Articles

^{31}P NMR Studies of Nucleotide Storage in the Dense Granules of Pig Platelets†

Kâmil Uğurbil,* Miriam H. Fukami,‡ and Holm Holmsen‡

ABSTRACT: Detailed ^{31}P NMR measurements have been conducted on pig platelet dense granules and aqueous mixtures of ATP, ADP, $MgCl_2$, and 5HT. The resonance line widths of the dense granule nucleotides were temperature independent above ~30 °C; below this temperature they exhibit a strong temperature dependence, becoming undetectably broad at ~5 °C. The temperature of transition is determined by the effective solute concentrations within the granules. Spin–spin relaxation time (T_2) and line-width measurements indicate that the ^{31}P resonances of dense granule nucleotides are not homogeneously broadened at 35 and 21 °C. However, the temperature-dependent changes in the intrinsic widths calculated from T_2 values parallel the changes in the measured line widths. From the T_1 and the T_2 data, a rotational correlation time of 13 ns is calculated for the dense granule nucleotides at 35 °C. Removal of 5HT and HA from the dense granules induce significant but relatively small changes in the temperature dependence of the resonance line widths. Analogous effects are seen with a gel phase separated from aqueous mixtures of ATP, ADP, and $MgCl_2$ in the presence or absence of 5HT. These results demonstrate that interactions involving the nucleotides and the divalent cations are predominant in determining the physicochemical state of the granule contents; in pig platelet dense granules the nucleotides and Mg^{2+} form a relatively fluid aggregate which serves as a matrix for 5HT and possibly HA binding. Incorporation of the amines into this matrix tends to increase its fluidity.

Dense granules of platelets are subcellular organelles which sequester high concentrations of nucleotides, divalent cations, and biogenic amines such as 5HT[1] and HA. The type of divalent cation accumulated is species dependent; it is almost exclusively Ca^{2+} in humans and predominantly Mg^{2+} in pigs.

Previous studies with intact platelets have demonstrated that nucleotides contained in the dense granules of human platelets are not detectable in ^{31}P NMR spectra (Uğurbil et al., 1979a; Costa et al., 1979a). In pig platelets (Uğurbil et al., 1979a; Costa et al., 1980), and subsequently in bovine platelets (Carrol et al., 1980), ^{31}P resonances from the granule nucleotides were shown to be detectable at 37 °C; however, the line widths were strongly temperature dependent, becoming undetectably broad at ~5 °C. Analogous results were obtained in ^{19}F NMR experiments with 4,6-difluoro-5HT taken up by the dense granules of pig and human platelets (Costa et al., 1979a, 1980): ^{19}F resonances from difluoro-5HT incorporated into human dense granules were not observed; however, the same molecule in pig dense granules was detectable with strongly temperature-dependent line widths. On the basis of these data, it was suggested that nucleotides together with 5HT exist as precipitate-like, high molecular weight aggregates in human dense granules (Uğurbil et al., 1979a; Costa et al., 1979a) and possibly in a more fluid aggregated state in pig platelet dense granules (Uğurbil et al., 1979a); the difference was ascribed to the difference in the type of divalent cation accumulated by each granule (Uğurbil et al., 1979a).

In this paper, we present detailed ^{31}P NMR measurements on and comparisons among whole pig platelets, isolated dense granules, and aqueous mixtures of nucleotides, Mg^{2+}, and amines; some of the phenomena associated with the ^{31}P resonances of these samples have previously been described qualitatively (Uğurbil et al., 1979a, 1981; Costa et al., 1979a, 1980; Carrol et al., 1980). The experiments presented here were aimed at understanding in greater detail the interactions responsible for the NMR properties displayed by the constituents of these vesicles. These experiments are also complementary to the ^{1}H NMR studies on similar samples which are reported separately (Uğurbil et al., 1984).

Materials and Methods

Platelet Suspensions for ^{31}P NMR. Platelet-rich plasma was prepared as described previously (Salganicoff et al., 1975) by using pig blood collected from a commercial abattoir (Penn Packing Co.) with acid–citrate–dextrose as the anticoagulant solution (Aster & Jandl, 1964). The platelet-rich plasma was diluted 1:1 with ice-cold 0.13 M NaCl/0.02 M Tris-HCl/0.003 M EDTA/0.005 M glucose, pH 7.4 (suspension medium), and centrifuged at 3000*g* for 15 min at 4 °C. The cells were resuspended in 20 volumes of cold suspension medium, sedimented as above, and finally resuspended in suspension medium at a concentration of 36–48 mg of platelet protein/mL.

Preparation of Platelet Organelles in Suspension. Platelet organelles which consisted of mitochondria, α-granules, dense

† From the Department of Biochemistry and Gray Freshwater Biological Institute, University of Minnesota, Navarre, Minnesota 55392 (K.U.), and the Department of Pharmacology and Thrombosis Research Center, Temple University, Philadelphia, Pennsylvania 19140 (M.H.F. and H.H.). *Received April, 26, 1983.* This research was supported by USPHS Grants HL-26089 and HL-14217.

‡ Present address: Department of Biochemistry, University of Bergen, N-5000 Bergen, Norway.

[1] Abbreviations: 5HT, 5-hydroxytryptamine (serotonin); HA, histamine; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; 2DG, 2-deoxy-D-glucose; 2DG-6P, 2-deoxy-D-glucose 6-phosphate; AA, antimycin A; fwhm, full width at half-maximum; GPC, glycerophosphocholine; NOE, nuclear Overhauser effect; CSA, chemical shift anisotropy; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

0006-2960/84/0423-0409$01.50/0

granules, and lysosomes were prepared essentially as described by Salganicoff et al. (1975) with the following modifications. The medium used for both washing and incubation consisted of 0.150 M NaCl/0.060 M Hepes/0.001 M EDTA, pH 6.5. The washed platelets were incubated with 20 μM rotenone, 25 mM 2DG, and 30 mM gluconic acid δ-lactone at 37 °C for 10 min prior to incubation with nagarse and ATP (Fukami et al., 1978). The homogenization or isolation medium consisted of 0.150 M KCl/30 mM Hepes/1 mM EDTA, pH 7.4; no bovine serum albumin was used in the isolation medium. The final suspension typically contained 50–60 mg of protein/mL. For some of the NMR measurements, the organelle suspension was diluted with 30 mM Hepes and 1 mM EDTA so that the final suspension was in 100 mM KCl, 30 mM Hepes, and 1 mM EDTA.

Reserpinized Pig Platelets. Shoats weighing 13–17 kg were injected once daily with 50 μg/kg of reserpine and 0.2 mg of atropine for 2–3 days until mildly toxic symptoms appeared. Subsequently, the administration of reserpine and atropine was discontinued for 1 or 2 days. If at the end of this period, toxic symptoms disappeared, a final regimen of reserpine and atropine was administered, and the animal was terminally bled after 24 h. No more than 7 days passed from initiation of treatment until sacrifice of the animal. Blood was collected from the carotid artery under sodium pentothal anesthesia and processed as described above.

Analysis of Nucleotide and Amine Content. Analysis of the nucleotides was performed by HPLC as described by Daniel et al. (1980) in cell extracts. These extracts were prepared by $HClO_4$ precipitation after granule and platelet suspensions were diluted 10- or 20-fold in suspension medium. Subsequently, the cell debris was removed by centrifugation, the extracts were neutralized with K_2CO_3, and the $KClO_4$ crystals were removed by centrifugation. During the extraction procedure, the samples were maintained between 1 and 5 °C.

Extracts for 5HT and HA were made by diluting an aliquot of the cell or organelle suspension 20-fold in 0.1 N HCl. 5HT was assayed by the σ-phthalaldehyde method described by Drummond & Gordon (1974). HA was assayed by a radioenzymatic assay using histamine methyltransferase (Shaff & Beaven, 1979).

Preparation of ATP–ADP–$MgCl_2$ Gels. For the NMR measurements of ATP–ADP–Mg^{2+} gel phase, the nucleotides plus $MgCl_2$ were first dissolved at an effective concentration of 0.8 and 1.6 M, respectively, at ambient temperature; they initially formed a clear solution with a pH <4. Upon adjusting the pH to ~5.3 with 2 M NaOH, a clear, more viscous and a denser phase was formed. This phase was allowed to settle to the bottom of the tube under gravity and was subsequently transferred to a 5 mm o.d. NMR tube for the NMR measurements. If $MgCl_2$ was replaced by $MgSO_4$, formation of the gel phase was inhibited at the nucleotide concentrations studied. For the preparation of nucleotide–Mg^{2+} gel with 5HT, 0.2 M 5HT was dissolved together with the nucleotides and $MgCl_2$ prior to adjusting the pH to ~5.3.

The distribution of nucleotides between the gel and the solution phases at different temperatures was determined. For these measurements, 1 M stock solutions of ATP or ADP were mixed with appropriate volumes of 4 M $MgCl_2$ to give a nucleotide:Mg^{2+} ratio of 1:2 and subsequently were diluted with H_2O to the desired concentrations. Each mixture was equilibrated either at 0 °C (in ice) or at 37 °C, and pH was brought to 5.7 by careful addition of small volumes of 10 M NaOH under vigorous shaking. The mixtures were kept at the appropriate temperatures for 60 more min, followed by centrifugation (1 min, 12000g). Subsequently, an aliquot of the supernatant was diluted 100–200-fold with 0.1 N HCl. The nucleotide concentration in this last sample was determined by the optical density at 257 nm by using E^{M}_{cm} of 14 700.

NMR Measurement. ^{31}P NMR measurements were conducted at 145.7 MHz on the Bruker WH360 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. ^{1}H decoupling was not employed during these measurements. Chemical shifts were measured by using internal GPC as a standard resonating at –0.49 ppm and are expressed relative to phosphoric acid. T_1 and T_2 values were calculated by a least-squares fit to data obtained by using the progressive saturation technique and the $(90°-\tau-180°-\tau)_n$ spin-echo pulse sequence, respectively.

The nucleotide content of the platelet organelles in suspension prepared for NMR measurements was calculated from the increase in the intensity of the P_i resonance after addition of a known quantity of Na_2HPO_4 (pH ~7) into the NMR sample. Spectra before and after the addition of P_i were obtained with 45° pulses and 10-s repetition time; the long repetition time was used to allow full relaxation of the P_i and the nucleotide resonances.

In suspensions of cells or subcellular organelles, magnetic field inhomogeneity in the NMR sample may constitute a significant fraction of the observed line widths. This is because these samples are not spun and because the magnetic susceptibility is not necessarily uniform throughout the sample. In measurements of line widths as a function of temperature, this inhomogeneous width contribution was accounted for by using the GPC and P_i resonances in suspension of intact cells and platelet organelles, respectively. As previously mentioned, GPC was added into the suspensions as a chemical shift standard; its T_2 was measured to be 0.18 s in a suspension of intact cells. In ^{31}P spectra of platelet organelles in suspension, a small P_i resonance stemming exclusively from P_i present in the extragranular volume was always present (Figure 1); in some cases, a small amount of P_i (<5 mM) was added into these samples. In one sample of organelles in suspension, the P_i resonance had a T_2 of 0.4 s. The intrinsic line widths[2] calculated from these T_2's are 1.8 and 0.8 Hz, for the GPC and the P_i resonances, respectively. The observed line widths for these peaks, however, were typically 25–30 Hz (fwhm, *excluding* the contribution from the exponential filter) in both intact cell and organelle suspensions. Consequently, the width of these peaks was taken as a measure of inhomogeneous widths in our samples and subtracted from the observed line widths of the nucleotide resonances. In the spectra of intact cell suspensions, a P_i resonance was also evident; however, this resonance was not a suitable measure of magnetic field inhomogeneity. This is because in intact cell suspensions both intra- and extracellular P_i was present, and in most cases the chemical shifts of these P_i resonance were not identical. Consequently, the line width of the observed P_i resonance stemming from the partially overlapping intra- and extracellular P_i peaks was greater than the line width of either resonance alone.

Results

In a ^{31}P NMR spectrum of intact pig platelets in suspension, ^{31}P resonances of both the cytoplasmic and the granule-con-

[2] Intrinsic line width = $1/(\pi T_2)$.

Table I: T_1, T_2, and Line Widths of the ^{31}P Resonances Observed from the Nucleotides Contained in the Dense Granules of Intact Pig Platelets at 145.7 MHz

	$(ATP_\gamma + ADP_\beta)$	ADP_α	ATP_α	ATP_β
T_1 (s),[a] 35 °C	0.95 ± 0.03	0.90 ± 0.07	0.86 ± 0.04	0.79 ± 0.03
T_1 (s),[a] 15 °C	1.04 ± 0.06			0.89 ± 0.08
T_2 (ms),[b] 35 °C	9.6	9.4	9.2	9.0
T_2 (ms),[b] 21 °C	6.2	*c*	7.6	5.4
$\Delta\nu_{1/2}$ (Hz),[d] 35 °C	60 ± 5			51 ± 5
$\Delta\nu_{1/2}$ (Hz),[d] 21 °C	95 ± 10			

[a] The errors quoted are standard deviations calculated from the least-squares fit to the progressive saturation data. [b] The standard deviation for the T_2 values obtained after the least-squares fit of the T_2 data was approximately ±5% at 37 °C and ±10% at 21 °C. Due to possible systematic errors in spin-echo T_2 measurements, we estimate the overall accuracy of the given T_2 to be no better than 25%. [c] T_2 values for the ADP_γ peak was not obtained due to overlap between this peak and the much larger ATP_γ peak at this temperature. Qualitatively, it appeared to have approximately the same T_2 as the ATP_γ peak. [d] The line width of the GPC resonance was taken as a measure of the field inhomogeneity contributions and was subtracted from the observed line widths. The line widths given represent the average of three measurements performed on three different samples. The errors given are the standard deviation from the mean for the three measurements.

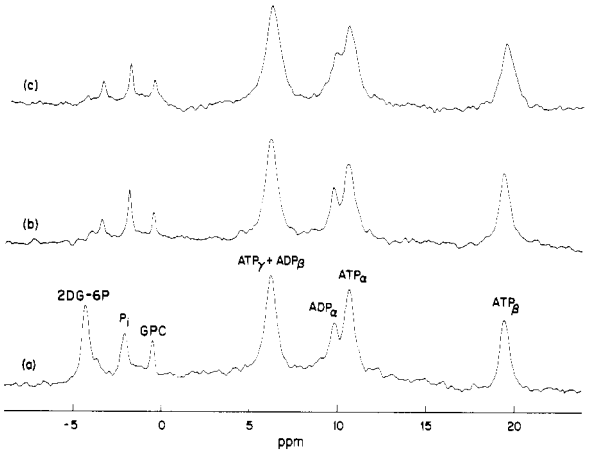

FIGURE 1: 145.7-MHz ^{31}P NMR spectra of intact platelets (a), suspension of platelet organelles (b), and purified dense granules (c) at 35 °C. The cytoplasmic nucleotides of intact platelets were depleted with 2DG and AA treatment prior to recording these spectra. The spectra were obtained with 45° pulses and 0.64-s repetition time and consist of 1000 scans for (a), 400 scans for (b), and 500 scans for (c). All samples contained GPC as the chemical shift reference. 2DG-6P is 2-deoxy-D-glucose 6-phosphate. The unlabeled peak at ~ −4.0 ppm in (b) and (c) is probably AMP and/or IMP.

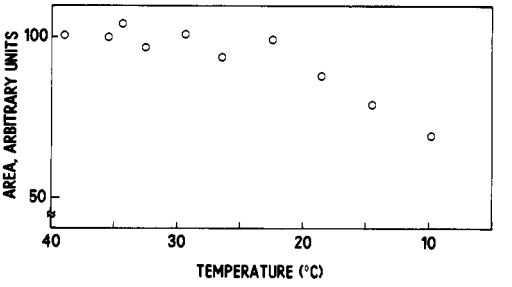

FIGURE 2: Integrated intensity of the $(ATP_\gamma + ADP_\beta)$ resonance of dense granule nucleotides as a function of temperature. Data were obtained from intact platelets lacking the cytoplasmic nucleotides by using 45° pulses and 3-s repetition time.

tained nucleotides are detected (Uğurbil et al., 1979a; Costa et al., 1980); the two sets of nucleotide resonances have different chemical shifts and are partially resolved from each other at 145.7 MHz. Upon selective depletion of the cytoplasmic nucleotides using metabolic inhibitors, well-resolved ^{31}P resonances can be observed from the nucleotides of the dense granules (Uğurbil et al., 1979a). Figure 1 illustrates three typical ^{31}P NMR spectra obtained at 35 °C from intact pig platelets lacking the cytoplasmic nucleotides (Figure 1a), from a suspension of platelet organelles (Figure 1b), and from a highly purified and stable preparation of dense granules (Figure 1c). Even though the preparation of subcellular organelles contains all cytoplasmic organelles, only the dense granules contain very high concentrations of nucleotides. The purified granules were prepared as described in Uğurbil et al. (1984) and were suspended in an ~600 mosM medium for stability. The high salt concentration causes the resonances to be broader in these samples (see Discussion below); otherwise, all three spectra look virtually the same, thus confirming the assignment of the resonances in the spectrum of whole cells (Figure 1a) and the suspension of all cytoplasmic organelles (Figure 1b) to the nucleotides contained in the dense granules. ^{31}P spectra from a suspension of platelet organelles were previously reported (Carrol et al., 1981; Uğurbil & Holmsen, 1981). This, however, is the first direct demonstration that a sample of highly purified dense granules yields the same set of resonances observed in intact platelets and in suspensions of organelles.

Specific assignments of each resonance is based on chemical shifts of ATP, ADP, and $MgSO_4$ at different solute concentrations (Uğurbil & Holmsen, 1981); in these mixtures, ^{31}P chemical shifts of ATP and, in particular, ADP were found to be strongly concentration dependent. At high concentrations (>200 mM at 37 °C), they approach the chemical shifts observed from intact dense granules (Uğurbil & Holmsen, 1981).

In the suspension of platelet organelles prepared for NMR studied, the absolute concentration of the nucleotides was measured on the same samples both by HPLC analysis of $HClO_4$ extracts and by NMR. For the NMR determinations, the integrated intensities of the nucleotide ^{31}P resonances were calibrated by using fully relaxed spectra obtained before and after addition of a known quantity of P_i into the samples. On two separate samples, total concentration of nucleotide di- and triphosphates was measured to be 6.0 and 5.9 mM by NMR, and 5.7 and 5.8 mM by HPLC analysis of $HClO_4$ extracts, respectively. This indicates that all of the nucleotides within pig platelet dense granules contribute to the NMR spectrum at 37 °C. In order to ascertain whether all of the nucleotides remain detectable in the NMR spectrum at lower temperatures, we measured the integrated intensities of the $(ATP_\gamma + ADP_\beta)$ resonance as a function of temperature in suspensions of normal platelets using fully relaxed spectra obtained with 45 °C pulses and 3-s repetition time (Figure 2). It is seen that the area of this peak remained constant as the temperature was lowered from ~37 to ~20 °C. Below 20 °C, however, the area diminished with decreasing temperature. This shows that below 20 °C, as the temperature is lowered, increasing number of nucleotides no longer contribute to the intensities of the observed ^{31}P resonances.

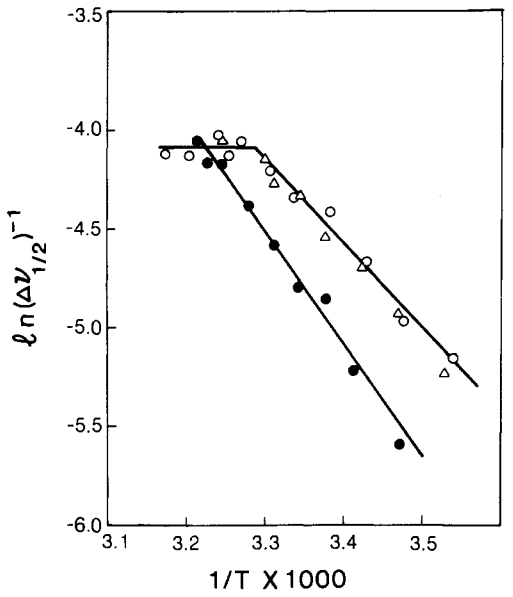

FIGURE 3: Plot of ln $(\Delta\nu_{1/2})^{-1}$ (where $\Delta\nu_{1/2}$ = fwhm) vs. T^{-1} of (ATP_γ + ADP_β) resonance observed from the nucleotides contained in the dense granules of intact normal platelets (○, △) and intact reserpine-treated platelets (●). Data were obtained by using platelets depleted of cytoplasmic nucleotides by treatment with 2DG and AA. The straight lines drawn through all the points for the reserpine-treated cells and through points at 30 °C and less for the normal cells were obtained by a least-squares fit to these data points. For the normal cells, only the (○) points were used for the fit.

Table I gives the T_1's, T_2's, and line widths ($\Delta\nu_{1/2}$) for the ^{31}P resonances of nucleotides contained in the dense granules of intact platelets in suspension. As discussed under Materials and Methods, the $\Delta\nu_{1/2}$'s given for the nucleotide resonances were obtained by subtracting the $\Delta\nu_{1/2}$ of the GPC resonance from the observed line widths of the nucleotide resonances. At both 35 and 21 °C, these $\Delta\nu_{1/2}$'s are approximately half the intrinsic line widths[2] calculated from the independently measured T_2's at the corresponding temperature (Table I).

Figure 3 shows the temperature dependence of the (ATP_γ + ADP_β) resonance line widths of granule-contained nucleotides in platelets from normal and reserpine-treated (discussed below) pigs. The ATP_β, ATP_α, and ADP_α resonances are also broadened with decreasing temperatures in a similar fashion to the (ATP_γ + ADP_β) peak (Uğurbil et al., 1979a). The most accurate data on the temperature dependence, however, are obtained from the (ATP_γ + ADP_β) resonance. The data presented in Figure 3 were measured by using intact cells in which the cytoplasmic nucleotides were depleted by using 2DG and AA. The variation of the line widths with temperature was completely reversible and quantitatively very reproducible between different samples prepared at different times. This reproducibility is illustrated in Figure 3, where two different sets of data from two different preparations of normal platelets were plotted together. Results obtained from similar measurements of line widths as a function of temperature using platelet organelles suspended in 100 mM KCl and 30 mM Hepes were virtually identical with those obtained from intact platelets in suspension (see Figure 8).

The ^{31}P resonances of dense granule nucleotides in reserpine-treated platelets were examined in order to determine the effect of the endogenous amines contained in the dense granule on the temperature dependence displayed by the phosphate moieties of the granular nucleotides. Our recent ^1H NMR studies (Uğurbil et al., 1984) demonstrated that, like the rabbit and guinea pig platelets (DaPrada et al., 1981), pig platelets sequester 5HT and HA within their dense granules (Uğurbil et al., 1984). From four separate determinations, 5HT and HA content of normal pig platelets was found to be 7.4 ± 1.3 and 12.4 ± 3.3 nmol/mg of platelet protein, respectively. Platelets obtained from reserpine-treated pigs were highly deficient in both amines. In one sample from a reserpine-treated pig, 5HT content was 0.3 nmol/mg of platelet protein. In a separate but identical preparation, 5HT and HA content was found to be 0.8 and 1.6 nmol/mg of platelet protein,[3] respectively.

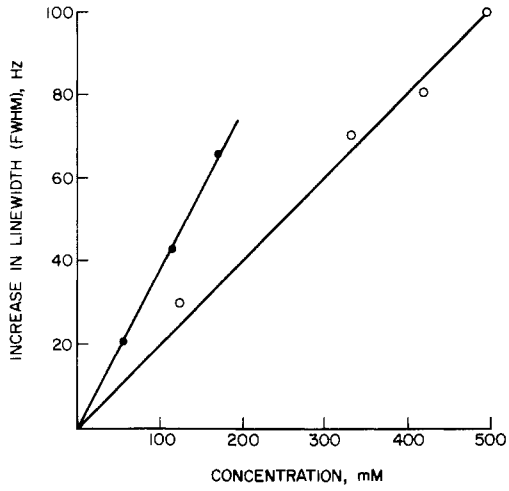

FIGURE 4: Increase in the line width of the (ATP_γ + ADP_β) resonance of dense granule nucleotides as a function of increasing concentrations of NaCl (●) and sucrose (○). Data were obtained at 28 °C by using platelet organelles suspended originally in 100 mM KCl and 30 mM Hepes, pH 7.4.

The ^{31}P NMR spectra obtained at 37 °C from a suspension of reserpine-treated platelets preincubated with AA and 2DG were identical with the spectrum shown for normal platelets (Figure 1a); the chemical shifts of the resonances and their relative intensities were not altered as a consequence of the reserpine treatment. The ^{31}P resonance line widths of the granule nucleotides in these amine-deficient platelets still exhibited a temperature dependence (Figure 3). At a given temperature, the line widths were greater in the absence of the amines. In normal platelets, the $\Delta\nu_{1/2}$'s of the granule nucleotides were independent of temperature above 31 °C, and a sharp transition occurred to a domain in which ln $(\Delta\nu_{1/2})^{-1}$ was linearly dependent on T^{-1}. We will refer to the temperature at which this type of transition in the line widths occurs as the transition temperature. In platelets from reserpine-treated pigs, the line widths were temperature dependent at all temperatures examined. If we assume that in the temperature-independent domain, the line widths would be the same in both the normal and reserpine-treated platelets, then extrapolation of the data yields ~37 °C as the transition temperature in the amine-deficient granules.

The ^{31}P line widths of the dense granule nucleotides also exhibited a dependence on the osmolarity of the medium surrounding the granules. Figure 4 shows the change in the

[3] In this case, the 5HT and HA content was assayed in a suspension of platelet organelles and was found to be 3 and 5.9 nmol/mg of protein, respectively. The numbers given in the text were converted to units of nanomoles per milligram of total platelet protein by using the following data: normally, 5HT and HA content was found to be 24.0 ± 5.5 and 33.2 ± 7.5 nmol/mg of protein, respectively (average of four preparations), in suspension of platelet organelles and 7.4 ± 1.3 and 12.0 ± 3.3 nmol/mg of total platelet protein, respectively, in whole cell suspensions. Thus, HA and 5HT content in units of nanomoles per milligram of protein in the platelet organelles is enriched on the average by a factor of 3 compared to a suspension of whole cells.

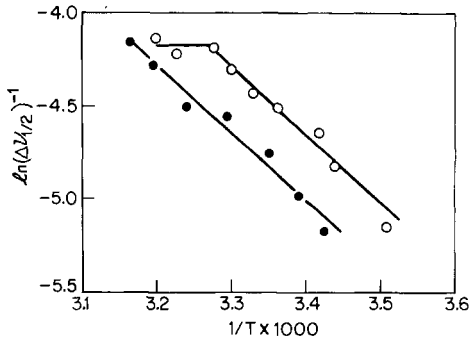

FIGURE 5: Plot of ln $(\Delta\nu_{1/2})^{-1}$ ($\Delta\nu_{1/2}$ = fwhm) vs. T^{-1} for the (ATP_γ + ADP_β) resonance observed from a suspension of platelet organelles in 100 mM KCl and 30 mM Hepes (O) and in 150 mM NaCl, 100 mM KCl, and 30 mM Hepes (●).

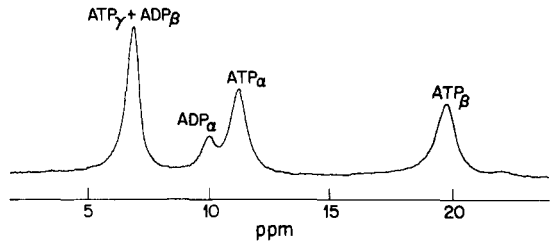

FIGURE 6: ^{31}P NMR spectrum at 145.7 MHz and 41 °C of the gellike phase separated from ATP, ADP, and $MgCl_2$ mixtures.

line width of the (ATP_γ + ADP_β) peak at 28 °C as NaCl or sucrose was added to a suspension of platelet organelles; the suspension medium initially contained 100 mM KCl and 30 mM Hepes (pH 7.4). The line widths increased linearly with increasing NaCl or sucrose concentration; however, the increase was approximately twice as rapid with NaCl compared to sucrose, indicating that the effect is osmotic in nature. Figure 5 shows the temperature dependence of the (ATP_γ + ADP_β) peak of the granule nucleotides observed from a suspension of platelet organelles under normal suspension conditions and in the presence of additional NaCl. This figure illustrates that the slope of the ln $(\Delta\nu_{1/2})^{-1}$ vs. T^{-1} plot was not affected by the additional NaCl; however, the transition temperature was shifted to higher temperatures.

The existence of a sharp transition from a temperature-independent to a temperature-dependent domain in ln $(\Delta\nu_{1/2})^{-1}$ vs. T^{-1} plots of granule nucleotides suggested the existence of a phase transition or a phase separation. It has previously been observed that in aqueous mixtures of ATP, ADP, and $MgCl_2$, a gellike, clear and viscous phase separates above a certain concentration. We have examined the temperature dependence of line widths in this gellike phase separated from ATP, ADP, and $MgCl_2$ mixtures. A small P_i peak was always present in this gel. The line width (fwhm) of this resonance was used as a measure of the inhomogeneous width and was subtracted from the widths of the nucleotide resonances. In some of these gel samples, GPC was included as a chemical shift reference. Figure 6 shows a spectrum obtained from such a gel at 41 °C. Chemical shifts are similar to those observed for ATP, ADP, and Mg^{2+} mixtures at high concentrations prior to gel formation (Uğurbil & Holmsen, 1981) and for the nucleotides in intact granules (Figure 1). A gel phase was also obtained from an aqueous mixture of ATP, and $MgCl_2$, in the presence of 5HT. At a given temperature, this gel was visibly less viscous than the one which lacked 5HT. The temperature dependence of the line widths observed from these gels in the presence and absence of 5HT are shown in Figure 7. It is clearly seen that the presence of 5HT results in narrower line widths at a given temperature as well as a reduction in the

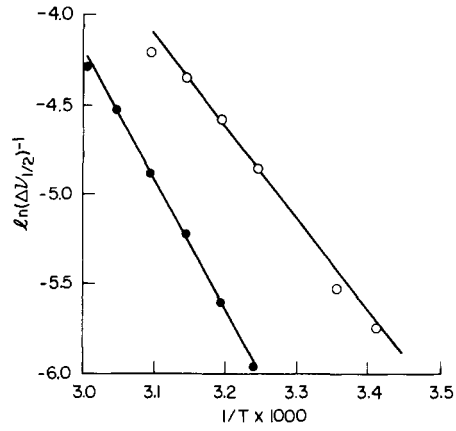

FIGURE 7: Plot of ln $(\Delta\nu_{1/2})^{-1}$ ($\Delta\nu_{1/2}$ = fwhm) vs. T^{-1} for the (ATP_γ + ADP_β) resonance of the gellike phase separated from aqueous mixtures of ATP, ADP, and $MgCl_2$ with (●) and without 5HT (O).

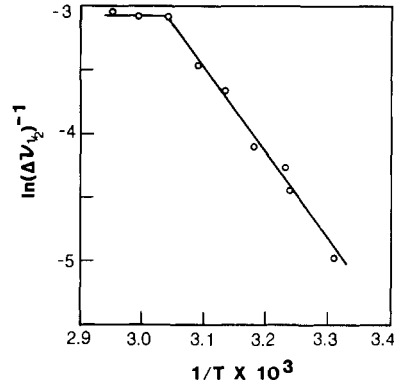

FIGURE 8: Plot of ln $(\Delta\nu_{1/2})^{-1}$ vs. T^{-1} for the ATP_γ resonance of the gellike phase separated from aqueous mixtures of ATP and $MgCl_2$.

slope of the ln $(\Delta\nu_{1/2})^{-1}$ vs. T^{-1} plot. At sufficiently high temperatures, the ^{31}P line widths observed from these gels became temperature independent. This is illustrated for a different sample of ATP–Mg^{2+} gel in Figure 8.

The concentration and temperature dependence of the separation of the denser gel phase was studied in aqueous mixture of nucleotides and Mg^{2+}. Figure 9 shows the concentration of ATP and ADP in the solution fraction ("supernatant") of aqueous mixtures of nucleotide and Mg^{2+}. At 37 °C ATP goes into solution up to ~200 mM concentration; further addition of ATP results in the separation of a gel phase and does not lead to an increase in the concentration of this nucleotide in the solution phase. At 0 °C a similar behavior is observed; however, the critical concentration above which a gel phase is separated is now ~100 mM. On the basis of these data, it can be inferred that a solution of ATP, ADP, and Mg^{2+} will exhibit a phase separation at a specific temperature which depends on the overall concentration of solutes. If the sample contains ~200 mM ATP (and 2 × Mg^{2+}), it will be a solution at or above 37 °C. But below 37 °C a gel phase will appear. At 0 °C, approximately half of the nucleotides of this sample will be in the denser gel phase.

Discussion

Our data demonstrate that above ~20 °C all of the nucleotides in dense granules of pig platelets contribute to the observed ^{31}P resonances. This is in direct contrast with observations on the Ca^{2+}-containing human dense granules, where virtually none of the nucleotides are detected in the ^{31}P NMR spectra (Uğurbil et al., 1979a; Costa et al., 1979a). Platelet dense granules of other species which sequester com-

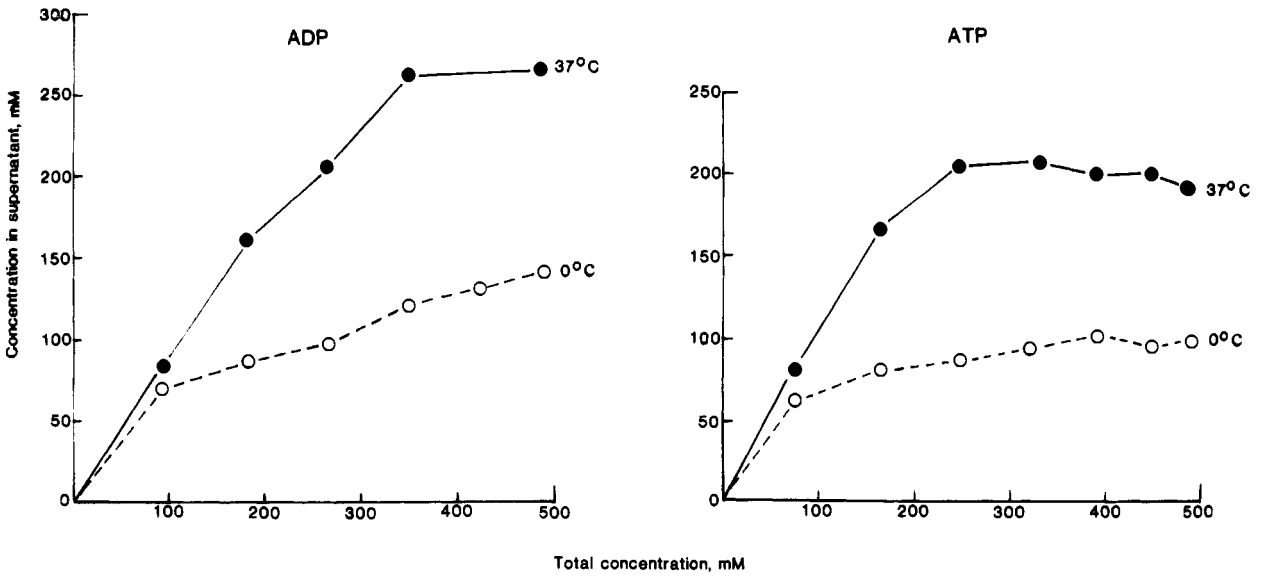

FIGURE 9: Partitioning of ATP and ADP between gel and solution phases as a function of average effective concentration in the gel plus solution phases (total concentration) at 0 and 37 °C in the presence of Mg^{2+} and at pH 5.7 (see Materials and Methods for details).

parable amounts of Ca^{2+} and Mg^{2+} (Schmidt & Carroll, 1982) may fall in between these two species and contain a mixture of NMR "detectable" and NMR "undetectable" nucleotides.

According to the T_2 and line-width measurements at 35 and 21 °C, the resonances are not homogeneously broadened, and approximately half of the observed line widths arise from inhomogeneities in the magnetic environment of the nucleotides. For the (ATP_γ + ADP_β) peak, at 35 and 21 °C, the intrinsic line widths calculated from the T_2 values are 33 and 55 Hz, respectively, whereas the observed widths are 60 and 95 Hz, respectively (Table I). Similarly, at 37 °C, the homogeneous width of the ATP_β peak is 35 Hz, 16 Hz less than the width measured from the spectra (Table I). These values for the observed and intrinsic widths are generally in good agreement with those reported at 30 °C for these resonances in pig platelets dense granules (Schmidt & Carrol, 1982). Since the nucleotide ^{31}P chemical shifts are sensitive to pH and solute concentrations (Uğurbil & Holmsen, 1981), the inhomogeneous contributions to line width probably reflect a variation of these parameters among the individual granules. Even though the measured line widths do not completely represent homogeneous widths, it is seen that the increase in line widths with decreasing temperature is paralleled by a quantitively similar increase in the intrinsic widths calculated from the T_2 values (Table I).

Chemical exchange between Mg^{2+} and/or Ca^{2+} bound and free forms of the nucleotides has been suggested as a possible explanation for line widths and their temperature dependence in ^{31}P NMR spectra of intact dense granules (Schmidt & Carrol, 1982); this clearly appears to be valid in the case of cattle and rabbit dense granules (Schmidt & Carrol, 1982). However, with pig dense granules, the T_2's and line widths for all three phosphates are approximately the same, and all show a change in both parameters with temperature. This is not expected from chemical exchange type of broadening of the ^{31}P resonances of nucleotides since α-phosphate line widths are virtually independent of chemical exchange between metal-free and -bound forms. Furthermore, ^{19}F resonances of the 4,6-difluoro-5HT taken up by the pig platelet dense granules (Costa et al., 1981) and ^1H resonances of the nucleotides and the amines (Uğurbil et al., 1984) contained in the pig platelet dense granules all exhibit similar temperature dependence of line widths. Therefore, we suggest that chemical exchange contribution to the observed line widths is of minor importance in pig platelet dense granules.

At high magnetic field strengths, and for moderately large molecules (e.g., tRNA and phospholipids), chemical shift anisotropy (CSA) is a dominant relaxation mechanism for ^{31}P nuclei (Gueron & Shulman, 1975; McLaughlin et al., 1975). Assuming this is the case for the phosphates of the nucleotides contained in the dense granules, the rotational correlation time τ_c can be calculated from the T_1 and T_2 data. When spin relaxations occur exclusively through CSA

$$T_1/T_2 = 7/6 + 2/3\omega^2\tau_c$$

where ω is the Larmor frequency of the ^{31}P nuclei (Abragam, 1961). This is a convenient equation to use because it does not specifically require a knowledge of the magnitude of the CSA. By use of the average of the T_1 and T_2 values for the (ATP_γ + ADP_β), ATP_α, and ATP_β (Table I), τ_c is calculated to be 1.3×10^{-8} s at 35 °C. This number is in good agreement with the τ_c of 0.4×10^{-8} s obtained from the ^1H NMR studies (Uğurbil et al., 1984). These τ_c values are several orders of magnitude longer than what would be observed for nucleotides in dilute solution. They are, however, typical of macromolecules with molecular weights in the 8000–15 000 range. For example, τ_c's of 12 and 17 ns have been reported (Oldfield et al., 1975) for horse heart cytochrome *c* and lysozyme. Homonuclear NOE measurements in suspensions of intact dense granules (Uğurbil et al., 1984) have demonstrated that the nucleotides form a structure in which they are in very close proximity of each other. In such an aggregate structure, τ_c will be long due to the high apparent molecular weight. The ^1H NMR data have also provided direct evidence that 5HT is part of these aggregates.

From 35 to 15 °C, T_1's remain the same within experimental error (Table I). From these T_1's and the T_2 measured for the (ATP_γ and ADP_β) resonance at 21 °C, a somewhat longer τ_c (1.6×10^{-8} s) is calculated at 21 °C. We could not measure T_2's at lower temperatures due to diminished peak heights, large line widths, and generally very rapid transverse relaxation. It is reasonable to assume that, as in going from 35 to 21 °C, T_2's continue to diminish, and the line widths continue to increase as the temperature is further decreased. This would imply even longer τ_c's at these lower temperatures. In fact, below 20 °C, some of the nucleotides do not contribute intensity to the observed ^{31}P peaks, indicating τ_c of $>10^{-6}$ for this population.[4]

Several explanations can be given for the reduction in the mobility of the dense granule nucleotides as the temperature is lowered. These include (1) an increase in the size of the aggregates at temperatures below the transition temperature, (2) a decrease in the motion of the phosphate moieties within these aggregates due to a reorganization of the aggregate structure below the transition temperature, or (3) a high activation energy of rotation within the dense granules below the transition temperature. On the basis of existing data, it is not possible to distinguish between these explanations. It may in fact be that all three are simultaneously responsible for the effect. Irrespective of the correct explanation, the interactions responsible for the decrease in the mobility of the nucleotides at lower temperatures has a discontinuous temperature dependence. Only below 31 °C the line widths are temperature dependent. A similar discontinuous change in line widths has also been reported for the ^{31}P resonances detected from nucleotides in the dense granules of bovine platelets; in this case, however, the transition temperature was ~20 °C (Carroll et al., 1980). Our data on granule suspensions at different osmolar environments (Figure 4) show that the temperature at which the discontinuity occurs depends directly on the effective solute concentration within these granules; it is also affected by the presence or absence of 5HT and possibly HA (Figure 3). The variations in transition temperature between the Mg^{2+} containing dense granules of different species can therefore be ascribed to possible differences in the amount of nucleotides and amines sequestered within these granules.

ATP and ADP form fluid gels at high concentrations and in the presence of Mg^{2+}. Appearance of such a phase in aqueous mixtures of these molecules depends on the effective solute concentration and temperature (Figure 9). In this gel phase, the ^{31}P resonances of the nucleotides exhibit a temperature dependence which is remarkably similar to that observed for the nucleotides within the granules. In fact, with this gel, if the temperature is increased to ~70 °C (in the absence of 5HT), the ^{31}P line widths also become temperature independent (Figure 8). These similarities suggest the possibility that nucleotides in the dense granules are in a state very similar to the nucleotide gels. Below the transition temperature they are in a viscous environment and yield ^{31}P resonances with temperature-dependent line widths; at a certain temperature, the fluidity in this environment increases discontinuously, and the nucleotide ^{31}P resonances become temperature independent.

Removal of endogenous 5HT and HA does not affect the ^{31}P chemical shifts of the dense granule nucleotides. It is clearly seen that in both amine-containing and amine-deficient dense granules, the nucleotide resonances display a temperature dependence; the differences in the ln $(\Delta\nu_{1/2})^{-1}$ vs. T^{-1} plots between the two are significant but relatively small. Furthermore, ^{1}H NMR studies have shown that the chemical shifts of H2, H8, and H1′ protons of the dense granule nucleotides were not altered upon removal of the 5HT and HA (Uğurbil et al., 1984). These observations indicate that interactions involving the nucleotides and the divalent cations are primarily responsible for the physical properties of the intragranular contents. Accumulation of the amines changes these properties only slightly. This is contrary to the earlier expectations which emphasized the affinity between the nucleotides and 5HT (Berneis et al., 1969a, 1970; Pletscher et al., 1971). Unlike the nucleotides and the divalent cations, intragranular endogeneous 5HT can traverse the granular membrane rapidly; consequently, its concentration in the granule can fluctuate (Costa et al., 1979b). If 5HT–nucleotide interactions were dominant and responsible for formation of aggregates, then the extent of aggregation and hence the osmotic imbalance across the dense granule membranes would fluctuate dramatically as well.

Many of the properties displayed by the nucleotides within the dense granules are remarkably similar to those displayed by aqueous mixtures of nucleotide, $MgCl_2$, and amines. The existence of these similarities suggests that other components of the dense granules such as proteins and lipids are of relatively minor importance in determining the physicochemical state of the nucleotides. Evidently, the existence and the nature of the storage complexes involving the nucleotides, the amines, and the divalent cations within the granules to a great extent stem from interactions among these solutes. As indicated by the recent NMR data, in human dense granules, Ca^{2+} and the nucleotides form a highly immobile aggregated state (Uğurbil et al., 1979a; Costa et al., 1979a), and 5HT is incorporated into these aggregates without significantly disturbing them (Costa et al., 1980); the nucleotides together with calcium probably form an amorphous solid matrix onto which 5HT molecules are adsorbed. In pig platelets, in the presence of Mg^{2+}, nucleotides form more fluid aggregates; 5HT and possibly HA are accumulated into this more fluid matrix (Uğurbil et al., 1984). Although the specific details of the NMR properties of dense granules from other species are different, these general conclusions probably remain valid.

Acknowledgments

We thank Larry Argenbright for helping us to bleed the reserpinized animals and Gail L. Pakstis for expert technical assistance.

Registry No. ATP, 56-65-5; ADP, 58-64-0; 5HT, 50-67-9; HA, 51-45-6; Mg, 7439-95-4.

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[4] Calculated by assuming that resonances with 300 Hz or larger line widths will not be detected and using a ^{31}P CSA of 140 ppm (Gueron & Shulman, 1975).

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

Kâmil Uğurbil,* Miriam H. Fukami,‡ and Holm Holmsen‡

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

† From the Gray Freshwater Biological Institute and the Department of Biochemistry, University of Minnesota, Navarre, Minnesota 55392 (K.U.), and the Department of Pharmacology and Thrombosis Research Center, Temple University, Philadelphia, Pennsylvania 19140 (M.H.F. and H.H.). *Received April 26, 1983.* This research was supported by USPHS Grants HL26089 and HL-14217.

‡ Present address: Department of Biochemistry, University of Bergen, N-5000 Bergen, Norway.

[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