrate that 5HT is first taken up in response to the inside acidic pH gradient across the granule membrane and is subsequently sequestered in a matrix formed by the divalent cations and the nucleotides.

Dense granules of platelets are membrane-enclosed vesicles which contain high concentrations of nucleotides, 5HT,[1] HA, and divalent cations (DaPrada et al., 1981). They share the general property of storing nucleotides and biogenic amines with other subcellular organelles such as the chromaffin granules of the adrenal medulla and the synaptic vesicles of neurons. The effective concentrations of the granule constituents within these vesicles can be remarkably high. In the interior of the platelet dense granules, the adenine nucleotides

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[1] Abbreviations: 5HT, 5-hydroxytryptamine (also known as serotonin); HA, histamine; NMR, nuclear magnetic resonance; fwhm, full width at half-maximum; TPS, 3-(trimethylsilyl)-1-propanesulfonic acid; NOE, nuclear Overhauser effect; FID, free induction decay; BSA, bovine serum albumin; HPLC, high-pressure liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

0006-2960/84/0423-0416$01.50/0

and the divalent cations exist in concentrations exceeding 0.5 and 1 M, respectively (Uğurbil & Holmsen, 1981). Chromaffin granules contain ~0.1 M ATP and sequester catecholamines at concentrations exceeding 0.5 M (Kirshner & Kirshner, 1971; Winkler, 1976). Yet, both types of granules are stable at ~300 mosM environments. The magnitude of osmotic imbalance that these granules are able to withstand have led to the conclusion that the granule interior cannot simply be an ideal solution and that some form of storage complex exists within these vesicles (Blaschko et al., 1956; Falk et al., 1956; Berneis et al., 1969a,b). This conclusion was supported by the observation that aqueous mixtures of biogenic amines and nucleotides aggregate at high solute concentrations and form structures with high apparent molecular weights (Berneis et al., 1969a,b, 1970; Pletscher et al., 1971).

Recently, direct evidence for the existence of storage complexes in vivo was provided by NMR experiments with intact platelets. These studies showed that ^{31}P resonances from the phosphate moieties of the nucleotides contained in human dense granules were undetectably broad (Uğurbil et al., 1979; Costa et al., 1979); it was concluded that the mobility of the nucleotides in the granule interior was highly restricted, most likely due to the existence of large molecular weight aggregates. ^{19}F NMR studies indicated that 5HT is probably part of such aggregates (Costa et al., 1979). Similar studies with pig platelets (Uğurbil et al., 1979, 1981; Costa et al. 1980), isolated dense granules from pig platelets (Uğurbil et al., 1984), and cattle and rabbit platelets (Carroll et al., 1980; Schmidt & Carroll, 1982) showed that the nucleotides within the dense granules of these platelets possess sufficient rotational mobility to yield relatively narrow ^{31}P resonances at 37 °C; however, the line widths were strongly temperature dependent, and the resonances became virtually undetectable at ~5 °C, again indicating that the intragranular conditions deviate substantially from an ideal solution state.

In this paper we present ^1H NMR measurements on isolated pig platelet dense granules where we monitor the aromatic protons of the amines and the nucleotides; ^{31}P NMR studies on both intact cells and isolated granules had shown that the properties of dense granules do not change upon isolation (Uğurbil et al., 1984). Unlike the ^{31}P measurements, however, in the ^1H NMR spectra of the isolated dense granules, we were able to detect and identify resonances from both the nucleotides and the amines; this has enabled us to obtain information of greater detail about the interactions between the different constituents of the granule interior.

Materials and Methods

Preparation of Dense Granules. Dense granules were studied either in a suspension which contained all subcellular organelles of platelets or after further purification. The former sample was prepared as described elsewhere (Uğurbil et al., 1984), except that, for the ^1H NMR studies reported in this paper, the organelles were further washed twice and resuspended in a medium where the H_2O was replaced by D_2O. The pH was adjusted between 7.2 and 7.4.[2] Typical samples used in ^1H NMR studies contained 50–60 mg of protein/mL, similar to the samples used for the ^{31}P measurements (Uğurbil et al., 1984).

Purified dense granules were isolated from the organelle mixture by using a sucrose gradient in D_2O which consisted of 1.5-mL steps increasing from 0.8 to 1.8 M sucrose in 0.2 M increments. All steps contained 0.6% BSA. The mixture of organelles prepared as described before (Uğurbil et al., 1984) was collected by centrifugation and resuspended in 0.4 M sucrose, 0.01 M Hepes, and 0.1 mM EDTA in D_2O; subsequently, this suspension was layered over the density gradient and centrifuged at 100 000 rpm for 60 min at ~5 °C in a Beckman ultracentrifuge with an SW-40 rotor. The pellet which contained predominantly dense granules was resuspended in ~15 mL of D_2O, containing 300 mM KCl, 30 mM monosodium phosphate, and 0.4% BSA (pH ≃ 7.4). After approximately a 20-min incubation in the D_2O-based medium, the suspension was centrifuged at 10 000 rpm for 10 min in a Sorval centrifuge with an SA-600 rotor, and the granules were collected as the pellet which was typically 0.1–0.2 mL in volume. This pellet was resuspended in 0.4 mL of the D_2O medium containing 300 mM KCl, 0.4% BSA, and 30 mM phosphate buffer. The sample was kept ice-cold prior to the NMR measurements which were started no later than 1 h after the final suspension. This preparation was found to be extremely stable in an ice bath for at least 10 h (longest time examined) and stable at 35–37 °C at least for 4 h (longest time examined).

Amine-Depleted Granules. These granules were studied in a suspension of organelles prepared as described in Uğurbil et al. (1984) from the platelets of reserpine-treated pigs. Reserpine treatment of pigs and isolation of their platelets are also described in Uğurbil et al. (1984).

Analysis of Nucleotide Amine and Metal Ion Content. Nucleotides were determined by HPLC analysis (Daniel et al., 1980) using $HClO_4$ extracts prepared as described in Uğurbil et al. (1984). 5HT and HA content was analyzed in HCl extracts (Uğurbil et al., 1984) by using the procedures of Drummond & Gordon (1974) and Shaff & Beaven (1979), respectively. Magnesium and calcium concentrations were measured by atomic absorption on a Perkin-Elmer 303 spectrometer by standard methods; $LaCl_3$ was added to all samples as a standard at a final concentration of 1%. Protein was determined as described by Miller (1959), and the values were corrected for 0.4% BSA which was present in the medium.

Electron Microscopy. The granule subfraction was fixed in 2.5% glutaraldehyde in Tyrode's salt solution at pH 7.4 for 30 min and postfixed in ice-cold 1% osmium tetraoxide also in Tyrode's solution for 5 min. The sample was ethanol dehydrated and embedded in Epon 812. Sections were stained with uranyl acetate followed by lead citrate.

NMR Measurements. ^1H NMR measurements were conducted at 360 MHz on a Bruker WH 360 instrument located at the Mid-Atlantic NMR facility, University of Pennsylvania, Philadelphia, PA, and on a Nicolet 360 instrument located at the Gray Freshwater Biological Institute, University of Minnesota. Samples were generally 0.25 mL in 5-mm NMR tubes. All chemical shifts were measured relative to TPS which was added to the NMR samples.

The spectra of intact granules presented were obtained after application of convolution difference procedure to the FIDs to eliminate a very broad base line that was present. For this procedure, the FID was multiplied by an exponential which caused 160-Hz line broadening; the resultant FID was multiplied by 0.85 and subtracted from the untreated original FID. The difference was multiplied by typically 20–30-Hz exponential filter and Fourier transformed. Spectra of extracts and suspension medium were not treated with the convolution difference procedure.

T_1 and T_2 values were calculated from a nonlinear least-squares fit to data obtained by progressive saturation and Hahn

[2] All pH values reported are direct pH meter readings, uncorrected for the deuterium isotope effect.

Table I: Assignments, Chemical Shifts, and the Spin Relaxation Times of the Resonances Observed in the 5–9 ppm Region of ^{1}H NMR Spectra of Intact Pig Platelet Dense Granules at 35 °C

	peak no.[a]							
	1	2	3	4	5	6	7	8
assignments	[b]	nuc (H8)	nuc (H2)	[c]	5HT[d] (H7 + H2)	5HT (H4)	5HT (H6)	nuc (H1′)
chemical shifts (ppm)[e]	8.41	8.16	7.73	7.10	6.79	6.50	6.33	5.78
T_1 (s)[f]	1.40 ± 0.18	1.10 ± 0.05	1.47 ± 0.26					1.06 ± 0.11
T_2 (ms)[f]	28.2 ± 3.8	19.7 ± 1.6	18.3 ± 1.5					15.5 ± 1.7

[a] Peak numbering correspond to that shown in Figure 1a. The chemical shifts are in ppm relative to TPS added into the granule suspension which is expected to remain outside the granules. It should be noted that most likely there will be differences in the diamagnetic susceptibility between the granule interior and the granule exterior. Consequently, these values need to be corrected. [b] Tentatively assigned to H2 of HA. [c] Primarily protein. May contain a contribution from H4 of HA. [d] Approximately half of the intensity in this resonance is assigned to 5HT; the rest of the intensity probably arises from proteins. [e] The values given are the averages from eight different samples; the errors (standard deviations from the mean) were ±0.03 for peaks 1, 6, and 7 and ±0.02 for the rest. [f] Numbers given represent the average of measurements on three different samples. The errors are the standard deviation from the mean for the three measurements. The error for each individual fit was ~5%.

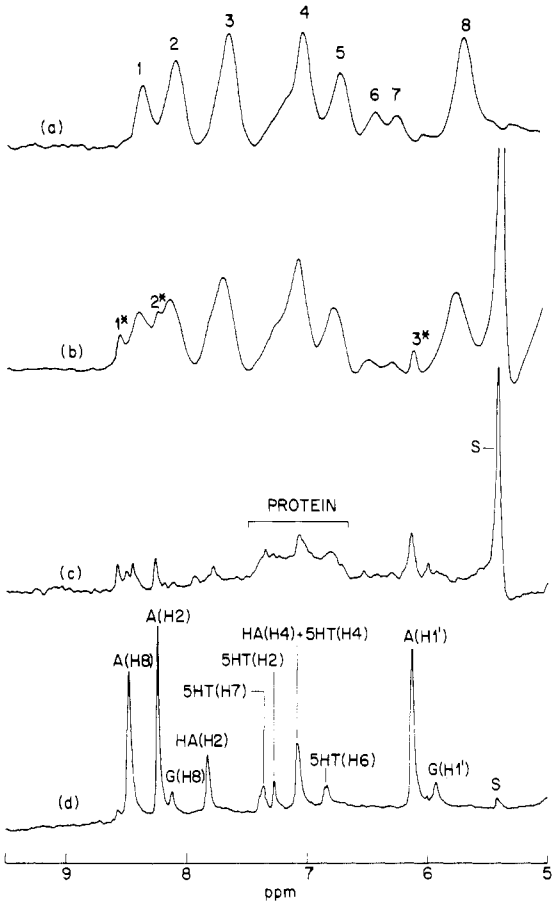

FIGURE 1: ^{1}H NMR spectra at 360 MHz and 35 °C recorded from (a) cytoplasmic organelles of pig platelets in suspension (400 FIDs obtained with 60° pulses and 2.2-s repetition time), (b) purified dense granules in suspension (200 FIDs obtained with 45° pulses and 3-s repetition time), (c) the suspension medium of sample used to obtain (b), after removal of the dense granules (100 FIDs, 45° pulses, and 5.5-s repetition time, pH 7.2), and (d) the acid extract of the dense granules recovered from the sample used for (b) (200 FIDs, 45° pulses, and 5.5-s repetition time, pH 7.5). Spectra a and b are convolution difference spectra obtained as described under Materials and Methods. For (c) and (d), the FIDs were processed with a 5-Hz exponential filter. Peak assignments for (a) and (b) are given in Table I. Peaks 1*, and 2*, and 3* in (b) stem from nucleotides present in the suspension outside the dense granules. In (d), A and G represent adenine and guanine nucleotides, respectively. Peak S is sucrose.

spin-echo pulse sequence, respectively. NOE measurements were performed by using a low-power preirradiation of different durations; immediately after this irradiation was gated off, a 90° nonselective pulse was applied, and the FID was collected. Subsequently, spins were allowed to relax with no

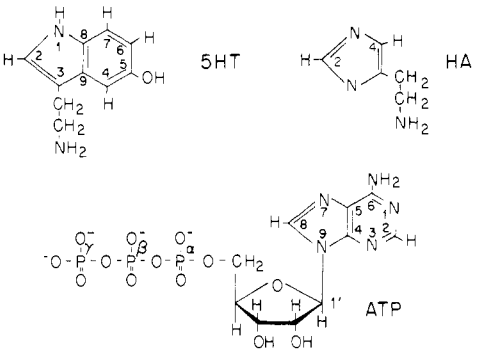

FIGURE 2: Molecular structures and the numbering of carbon atoms in 5HT, HA, and ATP.

irradiation, and the sequence was repeated. Control spectra were obtained by moving the frequency of the low-power irradiation pulse downfield to a position devoid of resonances, generally symmetrically opposite from peak 1. In control spectra, at the power levels used, no indirect saturation of the peaks was detectable even if the frequency of the saturation pulse was much closer to the resonances. The control and the NOE spectra were collected in alternate 16-scan blocks. The difference spectra were obtained by subtracting the two FIDs.

Results

Parts a and b of Figure 1 show the 5–9 ppm region of the 360-MHz ^{1}H NMR spectra obtained at 35 °C from a suspension of platelet organelles and purified dense granules, respectively. The former sample contains dense granules as well as mitochondria, α-granules, and lysosomes; however, only the dense granules contain large amounts of nucleotides and amines. The chemical shifts and the relative intensities of the resonances present in the intact granule spectra were highly reproducible. An indication of this reproducibility is provided by the spectra from different samples which are presented in the other figures.

The resonances 1–8, with the exception of the broad peak identified as 4, are assigned to the aromatic protons of the nucleotides and the amines and H1′ proton of the nucleotides (Figure 2) contained in the dense granules. Specific assignments are summarized in Table I together with the average chemical shift, T_1, and T_2 values measured for these resonances. The justification for these assignments are presented further on. The narrower peaks identified with an asterisk in Figure 1b and in all other figures arise from the nucleotides and amines not contained within the granules; this assignment is illustrated by the spectrum (Figure 1c) that was obtained from the suspension medium of this sample after the dense

Table II: Nucleotide, Amine, and Metal Ion Content of Purified Dense Granules of Pig Platelets[a]

amounts (nmol/mg of protein)[b,c]								ratios[d]			
ATP	ADP	GTP	GDP	5HT	HA	Ca^{2+}	Mg^{2+}	Ca^{2+}/Mg^{2+}	M/nuc[e]	Am/nuc[f]	A/G[g]
553 ± 197	307 ± 81	112 ± 48	56 ± 20	206 ± 35	308 ± 44	213 ± 57	1821 ± 344	0.12 ± 0.04	2.2 ± 1.2	0.5 ± 0.2	5.34 ± 0.95

[a] Dense granules were purified and suspended in the presence of BSA as described under Materials and Methods. [b] Protein was corrected for the BSA in the suspension medium. [c] Numbers given are the average of measurements in three independent preparations. [d] The ratios were calculated for each preparation and then average to obtain the mean and standard deviation from the mean. [e] Represents $(Ca^2 + Mg^{2+})$/(ATP + ADP + GTP + GDP). [f] Represents the ratio of (HA + 5HT) to the total pool of nucleotides. [g] Represents adenine nucleotide to guanine nucleotide ratio.

granules were removed by centrifugation. The resonances seen between 6.8 and 7.4 ppm in Figure 1c stem from the aromatic amino acid residues of BSA present in the medium used for suspending the purified granules and possibly from other proteins. Peak S is from sucrose carried over from the sucrose gradient used during purification.

Figure 1d illustrates a spectrum obtained from an extract prepared from the purified dense granules; the granules were collected as a pellet by centrifugation and were resuspended in 0.1 M DCl in order to disrupt the vesicular structure. The acid-insoluble matter was centrifuged out, and the pH was adjusted to ~7.5 with NaOD prior to recording the spectrum shown in Figure 1d. The very small sucrose resonance (peak S) in Figure 1d indicates that the suspension medium was effectively removed by the centrifugation. The dense granules are known to contain adenosine and guanosine di- and triphosphates and 5HT (Uğurbil & Holmsen, 1981; Table II); the resonances from the aromatic hydrogens of these molecules were easily identified in the extract spectrum (Figure 1d) on the basis of their characteristic chemical shifts. Specific assignments of the 5HT resonances were based on scalar spin–spin couplings. Two of the resonances were assigned to H2 and H4 protons of HA on the basis of a pH titration as well as addition of HA into the extract at different pH values. Presence of HA in the extract was also confirmed by conventional biochemical assays. Pig platelets have previously been reported to contain HA (DaPrada et al., 1981). Our observation directly demonstrates that the HA is sequestered within the dense granules and that it is present in amounts exceeding 5HT (Table II).

Upfield of 5 ppm (not shown), both the purified granules and the suspension of organelles displayed a broad envelope of resonances typically observed in ^1H NMR spectra of proteins and lipids; this contribution was significantly more prominent in the suspension of all cytoplasmic organelles, as expected.

It is evident from Figure 1 that the suspension of the platelet organelles is as suitable as the purified preparation for detecting those ^1H resonances of the dense granule contents that fall in the 5–9 ppm region of the spectra. In general, the dense granules in this unpurified suspension displayed greater stability under a variety of conditions such as exposure to different temperatures, different osmolarity, and NH_4Cl. Consequently, such studies, which are discussed further on, were mainly performed with the organelle suspensions rather than the purified granules.

The purified granules were initially prepared according to a previously published procedure (Salganicoff et al., 1975); the granules obtained by this procedure were found to be highly unstable. Figure 3 shows two spectra obtained from such a preparation immediately after it was brought up to 31 °C and after ~30-min incubation at 31 °C. The deterioration of the vesicular structure in this preparation is indicated by the appearance of the narrow resonances which arise from extravesicular nucleotides and amines. In order to obtain a

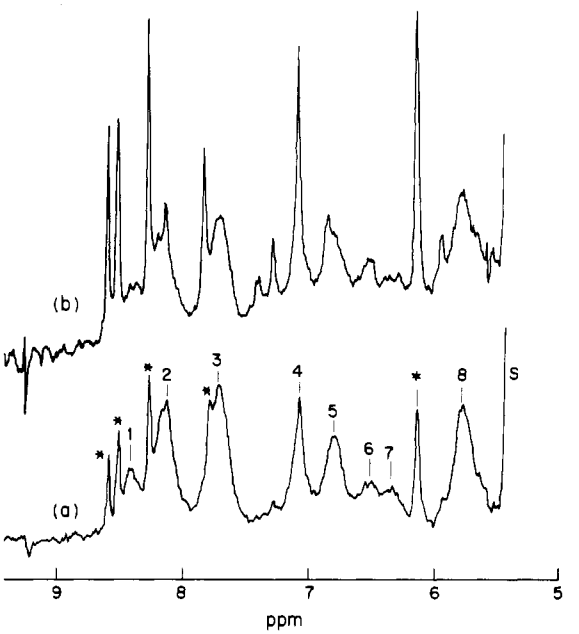

FIGURE 3: ^1H NMR spectra at 360 MHz of dense granules purified in the absence of BSA. (a) Immediately after raising the sample temperature to 31 °C. The sample was kept in an ice bath prior to raising the temperature. (b) ~30 min after raising the sample temperature to 31 °C. Both spectra are the sum of 400 FIDs obtained with 60° pulses and 1.8-s repetition time. These are not convolution difference spectra. Peaks labeled with an asterisk originate from molecules outside the dense granules. S is sucrose.

more stable preparation of purified dense granules, the following changes were made: (1) A sucrose gradient in D_2O with 1.8 M sucrose was used as the final step instead of higher sucrose concentrations. (2) BSA was included in the sucrose gradient and in the final suspension. (3) The concentration of KCl in the medium used for suspending the pure granules was increased approximately 2-fold to 300 mM. BSA was found to be crucial for stability, and its inclusion in the sucrose gradient also improved our yields. The high osmolarity of the suspension medium contributed to the long-term stability, although it was not by itself a sufficient condition for stability.

Figure 4 shows an electron micrograph picture obtained from a sample of purified granules prepared by our modified procedure; it demonstrates that this procedure yields a largely homogeneous sample of dense granules. The contents of the granules in this preparation were also determined and are given in Table II. Previous determinations of the amounts of nucleotides and the 5HT contained in pig platelet dense granules were performed with suspensions of platelet organelles or subsequent to secretion in suspensions of whole platelets (Uğurbil & Holmsen, 1981).

Peak Assignments. Resonances 1–8 shown in Figure 1a,b must arise from the dense granules since they are observed in the suspension both of organelles and of purified dense granules and they are absent in the spectrum obtained from the suspension medium after removal of the granules. These

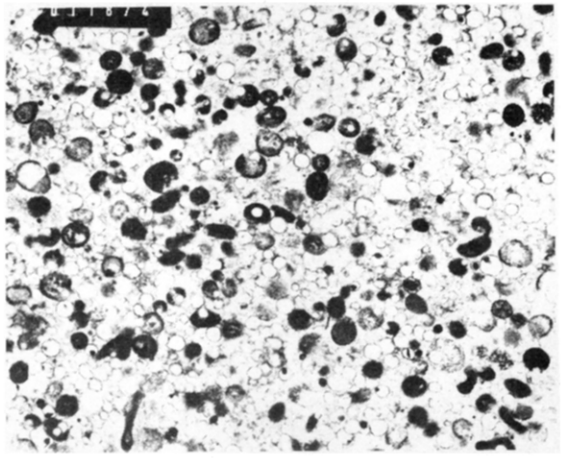

FIGURE 4: Electron micrograph of a sample of dense granules purified according to the procedure described under Materials and Methods. Magnification 7950×. The fraction is greatly enriched in dense granules of the bull's type compared to the starting material. However, there are also some mitochondria, α-granules, and empty membranes that had the same characteristics as the dense granule membrane.

resonances can contain contributions from the acid-soluble, small molecular weight compounds contained in the dense granules (i.e., nucleotides, HA, and 5HT), from the aromatic amino acids of proteins which are associated with the dense granules and/or which are present in the granule suspensions, and possibly from other, as yet unknown, acid-insoluble constituents. Dense granules are not known to contain soluble proteins in the vesicle interior (Costa et al., 1974). They do, however, have proteins associated with the vesicle membrane (Fukami et al., 1979).

The protein contribution to the ^{1}H NMR spectrum will primarily appear in the region of peaks 4 and 5 (between ~6.8 and 7.4 ppm) of the intact granule spectra (Wüthrich, 1976; Dwek, 1975). For example, such protein resonances are detected from the BSA in the suspension medium in the absence of the granules (Figure 1c). These peaks arise from the aromatic protons of tyrosines, tryptophans, and phenylalanines which are present in large numbers in proteins; the resonances of these protons also overlap with each other extensively and, consequently, are fairly intense. Therefore, chemical shift considerations suggest that peaks 4 and 5, and the downfield shoulder on peak 4 may contain contributions from proteins and peaks 1–3 and 6–8 must arise from the nucleotides and the amines detected in the extract spectrum. These general assignments are supported by the influence of temperature (Figure 7) and osmolarity of the suspension medium (Figure 8) on these resonances (discussed further on).

The specific assignments of peaks 2, 3, and 8 are based on the chemical shifts and the relative intensities of these resonances. The chemical shift ranges observed for the H8, H2, and H1′ protons of adenine nucleotides under a variety of conditions are given in Table III. In solution, H8 and H1′ protons of guanine nucleotides appear slightly upfield of adenine H2 and H1′ resonances, respectively (Figure 1d). Resonances from these protons may have a similar relationship when nucleotides are in the dense granule interior; however, line widths are such that the guanine and the adenine contributions cannot be resolved in the intact granule spectra. Peak 8 is clearly the only resonance which falls in the 6.18–5.6 ppm range observed for the H1′ proton. Its intensity is also consistent with the relative abundance of the nucleotides in the dense granule interior (Table II; Uğurbil & Holmsen, 1981). Therefore, peak 8 is assigned to H1′ of all the nucleotides. Peaks 2 and 3 are the only other resonances with

Table III: Chemical Shift Ranges of H2, H8, and H1′ Hydrogens of Adenine Nucleotides

	chemical shifts (ppm)		
	H8	H2	H1′
~3 mM ATP or ADP[a,b]	8.63–8.52	8.45–8.25	6.18–6.13
adenosine in a dodecanucleotide duplex[c]	8.04	7.30, 7.60[d]	
adenosine in poly(dA-dT)[e]	8.1	7.1	5.6
dense granules in pig platelets	8.16	7.73	5.78

[a] The sample contained twice as much Mg^{2+}. The chemical shift ranges reported correspond to the effects of pH. The two values given for each peak represent the chemical shifts at the end points of pH titration, at 35 °C; the higher numbers correspond to the protonated form. [b] ATP and ADP chemical shifts for these protons are not distinguishable. [c] From Patel et al. (1982). [d] The two values are for the two different adenosine residues in this duplex. [e] From Patel (1979); the shifts are for the helical form at a temperature well below the melting temperature.

Table IV: Chemical Shifts of H7, H2, H4, and H6 Hydrogens of 5HT in Dilute Solution and in an 5HT–ATP Complex, at 35 °C

	chemical shifts (ppm)			
	H7[a]	H2	H4	H6[a]
5HT[b]	7.41	7.28	7.09	6.87
5HT-ATP[c]	6.91	6.99	6.64	6.48

[a] Reported shifts correspond to the center of the doublet observed for these resonances. [b] Reported values are accurate to ±0.01 ppm. pH of the sample was 5.1, and the 5HT concentration was 5 mM. The 5HT chemical shifts in a 200 mM sample were larger by 0.05, 0.03, 0.05, and 0.04 ppm, respectively, for H7, H2, H4, and H6 protons. [c] Calculated by a nonlinear least-squares fit from the data shown in Figure 5 for bimolecular complex formation.

comparable intensities in the chemical shift range of adenine H8 and H2 protons and are assigned accordingly. Peak 3 probably contains a contribution from the H8 proton of the guanine nucleotides; in this case, peak 2 should have ~15–20% less (Table II) intensity in comparison to peaks 3 and 8. This is approximately what is observed. It should, however, be mentioned that because these are convolution difference spectra and because of the base-line problems due to overlapping peaks, accurate comparisons of intensities are not possible.

Peaks 6 and 7 and part of the intensity in peak 5 are assigned to H4, H6, and (H7 + H2) of 5HT, respectively, on the basis of three observations.

5HT is not a good metal chelator nor did it show a propensity for self-aggregation (see Table IV). However, such aromatic amines are known to associate with nucleotides through ring-stacking interactions with formation constants favorable for extensive complex formation (Granot, 1978; Granot & Fiat, 1977). Figure 5 illustrates 5HT chemical shifts in aqueous mixtures of 5HT and ATP as a function of increasing ATP concentration. These data were analyzed by assuming the formation of a bimolecular complex. Previous studies of Granot (1978) on similar biogenic amines justify this assumption. The chemicals shifts calculated for the 5HT aromatic protons in the ATP–5HT bimolecular complex are given in Table IV. The formation constant obtained from the data was 17 M^{-1}, comparable to values reported for catecholamines (Granot, 1978). Mg^{2+}, a cation which exists in large quantities within the pig dense granules, was found to weaken the binding but did not affect the final shifts of the 5HT hydrogens in the complex, again in agreement with ob-

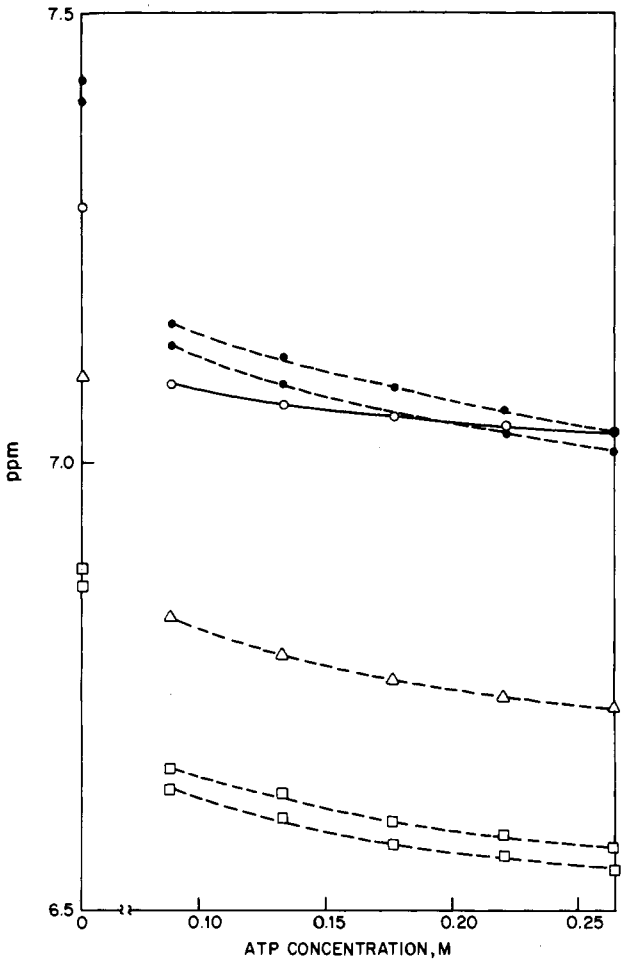

FIGURE 5: Chemical shifts of the 5HT aromatic hydrogens as a function of ATP concentration at 35 °C. (●) H7; (○) H2; (△) H4; (□) H6. H7 and H6 appear as well-resolved doublets due to scalar coupling to other; both peaks of the doublet were plotted for each resonance. 5HT concentration was 5 mM, and sample pH was 5.1.

servations with other amines (Granot & Fiat, 1977).

Comparison of values given in Table II and Table IV shows that the chemical shifts of peaks 5–7 of the intact granule spectrum are very similar to those calculated for the 5HT–ATP complex. It is more relevant to compare the differences in chemical shifts between the different 5HT protons; this is because the chemical shift standard in the granule suspension is located outside the granules in the suspension medium. Therefore, a correction may be required due to possible differences in the diamagnetic susceptibility of the dense granules and the suspension medium. In the solution studies, such a correction is not needed since the reference is truly "internal". In the 5HT–ATP complex, H4, H2, and H7 are at 0.16, 0.51, and 0.43 ppm, respectively, relative to H6 of 5HT (Table IV). In excellent agreement with these values, it is seen that relative to peak 7, whose chemical shift is 0.15 ppm higher than that calculated for H6 of 5HT in the 5HT–ATP complex, peaks 6 and 5 are at 0.17 and 0.46 ppm, respectively.

In addition, dense granules deficient in HA and 5HT were prepared from reserpine-treated pigs. ^{1}H NMR spectrum from these intact granules, and an extract prepared from them, is shown in Figure 6. The extract spectrum confirms the selective depletion of both amines by the reserpine treatment of the animal. Peaks 6 and 7 observed in spectra of normal granules (Figure 1b,c) are absent in the spectrum of intact, reserpine-treated granules (Figure 6). Peak 1 which also vanishes in response to amine depletion is outside the range

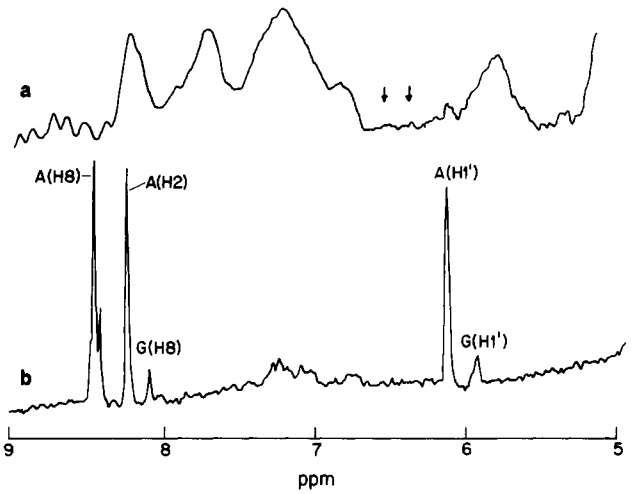

FIGURE 6: ^{1}H NMR spectra at 360 MHz of amine-deficient, intact dense granules from reserpine-treated pigs (a) and an acid extract prepared from them (b). The arrows indicate the positions of peaks 6 and 7 which are present in spectra of normal granules. Spectrum a is the sum of 1200 FIDs obtained with 60° pulses and 2.2-s repetition time. The FID was processed with the convolution difference procedure as described under Materials and Methods. (b) is the sum of 500 FIDs obtained with 60° pulses and 5.5-s repetition time. Both spectra were recorded at 35 °C. The pH of the extract was ~7.2. The intact granule sample was a suspension of organelles.

of chemical shifts expected of aromatic protons of 5HT, but not that of HA (discussed further on). There is a resonance in the spectrum of the amine-deficient granules that is at the position of peak 5 of normal granules. We are, however, unable to compare the intensities of this resonance and peak 5 observed in the spectrum of normal granules due to variations in the contents of the different samples.

Finally, when exogenous 5HT is added to the granule suspension, peaks 6 and 7 increase in intensity (Figure 9). Again, it is not possible to conclude anything about peak 5 from this experiment because it is overlapped by the exogenous 5HT peaks.

These three independent observations are consistent with assigning peaks 6 and 7 to H4 and H6 of 5HT, respectively. Similarity between the chemical shifts of peaks 5–7 and those calculated for the ATP–5HT complex (Table IV) suggests that peak 5 arises from the H7 plus the H2 protons of 5HT. This peak, however, must contain another contribution not related to the amines or the nucleotides; only part of its intensity is affected by increasing osmolarity of the suspension medium (see Figure 8 and discussion further on), and a resonance at this position is present in the amine-deficient granules.

HA has two aromatic hydrogens, H2 and H4. Their chemical shifts are pH dependent; at 35 °C, in dilute solution, H2 resonance moves from 8.65 to 7.72 ppm and H4 from 7.40 to 7.05 ppm as the aromatic ring of HA is converted from imidazolium ion to imidazole form with increasing pH. In solution studies, HA did not show a strong affinity for ATP at pH ~5.5 (which approximates the intragranular pH) and in the presence of Mg^{2+}; no difference in the chemical shifts of H2 and H4 resonances of 25 mM HA were observed in the presence or absence of ~225 mM ATP and ~350 mM Mg^{2+}. If this is also true in the interior of the dense granules, it suggests that HA protons will appear downfield of ~7 ppm. Peak 1 is a resonance which disappears upon removal of the amines and is not in the correct chemical shift range for 5HT. Its position, however, is within the range expected for H2 of HA. Its intensity relative to the nucleotide peaks, within the uncertainties introduced by convolution difference procedure and the overlap with peak 2, is consistent with the HA content

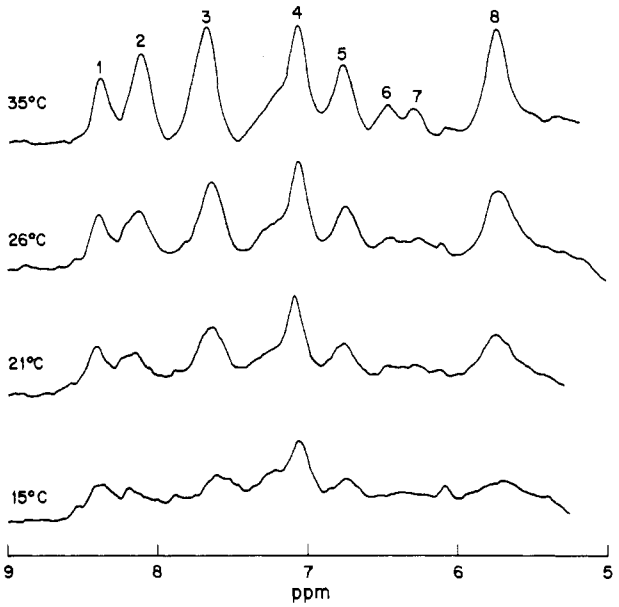

FIGURE 7: Temperature dependence of the ^1H resonances observed from intact dense granules of pig platelets. The sample was a suspension of platelet organelles. All parameters except for temperature were identical with those given for Figure 1a.

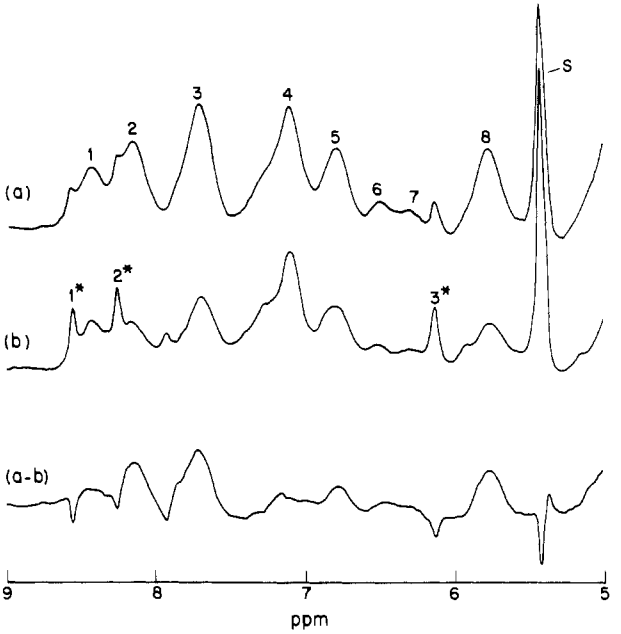

FIGURE 8: ^1H NMR spectra at 360 MHz of purified dense granules before (a) and after (b) addition of KCl into the suspension; (a – b) is the difference. The suspension medium initially contained 300 mM KCl, 30 mM monosodium phosphate, and 0.4% BSA. After spectrum a was recorded, KCl concentration was increased to 600 mM by the addition of a small amount of a concentrated KCl solution. Both spectra are the sum of 400 FIDs obtained at 35 °C with 60° pulses and 2.2-s repetition time. All three are convolution difference spectra.

of the granules (Table II). It is, therefore, tentatively assigned to the H2 proton of HA. On the basis of the chemical shifts, it is possible that the resonance from the H4 proton of HA is part of the large and broad peak labeled 4. However, no evidence for this exists.

Summarizing the peak assignments, it is concluded that peaks 2, 3, and 8 arise from the aromatic protons and ribose H1′ of nucleotides and 6, 7, and part of 5 from aromatic hydrogens of 5HT. These are the only reliable assignments, with several independent pieces of evidence justifying them. Assignment of peak 1 is tentative and remains to be tested by further experiments. It is clear that peak 4 and part of peak 5 contain contributions from proteins; a HA resonance may also fall within the range of these peaks.

Effect of Temperature. It has been shown that in pig platelet dense granules, the line widths of the nucleotide ^{31}P resonances are strongly temperature dependent (Uğurbil et al., 1979; Costa et al., 1980). A similar temperature dependence was also shown for the ^{19}F resonances of 4,6-difluoro-5HT taken up by pig platelet dense granules (Costa et al., 1981). Figure 7 illustrates the effect of temperature on the 5–9 ppm region of the ^1H NMR spectrum of pig platelet dense granules. The resonances assigned to the nucleotides and 5HT show the largest effect, with smaller effects observed for peaks 1 and 4. Temperature can also affect the line widths of the resonances originating from the proteins present in the relatively viscous suspension or associated with the granule membranes. Therefore, it is not surprising that all of the resonances display some degree of dependence on temperature.

In the ^1H NMR spectra (Figure 7), resonances lose intensity as well as become broader when the temperature is lowered. This in part is due to the convolution difference procedure used with these spectra.

Effect of Suspension Medium Osmolarity. The line widths of the ^{31}P resonances of nucleotides contained in pig platelet dense granules show a marked dependence on the osmolarity of the medium in which the granules are suspended (Uğurbil et al., 1984); this phenomenon was attributed to osmotic pressure dependent changes in the internal volume of the granules with consequent changes in the effective solute concentration within the dense granules.

Figure 8 illustrates the effect of increasing osmolarity of the suspension medium on the ^1H NMR spectra of intact granules. The spectra in Figure 8a,b were obtained before and after addition of KCl into a suspension of purified dense granules. The difference of the two spectra is also shown in Figure 8. Narrow peaks 1*, 2*, and 3* and the corresponding unlabeled peaks in Figure 8a are from the nucleotides in the medium. Resonances assigned to the granule-contained nucleotides and 5HT, and peak 1, which is tentatively assigned to HA, all lose intensity in response to the addition of KCl into the suspension medium; unlike the ^{31}P resonances of the nucleotides, line widths of the ^1H peaks do not increase substantially. Little effect is observed on peak 4, and peak 5 loses fractionally less of its intensity than the other peaks which are affected; unlike temperature, increasing KCl concentration approximately 2-fold is not expected to affect the line widths or the intensities of resonances stemming from proteins. These observations are consistent with the peak assignments discussed earlier. They are also indicative of the potentially heterogeneous nature of the physicochemical properties within the dense granules.

Some of the granules apparently break up upon addition of KCl. This is indicated by the small increase in the narrow peaks (Figure 8b) coming from the nucleotides in the medium and the presence of negative peaks at these positions in the difference spectrum. However, this is not sufficient to account for the loss of intensity in the resonances associated with the contents of the granules.

T_1 and T_2. T_1 and T_2 of the observed resonances were measured in suspension of platelet organelles at 35 °C by using progressive saturation and Hahn spin-echo pulse sequence, respectively. They are given in Table I. These data illustrate that the T_1 and T_2 of the resonances observed from the contents of the dense granules is approximately the same for each resonance and that $T_1 \gg T_2$ in each case. In addition, it is seen that intrinsic line widths [which are equal to $1/(\pi T_2)$]

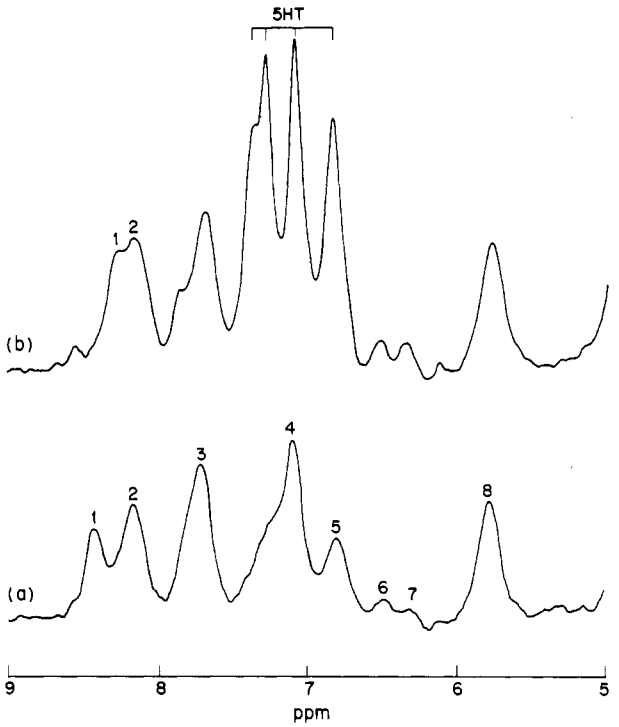

FIGURE 9: Effect of exogenous 5HT on the ^1H resonances observed from dense granules. (a) Before addition of 5HT (control spectrum); (b) after addition of 10 mM 5HT into the suspension. Both spectra are 600 FIDs recorded at 35 °C with 90° pulses and 3-s repetition time and were processed with the convolution difference procedure as described under Materials and Methods. The sample was a suspension of platelet organelles. The four resonances identified as 5HT arise from 5HT outside the dense granules.

calculated from the T_2 values are less than the observed line widths. The line widths (fwhm) measured for the nucleotide peaks 2, 3, and 8 before the application of the convolution difference spectra are ~45 Hz;[3] the intrinsic widths are 16, 17, and 21 Hz, respectively.

5HT Uptake. Using the ^1H NMR spectra, we have monitored 5HT uptake by the dense granules both in suspension of organelles and in the purified preparation in the absence of exogenous ATP. This is illustrated in Figure 9 for a suspension of organelles. Identical results were obtained with a purified preparation.

Upon addition of 5HT into the suspension, we observe an increase in the intensity of resonances 6 and 7, consistent with the assignment of these peaks to the 5HT protons. In addition, peak 1 shifts upfield and largely overlaps peak 2. The magnitude of this upfield shift was found to be proportional to the amount of 5HT added. Furthermore, the effect was reversible; if the granules were collected by centrifugation, washed, and resuspended in 5HT free medium, peak 1 shifted downfield toward its original position. In the sample where this experiment was performed, peak 1 shifted from 8.41 to 8.2 ppm upon 5HT addition and completely overlapped peak 2. Subsequently, when the granules were collected, washed, and resuspended in a 5HT-free medium, peak 1 was observed at 8.35 ppm.

In identical NMR measurements, analogous shifts in peak 1 were not observed upon incubation with HA. Our subsequent studies (M. H. Fukami, K. Uğurbil, and H. Holmsen, unpublished results) indicate that this may be caused by the extremely slow uptake of HA by the dense granules.

[3] The width of the sucrose resonance was subtracted from the observed widths to account for the contribution of magnetic field inhomogeneity over the sample.

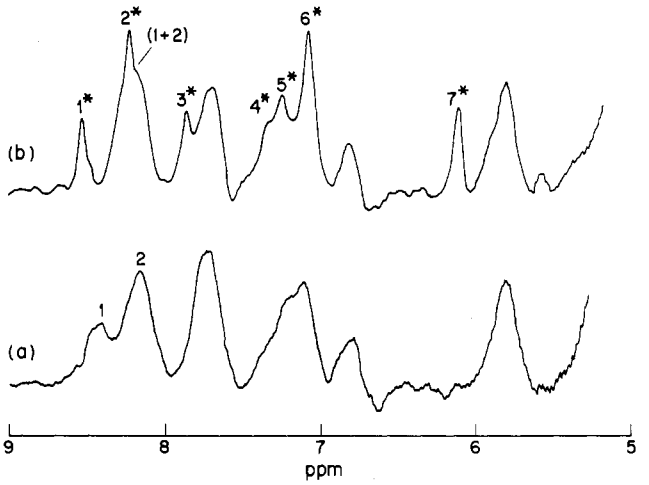

FIGURE 10: Effect of NH_4Cl addition on the ^1H resonances observed from pig platelet dense granules. (a) Before and (b) after addition of 10 mM NH_4Cl. Peaks labeled with an asterisk in (b) stem from nucleotides, HA, and 5HT outside the granules. The sample was a suspension of platelet organelles. Both spectra are convolution difference spectra. They represent the sum of 300 FIDs obtained at 35 °C with 60° pulses and 3.2-s repetition time.

The upfield shifts observed in peak 1, in response to 5HT addition, are ascribed to alkalinization of the dense granule interior concomitant with 5HT uptake and accumulation. This conclusion is based on the observation that NH_4Cl has the same effect on the ^1H NMR spectrum (Figure 10). Ammonium is known to diffuse across membranes in the neutral form as NH_3 and hence can cause alkalinization of vesicle or cell interiors; if NH_4^+ can also diffuse across the membrane, even at substantially diminished rates compared to NH_3, the alkalinization of the vesicle or cell interior would ultimately stop when the transmembrane pH gradient is collapsed. The dense granules were suspended in medium with a pH of ~7.5, and the interior of the dense granule is known to be relatively acidic. pH values of ~5.4 and ~5.7 were reported for the dense granules of pig platelets based on methylamine distribution measurements (Carty et al., 1981; Johnson et al., 1978); in these measurements, NH_4Cl was also shown to substantially diminish the pH gradient across the dense granule membrane.

Addition of NH_4Cl causes some disintegration of the dense granules, as indicated by the appearance of narrow peaks labeled 1* through 7* (Figure 10). The purified granules in particular exhibited extreme sensitivity to NH_4Cl; at ~10 mM concentrations of NH_4Cl, ~60% of the granules appeared to disintegrate.

NOE Measurements. Homonuclear NOE measurements were performed in order to obtain information on the motion and organization of the molecules within the dense granules. The largest effects were seen among peaks 2, 3, and 8. This is illustrated in Figure 11. Figure 11a is the control spectrum where the frequency of the saturation pulse was set 950-Hz downfield of peak 1, at a position devoid of resonances. Parts b and c of Figure 11 are NOE difference spectra where the reduction in the intensities due to NOE and the reduction in the intensity of peak 8 (H1′) due to direct saturation appear as positive peaks. Saturation times were 0.15 s in Figure 11b and 0.5 s in Figure 11c. Large negative NOE's are evident in peaks 2 and 3 and the DHO resonance as well as a group of resonances just upfield of the DHO peak; the chemical shifts of these resonances are in the region of nucleotide ribose protons other than H1′. An NOE on these ribose protons would be expected upon saturation of ribose H1′.

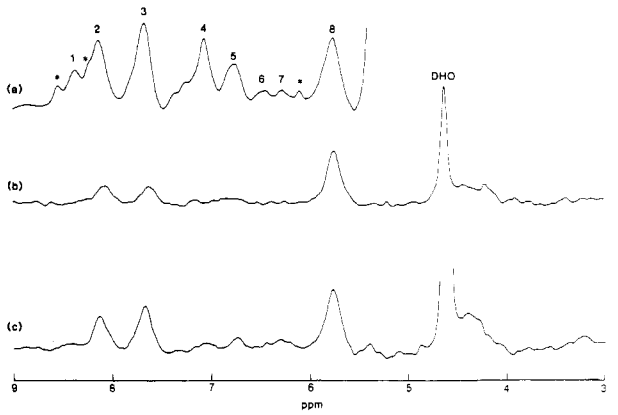

FIGURE 11: NOE at 35 °C, on the ^1H resonances observed from dense granules upon saturation of the nucleotide H1′ resonance (peak 8). (a) Control spectrum; (b) NOE difference spectrum with 0.15-s saturation of H1′; (c) NOE difference spectrum with 0.5-s saturation of H1′. All three spectra were taken from a set of measurements where 10 spectra were recorded by cycling through five different saturation times and a control for each in 16 scan blocks until 240 FIDs were accumulated for each. The difference spectra were obtained by subtracting the FIDs. All are convolution difference spectra. The sample was a suspension of purified dense granules. After a low-power saturation pulse of different durations, a nonselective 90° pulse was applied, and the FID was recorded. A 3.2-s delay was used in all cases between the end of data acquisition and the onset of the low-power saturation pulse. In control spectra the frequency of the saturation pulse was moved 950 Hz downfield of peak 1 to a position devoid of any resonances.

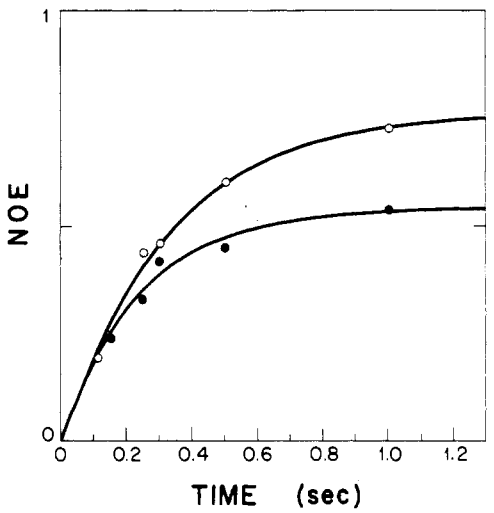

FIGURE 12: NOE on peaks 2 (●) and 3 (○), during saturation of peak 8 for different durations; this was obtained from the same set of data as Figure 11.

In order to establish steady-state levels of NOEs, irradiation times in excess of 1 s were required. Time dependence of the NOE buildup on peaks 2 and 3 during saturation of peak 8 for different durations is shown in Figure 12. The solid lines were obtained by fitting the data to an equation of the form $A(1 - e^{-\rho t})$ where ρ is the spin–lattice relaxation rate constant (Noggle & Schirmer, 1971). The values of ρ obtained for peaks 2 and 3 were 0.25 ± 0.02 and 0.33 ± 0.02 s^{-1}, respectively. Steady-state NOE factors calculated from these data were 0.6 and 0.7 for peaks 2 and 3, respectively. These are very large effects. Upon prolonged saturation of the H1′ peak, NOE's were also observed on the other resonances of the intact granule spectra.

NOE during saturation of resonances other than H1′ was also examined. Irradiation of nucleotide peaks 2 and 3 for periods 0.1 s and longer showed a negative NOE on H1′, as expected. Irradiation of the 5HT resonance peaks 5–7 resulted

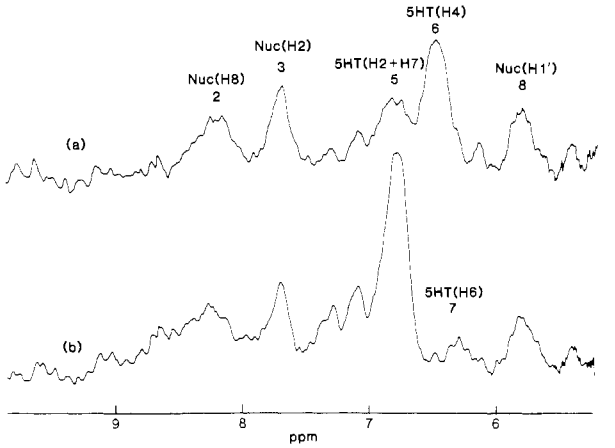

FIGURE 13: NOE difference spectra obtained from dense granules upon saturation of the 5HT H4 resonance (a) and 5HT (H7 + H2) resonances (b). Peaks are numbered according to the notation used in Figure 1a. Saturation pulse was 1 s long. All other parameters as in Figure 11.

in negative NOE's on the nucleotide peaks 2, 3, and 8. This is illustrated for saturation of peaks 5 and 6 in Figure 13. In this case, however, irradiation times of ~0.5 s or longer were required to detect the effect.

Discussion

The ^1H NMR results presented in this paper, together with the ^{31}P NMR studies reported separately (Uğurbil et al., 1984), demonstrate that NMR studies on dense granules can be conducted by using either a suspension of platelet organelles or suspension of purified granules; among the various organelles of the platelet cytoplasm, only the dense granules contain large amounts of nucleotides and amines and are the source of ^1H and ^{31}P signals detected from these molecules. The applicability of the conclusions obtained from studies with these preparations to the situation within intact platelets had already been shown by ^{31}P NMR (Uğurbil et al., 1984).

For most of the NMR measurements, the suspension of the platelet organelles is the preferable sample. In this preparation, the dense granules have not been exposed to high sucrose concentrations, they are in an environment that approximates the intracellular conditions more closely, and they are less susceptible to breakage when exposed to ammonium salts or 5HT. These advantages do not necessarily prevail in studies with other techniques. For example, in measurements of intragranular pH by weak acid or base distribution, a purified preparation is essential because it is impossible to distinguish between the different types of organelles. In these studies, however, caution must be exercised regarding the integrity of the purified granules in the sample. Clearly, stability of such highly purified preparations require the presence of large amounts of protein during and after the isolation procedure, as demonstrated by our data.

Previous studies had shown that the line widths and integrated intensities of the ^{31}P resonances of the nucleotides contained in the dense granules and ^{19}F resonances of 4,6-difluoro-5HT taken up by the dense granules are sensitive to temperature (Uğurbil et al., 1979; Costa et al., 1980). Our results show that a similar dependence is also displayed by the aromatic hydrogens of the nucleotides and the amines. These ^1H resonances are also affected by the osmolarity of the suspension medium just as the ^{31}P resonances of the nucleotides are (Uğurbil et al., 1984); however, the ^1H resonances predominantly lose integrated intensity whereas ^{31}P resonances become broader as the osmolarity increases. These observa-

tions were used for the assignment of the ^1H resonances in this paper; possible causes of these dependences are discussed elsewhere (Uğurbil et al., 1984).

In the absence of unpaired electrons, the primary spin relaxation mechanism for protons is dipole–dipole coupling to each other. Previously, we were unable to detect any paramagnetic ions in dense granules and had excluded this as a possible source of spin relaxation (Uğurbil et al., 1979). Presence of free radicals associated with the amines and the nucleotides in the granule interior is unlikely, given the content of the granules. For protons undergoing spin relaxation through dipole–dipole coupling to each other, the ratio of T_1 and T_2 is independent of the internuclear distance and is given by the expression

$$T_1/T_2 = \tfrac{3}{4}\omega^2\tau_c^2 \quad (1)$$

in the limit $\omega\tau_c \gg 1$, where τ_c is the rotational correlation time and ω is the Larmor frequency (Solomon, 1955). The fact that $\omega\tau_c \gg 1$ limit is applicable to the protons of dense granule contents is shown by the large and negative homonuclear NOE's observed for these spins.

Using an average of T_1 and T_2 values measured for the nucleotide resonances (peaks 2, 3, and 8) at 35 °C (Table I), from eq 1, we calculate a τ_c of 4 ns. This is in good agreement with the τ_c value of 13 ns obtained from the ^{31}P NMR data (Uğurbil et al., 1984). Equation 1 and the analogous expression used for calculating τ_c from the ^{31}P data are valid for isotropic motion and ignore the possible presence of local or internal motion. Hence, the correlation times calculated do not characterize exactly the dynamics of the molecules within the granules; however, they do provide a very useful measure of the average mobility of these molecules.

Several conclusions can be derived from the ^1H NMR data about the interaction among the nucleotides and the amines within the dense granules. The chemical shifts of the aromatic 5HT hydrogens relative to each other in intact granules are in excellent agreement with those calculated for the 5HT–ATP bimolecular complex. Since the 5HT resonances undergo large but unequal upfield shifts upon binding of 5HT to ATP (Table IV), their relative positions are a reliable indicator of complexation with nucleotides. Therefore, the chemical shift data demonstrate that 5HT contained in the dense granules is predominantly bound to the nucleotides. This conclusion is also supported by the existence of an intermolecular NOE observed between 5HT and the nucleotide hydrogens (Figure 13).

The upfield shifts induced by ATP (or ADP) in 5HT resonances arise from ring-current effects in the complex where the 5HT and nucleotide aromatic rings are expected to stack with each other; although it is possible to determine the geometry of the 5HT–nucleotide complex from these ring-current shifts, we have not performed such calculations. However, the geometry of this complex is probably very similar to the complex formed between ATP, ADP, and dopamine, a biogenic amine which has an aromatic moiety. The structure of dopamine–ATP complex was obtained from an analysis of ring-current shift data and involves stacking of aromatic rings as well as hydrogen bonding between the –OH of the amine and nitrogens of the adenine ring (Granot, 1978).

It is clear from the chemical shift data (Table III) that the resonances of the aromatic protons and ribose H1′ proton of the nucleotides contained in the granules are also shifted upfield relative to their position in dilute solution. The magnitudes of the shifts are not accurately determined from the present data. The reason for this is 2-fold: First, as previously mentioned, the granule chemical shifts are referred to a standard in the medium which is "external" to the contents of the granules; consequently, they may need to be corrected before comparing them with shifts obtained relative to a truly internal standard. Second, the chemical shifts of the nucleotide protons of interest are pH dependent through the pK of the aromatic ring; although measurements on the intragranular pH have been reported, we cannot assume that the pK is unaltered within the unusual environment of the granule interior. Our data, however, provide some estimates of these effects. On the basis of the 5HT chemical shifts, a correction of ~0.16 ppm is tentatively suggested to account for the diamagnetic susceptibility of the dense granules. The chemical shifts of the 5HT protons relative to each other in intact granule samples is in excellent agreement with those calculated for the 5HT–ATP complex on the basis of solution studies. However, the shifts of these protons in the granules relative to extragranular TPS is higher from the calculated 5HT–ATP shifts by 0.15 and 0.16 ppm for the H6 and H4 protons, respectively, and by 0.16 ppm for the (H7 + H2) protons; for the last number, an average was taken because H7 and H2 are not resolved from each other in the spectra of intact granules. Thus, there exists a uniform ~0.16 ppm difference for each 5HT proton between the two sets of chemical shifts. This difference is within the range expected for diamagnetic susceptibility corrections; however, further experiments are needed to definitively confirm its origin.

In dilute solution (3 mM) and in the presence of 2-fold higher concentrations of Mg^{2+}, the pH dependence of the aromatic proton resonances of ATP gave a pK of ~4.2. Upon addition of NH_4Cl to dense granule suspensions, the nucleotide peaks 2, 3, and 8 do not shift (Figure 10). Since ammonium is thought to collapse the inside acidic pH gradient across the dense granule membrane (Johnson et al., 1978; Carty et al., 1981; Wilkins & Salganicoff, 1981), this observation suggests that the aromatic rings of the nucleotides in the granule interior are predominantly in the neutral form. This is consistent with the pH values of ~5.4 and ~5.7 reported for the pig platelet dense granules (Carty et al., 1981; Johnson et al., 1978) and suggests that the pK cannot be higher than 4.2 within the dense granules. Therefore, the shifts measured by the dense granules should be compared to the lower of the two values given for 3 mM ATP and ADP in Table III. Using these numbers and the 0.16 ppm correction, we obtain upfield shifts of 0.2, 0.36, and 0.2 ppm, respectively, for the H8, H2, and H1′ resonances of adenine nucleotides within dense granules relative to their position in dilute solution. These are significant shifts, although not as large as those observed with polynucleotide duplexes (Table III).

As discussed further on, the homonuclear NOE measurements on the nucleotide peaks indicate that the nucleotides form a molecular complex in which they are in very close proximity of each other. In such a structure the spins of one nucleotide will experience shifts due to the local magnetic fields generated by the ring currents of the nearby nucleotides. This is probably the primary source of the upfield shifts undergone by the nucleotide protons in the granule interior. Those nucleotides which are stacked with 5HT will also experience upfield shifts due to the 5HT ring currents. However, the nucleotide content of the dense granules is far in excess of the 5HT content (Table II). Consequently, in case of either slow or fast exchange, 5HT effects may not contribute any significant shifts to the resonances of the nucleotide pool. If slow exchange prevails, only a small fraction of the nucleotides will undergo 5HT-induced shifts. In any case, the measurements on dense granules from reserpine-treated pigs provide direct

experimental evidence demonstrating that, within the accuracy of our measurements, interactions either with 5HT or with HA are not responsible for the chemical shifts of the nucleotides; the chemical shifts of the nucleotide peaks 2, 3, and 8 remain virtually unaltered upon selective removal of the two amines.

Irrespective of the origin of the upfield shifts of the nucleotide resonances in spectra of intact granules, it is clear that they reflect the state of the nucleotides within the dense granules. Therefore, the lack of changes in the position of the nucleotide resonances upon the removal of amines demonstrates that the organization of the nucleotides is not dependent on the presence of the amines. This is contrary to original expectations which emphasized nucleotide–amine binding (Berneis et al., 1969a,b; Pletscher et al., 1971) and in agreement with our previous conclusion (Uğurbil & Holmsen, 1981; Uğurbil et al., 1984) based on ^{31}P NMR studies that the interactions between the nucleotides and the amines are *not* the dominant factor responsible for the state of the nucleotides within the granules. This conclusion is remarkable, given the fact that the two amines *together* are 50% as abundant as the nucleotides within the dense granules (Table II); it is, in fact, difficult to explain these results if most of the HA, like 5HT, is bound to the nucleotides. Therefore, we suggest that HA does not interact strongly with the nucleotides and is largely not bound to them in the dense granules through ring-stacking interactions. This would be consistent with the very low affinity displayed by HA toward ATP in aqueous mixtures at pH 5.5 and in the presence of Mg^{2+}.

Information about the arrangement of the nucleotides within the dense granules is derived from the NOE studies. Upon irradiation of the H1′ resonance, a large and approximately equal reduction is observed in the intensities of peaks 2 and 3. As discussed previously, peak 2 is assigned to the H8 of adenine nucleotides, and peak 3 arises from H2 of adenine and possibly from H8 of guanine nucleotides; the relative amounts of the nucleotides (Table II) indicate that the guanine contribution should be <20% of the overall peak intensity. For short irradiation times, in the limit $\omega\tau_c \gg 1$, the magnitude of the NOE is approximated by the simple expression

$$\eta = -\frac{\gamma^4\hbar^2}{10r^6}\tau_c t \qquad (2)$$

where η is the fractional reduction in intensity (NOE), r is the distance between the spins, and t is the irradiation time (Wagner & Wüthrich, 1979). This approximation is valid for the NOE's observed with 0.1–0.2-s saturation periods used in our measurements where H1′ was irradiated; the time dependence of the NOE buildup on peaks 2 and 3 during saturation of H1′ resonance showed a linear dependence up to saturation pulse length of 0.3 s (see Figure 12), and the saturation times required for establishing steady-state levels were in excess of 1 s.

Given the r^{-6} dependence, the approximately equal NOE's on peaks 2 and 3 upon saturation of H1′ indicate that the H8–H1′ and H2–H1′ separation should be approximately the same. If we consider the effect to be exclusively intramolecular, the only geometry compatible with the data occurs in the anti conformation where the H8–H1′ and H2–H1′ distances are 3.8 and 4.4 Å,[4] respectively; in all other conformations, the discrepancy between the H8–H1′ and H1–H1′ distances increases. However, the distances in the anti conformation are too large to account for the magnitude of the observed effects. Because of the r^{-6} dependence, NOE's are generally not observed for $r > 4$ Å. For example, in lysozyme where τ_c is $\sim 2 \times 10^{-8}$ s (Oldfield et al., 1975), 0.25-s saturation pulses produce <1% NOE's for distances over 4 Å (Paulsen et al., 1980), and effects comparable to that observed with peaks 2 and 3 of dense granules occur only for interproton distances of 2 Å or less. Similar results were obtained with tRNA (Tropp & Redfield, 1981) for which τ_c values between 15 and 30 ns have been reported (Schmidt et al., 1980; Komorski & Allerhand, 1972). Furthermore, 3.8- and 4.4-Å separations indicate that upon saturation of H1′ for short periods, the NOE on the H8 resonance should be 2.4 times larger than on H2; this clearly was not the case even considering the ambiguities caused by the guanine nucleotides on the intensity of peak 3. Therefore, we conclude that the NOE's observed on peaks 2 and 3 upon irradiation of H1′ are predominantly *intermolecular* effects which indicate that H1′ of one nucleotide is very close to the H2 and H8 of another. An estimate of the internuclear H1′–H2 and H1′–H8 separation can be calculated from eq 2. The ratio η/t for peaks 2 and 3 obtained for short saturation pulses of 0.1–0.2-s duration in three different samples was 1.8 ± 0.2 and 1.5 ± 0.1, respectively, at 35 °C. By use of these numbers and a τ_c of 4 ns, eq 2 yields 2.3 Å for the internuclear distance from an H1′ to an H8. If we neglect the minor guanine contribution to peak 3, the distance calculated for the internuclear separation between H1′ and H2 hydrogens is larger by 0.1 Å. The τ_c of 13 ns obtained from the ^{31}P data (Uğurbil et al., 1984) yields 2.7 and 2.8 Å for the H8–H1′ and H2–H1′ internuclear distances, respectively. Because the τ_c's used in these calculations were obtained by using equations applicable to isotropic motion only, these distances should not be taken as more than a semiquantitative demonstration that, on the average, the ribose H1′ hydrogen is indeed very close to the H2 and H8 hydrogens within the environment of these vesicles.

The NOE data on the nucleotide peaks provide the first direct evidence for the association of the nucleotides with each other to form a high molecular weight complex within the dense granules. Indirect evidence for the existence of such aggregates was provided by the ^{31}P NMR data (Uğurbil et al., 1979, 1981, 1984; Costa et al., 1979, 1980; Carrol & Schmidt, 1981). Aggregation of the highly charged nucleotide di- and triphosphates cannot possibly occur to any great extent without a mechanism by which the repulsive forces between the negatively charged phosphate moieties are substantially reduced. Therefore, the divalent cations which are present in such large quantities within the dense granules and which have well-known affinities for the phosphate moieties of the nucleotides are probably part of the nucleotide aggregates. We know from the ^{31}P NMR (Uğurbil et al., 1984; Uğurbil & Holmsen, 1981) and ^{1}H NMR measurements (Figure 6) on reserpine-treated, amine-deficient granules that neither 5HT nor HA is necessary for the formation of these nucleotide aggregates. As previously suggested (Uğurbil et al., 1979; Uğurbil & Holmsen, 1981), the interaction of the nucleotides with the different cations sequestered in the dense granules of different species probably accounts for the size of these aggregates. In human dense granules, where the divalent cation is Ca^{2+}, the aggregation is extensive, and the nucleotides together with the Ca^{2+} and possibly 5HT exist in a precipitate-like state; hence, ^{31}P resonances are not detected from the intact dense granules (Uğurbil et al., 1979; Costa et al., 1979). In pig dense granules which contain Mg^{2+}, aggregate size is much smaller, and ^{1}H and ^{31}P resonances can be detected from them at 35 °C. In other animals where a mixture of Ca^{2+} and

[4] These distances were kindly supplied by A. Wang, Massachusetts Institute of Technology, Cambridge, MA, for the C3-endo RNA pucker form.

Mg^{2+} is found, a heterogeneous mixture of nucleotide populations with different degrees of aggregation may exist.

An estimate of the size of the aggregates at 35 °C within the pig platelet dense granules can be obtained from the correlation times. For a hard sphere, the rotational correlation time is directly proportional to the molecular weight (Dwek, 1975). For numerous biological macromolecules, correlation times have been measured by using primarily ^{13}C NMR (Oldfield et al., 1975; Komorski & Allerhand, 1972) and other techniques (e.g., Tao et al., 1970). Given these data, τ_c's of 13 and 4 ns calculated from the ^{31}P (Uğurbil et al., 1984) and ^{1}H NMR data, respectively, for the dense granules nucleotides suggest an average molecular weight of ~10 kilodaltons at 35 °C; this implies that, on the average, the aggregates contain approximately 20 molecules of nucleotides and 5HT, with nucleotides being the dominant component. The existence of most of the nucleotides and the amines as such aggregates can reduce the effective osmolarity within the granules and eliminate the osmotic imbalance that would exist across the vesicle membrane if the components were free in solution.

Uptake and accumulation of biogenic amines into specialized subcellular vesicles have been a subject of general interest. Our results provide a direct demonstration that 5HT uptake causes alkalinization of the dense granule interior (Figure 9), indicating that 5HT uptake is coupled to the inside acidic pH gradient which exists across the dense granule membrane. This is in agreement with previous studies of 5HT transport by pig platelet dense granules (Wilkins & Salganicoff, 1981; Rudnick et al., 1980; Carty et al., 1981; Johnson et al., 1978). 5HT may simply be transported in the neutral form and acquire an H^+ ion once it is in the interior of the dense granule. This would be equivalent to extrusion of an H^+ per 5HT molecule taken up. A higher stoichiometry of coupling to H^+ extrusion as well as coupling to movement of other ions is also possible.

In the absence of proton pumps to maintain the granule interior acidic, 5HT uptake coupled to H^+ extrusion would be limited by the H^+ concentration within the granules. Given the dense granule volume,[5] at a pH of 5.5 only ~8 H^+ ions exist within each dense granule. After a few 5HT molecules traverse the dense granule membrane, these protons would be mostly consumed, raising the granule pH and halting further uptake of 5HT. However, another source of H^+ clearly exists within the dense granules in the compound which gives rise to peak 1 (tentatively identified as HA). This compound is capable of donating H^+ ions in the physiological pH range as demonstrated by Figures 9 and 10; therefore, it acts as a buffer, and alkalinization of the granule interior occurs only after substantial uptake of 5HT. In this case, the extent of 5HT accumulation is limited by the amount of this H^+-donating compound present within the dense granules in the protonated form.

Evidence for the existence of ATP-linked H^+ pumps on the dense granule membrane exists (Wilkins & Salganicoff, 1981; Rudnick et al., 1980; Keyes et al., 1982; Carty et al., 1981). However, it is not known whether these pumps can maintain the granule interior acidic during a rapid influx of 5HT. The presence of the H^+-donating compound within the granules, however, assures large 5HT uptake, even in the absence of an ATP-driven H^+ pump. It is, therefore, suggestive that both H^+ pumps and the presence of an H^+ source in the granule interior may be physiologically important for efficient 5HT uptake. Upon a sudden increase in 5HT concentration outside the dense granules, rapid 5HT influx may initially occur due to the availability of H^+ within the dense granules; subsequently, H^+ pumping can regenerate and maintain an inside acidic pH gradient and ensure further 5HT uptake.

It is interesting to note that the 5HT rapidly accumulated in the granules upon addition of exogenous 5HT can be washed away as indicated by the reversibility of the shift in the position of peak 1. However, this procedure or even longer incubations in dilute, relatively acidic (pH ~5) suspensions did not deplete the 5HT within the dense granules; in all cases examined, 5HT was present in the dense granules and was detectable by NMR or chemical assays. This observation divides the 5HT population within the dense granules into two: those that will exchange rapidly with exogenous 5HT and those that will exchange slowly. From our experiments we are unable to be more specific about the times or the amounts involved. The latter pool gives rise to peaks 6 and 7 and part of peak 5 and is bound to the nucleotides. The former pool accumulates in response to the inside acidic pH gradient and causes alkalinization of the granule interior; 5HT molecules in this pool probably bind to the nucleotides as well. This binding, however, must be more reversible than the binding of the other 5HT pool; this is possible if the amines interact with small aggregates containing fewer nucleotides than the average or with nucleotides located on the surface of larger aggregates.

In the absence of specific and reliable assignments, it has not been possible to obtain extensive information on HA. The NMR data and our uptake studies (M. H. Fukami et al., unpublished results) indicate that HA behaves very differently from 5HT; it is taken up very slowly relative to 5HT and, unlike 5HT, may not be bound to the nucleotides extensively. If the tentative assignment of peak 1 to HA is correct, it indicates that HA predominantly exists in the charged imidazolium form within the granules.

Summary. The ^{1}H NMR data together with the earlier ^{31}P and ^{19}F studies can be summarized as follows. In the dense granules, nucleotides together with the divalent cations form high molecular weight aggregates. The direct evidence for this is the ^{1}H NMR data presented here, and the earlier ^{31}P NMR data are consistent with this conclusion. In Mg^{2+}-containing pig dense granules, the aggregates consist, on the average, of approximately 20 molecules of nucleotides and 5HT at 35 °C; the aggregate size possibly increases at lower temperatures or at higher osmolarity of the extragranular medium. In humans, where Ca^{2+} is the dominant cation, aggregation is extensive, and the resultant structure is solidlike; this is suggested by the ^{31}P NMR data (Uğurbil et al., 1979; Costa et al., 1979) and the behavior of aqueous mixtures of nucleotides and Ca^{2+} ions (Uğurbil et al., 1979). Both in human and in pig platelet dense granules, 5HT is incorporated into these aggregates as shown by ^{1}H NMR data presented here and ^{19}F NMR data on human and pig platelets (Costa et al., 1979, 1980); but the ^{31}P NMR (Uğurbil & Holmsen, 1981; Uğurbil et al., 1984) and ^{1}H NMR data on amine-deficient granules demonstrate that the incorporation of the amines represents a small perturbation on the nature and size of these aggregates. Thus, the nucleotide–cation aggregates appear to act as a matrix to which amine binding occurs. In pig platelets, a large part of the 5HT [i.e., all the 5HT accounted for in chemical assays of isolated granules (Table II)] is bound to the nucleotides and is part of the nucleotide–cation aggregates; it does not rapidly exchange with exogenous 5HT as indicated by the ^{1}H NMR data presented here. On a much shorter time scale, as shown by the ^{1}H NMR data, 5HT can be taken up in response to an inside-acidic pH gradient which exists across the dense granule membrane; this 5HT probably

[5] The volume of the dense granule is calculated by assuming that it is spherical and has an average diameter of 198 nm (Costa et al., 1974).

binds to the nucleotides also, possibly to those molecules located on the surface of the larger nucleotide aggregates, or to nucleotides which are present singly or in clusters containing a few molecules. In human dense granules, most of the binding probably occurs in the form of adsorption onto the nucleotides located on the surface of the solidlike aggregates. These conclusions are probably applicable to most subcellular vesicles which contain large amounts of nucleotides and even larger amounts of a divalent cation. The properties of these organelles are distinctly different from the properties of other subcellular vesicles such as the chromaffin granules where divalent cation content is far less than that of nucleotides and where a highly charged protein is also present in great abundance (Winkler, 1976). In these vesicles, the interactions between the protein and the small molecular weight constituents appear to be responsible for the accumulation of amines and the reduction of osmotic imbalances (Sharp & Richards, 1977).

Acknowledgments

We thank Dr. G. J. Stewart of the Thrombosis Research Center, Temple University, Philadelphia, PA, for the electron micrographs and G. L. Pakstis for excellent technical assistance.

Registry No. 5HT, 50-67-9; ATP, 56-65-5; ADP, 58-64-0; GTP, 86-01-1; GDP, 146-91-8; HA, 51-45-6.

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