Phosphorus Nuclear Magnetic Resonance of Bovine Platelets†

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ABSTRACT: ^{31}P nuclear magnetic resonance (NMR) spectra of bovine platelets at 28 °C display prominent peaks from adenine nucleotides in two different environments, the metabolic and storage pools. Addition of 20 mM 2-deoxy-D-glucose and 0.1 mM 2,4-dinitrophenol depletes metabolic ATP, resulting in loss of nucleotide signals at 5, 10, and 19 ppm and leaving peaks at 6.5, 10.5, and 19 ppm that are assigned primarily to the dense granule storage pool. The ATP/ADP concentration ratio of the remaining pools is 1.9 ± 0.2, with chemical shifts which reflect a more acidic environment for the intragranular nucleotides than for the cytosol. Isolated bovine dense granules give spectra with similar peak positions to those ascribed to the storage pool after metabolic depletion of whole cells. Storage pool nucleotide spectra are highly temperature dependent. Below 20 °C, the β-ATP peak broadens and decreases in area to the point that it is almost undetectable at 0 °C, probably reflecting formation of progressively higher molecular weight aggregates at the lower temperatures. The release reaction was followed by ^{31}P NMR after addition of thrombin at 28 °C. Spectra of the cell suspension taken at 5-min intervals were compared to spectra of perchloric acid extracts produced in parallel. A new finding of these experiments is that the released nucleotides rapidly undergo a transition to a "NMR silent" form as part of the bovine platelet release reaction. Metabolic breakdown and external paramagnetic ion interactions did not explain the loss of signal from the storage pool. During release reaction, the nucleotides may be broadened out beyond detection due to immobilization by divalent cation and/or platelet membrane binding.

Circulating platelets in blood respond to the binding of a variety of stimulants such as collagen, ADP, and thrombin by changing shape, aggregating, and releasing granule contents. The primary effect of this platelet response is the formation of a dense mass of platelets known as a hemostatic plug at the site of blood vessel injury (Weiss, 1975). The platelet release reaction has been shown to involve secretion from different organelle populations consisting of dense granules, α granules, and lysosomes in a process requiring metabolic energy (Holmsen et al., 1969). While the latter two granule populations release a variety of proteins and enzymes (Holmsen & Day, 1970); Kaplan et al., 1979), the bulk of the dense granule releasate is composed of ATP, ADP, serotonin, pyrophosphate, and divalent cations (Fukami & Salganicoff, 1977). These compounds are probably stored within dense granules in aggregates (Berneis et al., 1969), but the nature of the complex is poorly understood.

As applied to the study of cells and tissues, phosphorus-31 NMR[1] is unique, not only in the ability to follow, in situ, changes in levels of intracellular phosphorylated compounds but also in the ability to report on the chemical environments of different pools of these compounds (Shulman et al., 1979). Application of this technique to platelets would seem particularly useful in sampling the environment of phosphorylated compounds in both the metabolic and storage pools, especially adenine nucleotides, and recent studies of human (Costa et al., 1979; Ugurbil et al., 1979) and pig platelets (Johnson et al., 1978a; Ugurbil et al., 1979) confirm this expectation.

In the present paper, we have focused on the storage pool of bovine platelets and have explored the fate of dense granule nucleotides during the release reaction. Intact cow and pig dense granules were isolated and their NMR spectra compared to storage pool nucleotide signals in whole platelets. Acid extracts of platelets before and after thrombin addition were examined by ^{31}P NMR to monitor metabolic changes during the release reaction.

Experimental Procedures

Preparation of Platelets (All Steps at 18–20 °C). Cow or pig blood was collected at a local slaughterhouse and immediately anticoagulated with 1 volume of 3.8% sodium citrate–50 mM glucose, at pH 5.8, to 9 volumes of blood. The blood was spun in a Beckman Model J-6 centrifuge at 3600 rpm (4000*g*) for 12 min with the rotor allowed to stop without breaking. After the plasma supernatant was removed, the adherent platelets on the red blood cell interface were collected by syringe and further centrifuged in a Sorvall SS-34 rotor at 8000 rpm (7700*g*) for 10 min to give a thick platelet suspension layered between the plasma supernatant and the red blood cell pellet. This crude platelet preparation could be utilizied directly for ^{31}P NMR studies or further purified as below.

The platelet layer was removed by lab spoon and resuspended in 200 mL (per 12 L of starting whole blood) of Tangen–Hepes–BSA, pH 7.4 (145 mM NaCl, 5 mM KCl, 0.1 mM $MgCl_2$, 0.05 mM $CaCl_2$, 5 mM Hepes, 5.5 mM glucose, and 1 mg/mL BSA.) A 10-mL solution of 3.8% sodium citrate–20 mM glucose buffer, pH 7.4, was added to aid resuspension and the mixture gently stirred for 10 min. The suspension was then centrifuged in a Sorvall SS-34 rotor at 1000 rpm (121*g*) for 8 min (brake off) to pellet leukocytes and red blood cells. The platelet suspension was carefully removed by syringe and pelleted in the SS-34 rotor at 8000 rpm for 10 min.

Isolation of Platelet Dense Granules. Platelets were isolated from 10 L of anticoagulated blood as described above except

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[1] Abbreviations used: NMR, nuclear magnetic resonance; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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that the crude platelet layer was resuspended in Tangen–Hepes–BSA–10% glycerol solution to which 0.05 volume of 3.8% citrate–50 mM glucose, pH 7.4, was added. After the final purified platelet pellet was obtained as described above, the platelets were hypotonically swollen in Tangen–Hepes–BSA plus 5 mM EDTA–5 mM EGTA, pH 7.4, buffer at 0 °C (all subsequent steps at 0–4 °C). This suspension was put through a French press at a pressure of 800 psi, and the effluent from the press was centrifuged in the SS-34 rotor at 15 000 rpm (27000*g*). The supernatant was discarded and the pellet resuspended by homogenization in 50 mL of an actomyosin dissociation buffer (Wang, 1977) composed of 0.3 M KCl, 2 mM ATP, 0.5 mM $MgCl_2$, 50 mM imidazole, and 0.1 mM phenylmethanesulfonyl fluoride, pH 6.9 The suspension was centrifuged in the SS-34 rotor for 10 min at 3000 rpm (1100*g*) to remove intact cells and large debris. The supernatant was then centrifuged at 15 000 rpm (27000*g*) to pellet the organelle fraction which was resuspended in a small volume of 0.25 M sucrose–20 mM Tris-HCl–5 mM EDTA–5 mM EGTA, pH 7.95, and analyzed by ^{31}P NMR and electron microscopy.

NMR Methods. ^{31}P NMR spectra were taken at 109.4 MHz with a 63.4-kG Bruker superconducting magnet, Nicolet 1180 computer, and home-built quadrature detection transmitter/receiver. In most experiments, a 50° pulse angle of 9-μs width was used with a pulse repetition period of 0.34 s. Such a short time leads to some saturation, but experiments at longer wait times showed that the nucleotide peaks maintained the same relative intensity. Thus, quantitation is valid in terms of proportions among the nucleotides (ADP and ATP). For spectra of acid extracts, the pulse repetition time was made much longer (10 s) to provide approximate nonsaturating conditions for all phosphate species.

The signal-to-noise ratio with the standard, 10-mm saddle-shaped coil probe is adequate for spectra on quiescent cells where many minutes may be spent time averaging. But platelets can undergo rapid metabolic and structural changes, so for several experiments, we needed better performance. A home-built 15-mm diameter coil of solenoid geometry, mounted crosswise to the field direction, supplied the necessary boost. For theoretical reasons (Hoult & Richards, 1976), the solenoid coil is superior, and some practical examples have been described (e.g., Oldfield & Meadows, 1978). For cow platelets, which are available in large quantities, we used a polycarbonate tube holding 3.5 mL. The tube does not spin and is large enough that the H_0 field homogeneity cannot be fully corrected with our present magnet. Even though this introduces extra broadening, platelet peaks still have natural line widths at least twice the instrumental width. In some cases, when the extra signal-to-noise performance was not required, the standard 10-mm probe was used (nonspinning).

Spectra were acquired without a field frequency lock since drift in the field is negligible. Chemical shifts are referenced to external 85% H_3PO_4 at 0 ppm. In extracts, the glycerophosphorylcholine peak was used as an internal standard at −0.5 ppm. Except for extract spectra, an exponential weighting function corresponding to 20-Hz width was applied to the free induction decay to improve the signal-to-noise ratios. Spectra were recorded at 28–30 °C in all cases except in the study of temperature dependence where the standard Bruker control unit and 10-mm variable temperature probe were used to thermostat the sample to within ±1 °C.

Electron Microscopy. Platelet preparations were routinely fixed in solutions of 145 mM NaCl, 5 mM KCl, 0.05 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 5.5 mM glucose in 5 mM Hepes buffer, pH 7.4, with 1% glutaraldehyde before and after treatment and NMR spectra acquisition. Samples were postfixed in 2% osmium tetraoxide and then dehydrated in a graded series of ethanol solutions, transferred into propylene oxide through a graded series, and embedded in Epon. The plastic-embedded samples were stained with 2% uranyl acetate and then with 0.2% lead acetate after sectioning. Sections were examined with a JEOL Corp. JEM 100-CX transmission electron microscope.

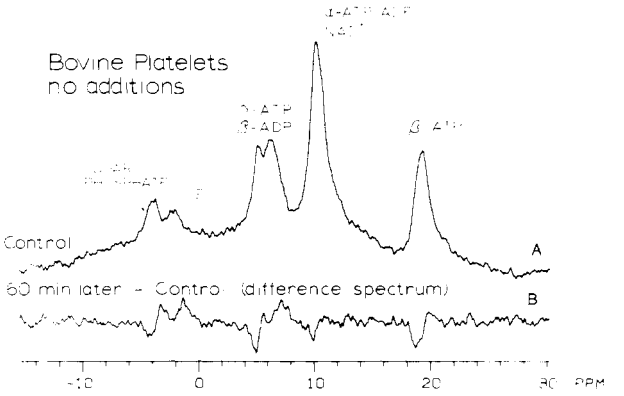

FIGURE 1: ^{31}P NMR spectrum of bovine platelet suspension. (A) (Control) Result of a 5-min time average on 3.5 mL of a freshly prepared "crude" platelet suspension in the sideways-oriented coil. Pulse parameters: 50° pulse width, 0.34-s repetition period, 1000 scans, 20-Hz digital line broadening. (B) Difference spectrum generated by subtracting digitally spectrum A from another spectrum taken under identical conditions 60 min later, with the sample left in the probe at 28 °C.

Protein Determination. Protein concentrations in the platelet suspension were determined by the biuret method (Jacobs et al., 1956).

Acid Extracts. One volume of 35% $HClO_4$ was added to 3.5 volumes of platelet suspension, and the solution was rapidly mixed. After standing 15 min, the suspension was centrifuged, and the clear supernatant was neutralized with KOH. The resulting $KClO_4$ precipitate was then centrifuged out. For NMR spectra, samples were lyophilized and redissolved in D_2O. EDTA (2.5% w/v) was added and the pH readjusted with DCl or NaOD. pH values reported are uncorrected meter readings, commonly denoted pH*. Noise-modulated broadband ^1H decoupling was used for extract spectra; the probe temperature was 35 °C.

Phosphate Analysis. Total phosphate concentrations in perchloric acid extracts of platelet samples were determined spectrophotometrically (Ames & Dubin, 1960) after ashing aliquots of the extracts in the presence of 0.2 mL of 10% magnesium nitrate dissolved in ethanol and digesting the samples 20 min at 100 °C in 0.5 mL of 1 N hydrochloric acid.

Results

Spectra of Bovine Platelets. ^{31}P NMR spectra of fresh bovine platelet suspensions show prominent nucleotide resonances (Figure 1). Near 20 ppm, the peak comes from the β phosphorus of trinucleotides; in the case of platelets, ATP overwhelmingly dominates. Between 5 and 7 ppm is a band of peaks from γ-ATP and β-ADP (with a minor contribution from pyrophosphate). From the area ratio for 20 ppm/5–7 ppm of 0.7 ± 0.05, the approximate concentration ratio of total observable adenine nucleotides in this sample is ATP/ADP = 2.4 ± 0.2. This ratio varies from preparation to preparation, ranging between 0.9 and 2.5. $HClO_4$ extracts of fresh platelets also reflect this variation. Inorganic pyrophosphate (PP_i) can be seen separately in extracts, but for bovine platelets it

contributes only 2–10% of the 5–7 ppm region. Contained in the large peak at 10.5 ppm are mostly α-ATP and α-ADP along with several nucleotide diphospho sugars, including NAD^+ as well as CDP-choline. Inorganic phosphate and monophosphate esters are separately observable at −2 and −4 ppm, respectively.

While the spectrum shown in Figure 1 is from a crude preparation which contained about 10% red blood cells and 1–2% leukocytes, as determined by hemocytometer counting, identical spectra are given by highly purified washed bovine platelets. This result is due in part to the relatively high nucleotide content of the platelets and in part to the low signal level from the red blood cells under the pulse time conditions used in collecting the data.

A broad "hump" underlies the nucleotide and monophosphate spectra. This feature most likely arises from phospholipids of the platelet and granule membranes (Costa et al., 1979). The hump appears to be much more prominent in platelets than in other cell studies by ^{31}P NMR such as *E. coli* (Navon et al., 1977a), Ehrlich ascites tumor cells (Navon et al., 1977b), and HeLa cells (Evans & Kaplan, 1977), probably reflecting the relatively high concentration of phospholipids in platelets. The raised base line was taken into account in measuring areas of nucleotide peaks.

Electron micrographs of a portion of a fresh platelet NMR sample show discoid cells with numerous intact granules. Micrographs of typical fields from thin sections of platelet samples prior to and after collection of the NMR spectrum shown in Figure 1A clearly show normal, competent platelets. There appear to be some changes from a discoid to a more spherical shape after 1 h in the NMR probe at 28 °C and a marked depletion of glycogen granules. However, dense granules and α granules appear equally as abundant as in freshly fixed samples, and there is little spontaneous shape change, aggregation, or release.

After only a few minutes in the NMR tube, the platelets are anaerobic. With their large store of glycogen, they can still maintain glycolysis, however. Figure 1B is a *difference spectrum* obtained by subtracting the spectrum in Figure 1A from one taken under identical instrumental conditions 1 h later. Some ATP loss (7% of the total visible in the control) shows up as the negative peaks at 19 and 5 ppm; the smaller negative peak at 10 ppm suggests that ATP loss has been accompanied by ADP gain since the α-ATP and α-ADP peaks are nearly coincident. The pH has dropped due to lactic acid production, as shown most clearly by the upfield shift of the sugar phosphate peak (seen in the difference spectrum as a dispersion-like signal at −3.5 ppm). Overall, the changes in platelet nucleotide and metabolite levels are relatively small at 28 °C during a 60-min incubation under anaerobic conditions.

Effects of Metabolic Inhibitors. The metabolic poisons 2-deoxy-D-glucose and 2,4-dinitrophenol, the latter a classic uncoupler of oxidative phosphorylation (Pullman et al., 1960), assure that virtually all metabolic nucleotides are depleted (Holmsen et al., 1974). When bovine platelets are incubated with 20 mM 2-deoxy-glucose and 0.1 mM dinitrophenol, part of the adenine nucleotide signals in the ^{31}P NMR spectra disappears and new peaks for 2-deoxyglucose 6-phosphate and P_i are seen (Figure 2). The reaction is essentially complete in 30 min. The important observation here is that *only part of the ^{31}P nucleotide spectrum of platelets is lost.* The remaining nucleotide peaks (top of Figure 2) have been previously ascribed to the storage pool ATP and ADP in pig platelets (Ugurbil et al., 1979). They are broader and, although

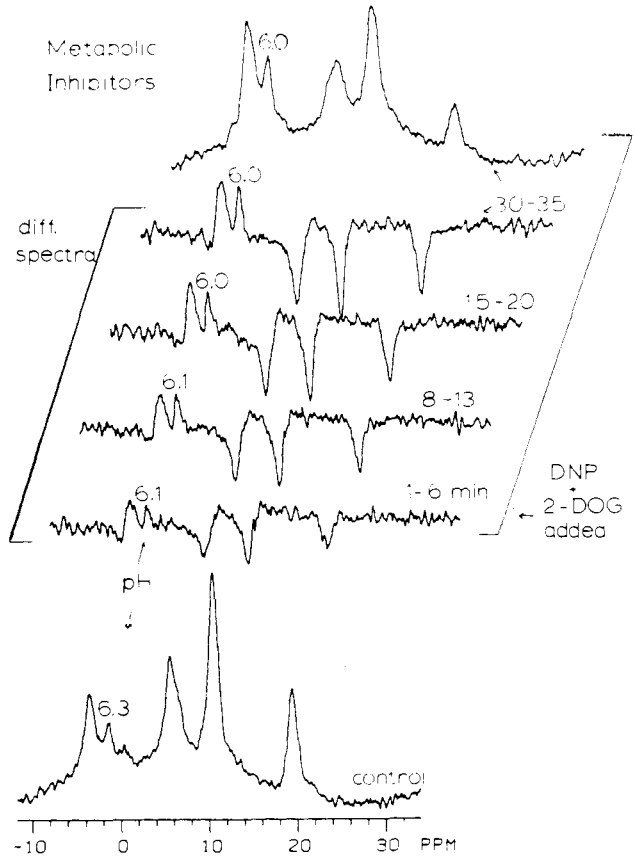

FIGURE 2: Effect of metabolic inhibitors on bovine platelet adenine nucleotides. After a 5-min NMR acquisition on fresh platelets (labeled "control") by using the spectral and sample conditions of Figure 1, 20 mM 2-deoxyglucose and 0.1 mM 2,4-dinitrophenol were added. A series of 5-min runs were then made, with the difference spectra between these runs and the control shown in the figure as the four middle traces. Inverted peaks represent signal lost from the original spectrum. The top trace is a normal spectrum taken 30 min after addition of the inhibitors.

the figure does not clearly show it, have different chemical shifts than metabolic ATP. In this experiment, approximately 50% of the ATP peak at 20 ppm remains, suggesting that about $^1/_2$ of the ATP seen in the preinhibition NMR spectrum is in the storage pool. Since ADP contributes only to the peaks at 6 and 10 ppm, its concentration relative to ATP can be calculated. After metabolic inhibitor treatment, the ATP/ADP ratio is 1.9 ± 0.2.

The β and γ peaks of metabolic ATP lie downfield of the corresponding granular nucleotides. Under the sample conditions we use for bovine platelets, the β peak is never resolved into two distinguishable resonances, but the γ phosphorus often is, as can be seen in Figure 1 where the metabolic γ-ATP is at 5 ppm. As the pH drops in the platelet cytosol, this resolved peak moves upfield and tends to merge with the granule ATP/ADP band, as evidenced in the preinhibitor spectrum of Figure 2. The initial upfield position of storage γ-ATP and β-ADP near 6.5 ppm suggests that the bovine platelet dense granule environment is more acidic than the cytosol, a result which parallels other findings with pig platelets (Ugurbil et al., 1979; Johnson et al., 1978a).

Temperature Dependence of Storage Nucleotides. Ugurbil et al. (1979) discovered that pig platelet dense granule nucleotide peaks broadened and lost area as the temperature was lowered from 30 to 10 °C. We find a similar result for bovine platelets but with some important differences in details. The effects are more easily seen after metabolic inhibitors have

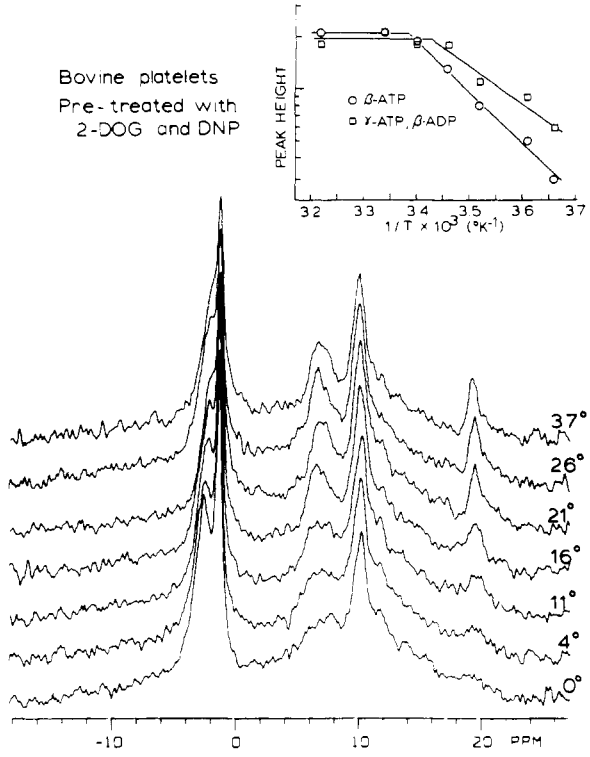

FIGURE 3: Temperature dependence of storage pool adenine nucleotide spectra. Bovine platelets were first incubated with 2-deoxyglucose and 2,4-dinitrophenol (conditions as in Figure 2). Then spectra at the indicated temperatures were collected with the standard 10-mm probe by using 50° pulse widths, 0.34-s repetition time, 4000 scans. Peak heights above the broad phospholipid base-line "hump" were plotted for the resonances at 19 ppm (β-ATP) and 6–8 ppm (γ-ATP, β-ADP) as a function of inverse temperature (inset).

depleted the mitochondrial and cytosolic ATP and ADP (Ugurbil et al., 1979). Figure 3 shows spectra of bovine platelets after treatment with 2-deoxyglucose and 2,4-dinitrophenol. At 0 °C, the β-ATP peak is barely detectable. It increases in both height and area up to 21 °C and then levels off. The behavior of the γ-ATP and β-ADP bands is more complicated. The downfield side (at about 6.5 ppm) of this composite peak changes more than the upfield side (at about 7.5 ppm). In fact, the changes at 6.5 ppm closely parallel those of the β-ATP peak at 19.5 ppm. It is possible that dense granule ATP and ADP resonances are being separately monitored at 6.5 and 7.5 ppm, respectively, with the ATP showing a more pronounced temperature dependence. Other explanations such as different populations of dense granules in the sample might also be consistent with the data.

Since both the peak heights and peak areas decrease as the temperature is lowered, it is difficult to ascribe significance to the slope of the plot of peak height vs. inverse temperature. However, the "break" in the curve near 16–20 °C is seen in plots of both peak height and area. The α phosphate peak near 10 ppm is the most persistent nucleotide resonance at low temperature. Less area is lost from this peak than from the 6–8 ppm band, and the line width even decreases slightly, indicating that α phosphates are not as sensitive as the terminal nuclei to whatever factors cause broadening at low temperature.

Isolated Platelet Dense Granules. In seeking a method for preparing bovine platelet dense granules, we adapted a version of the procedure developed by Salganicoff and co-workers (Salganicoff & Fukami, 1972; Johnson et al., 1978b) for human and pig preparations. We substituted a modified glycerol swelling (Barber & Jamieson, 1979) to selectively

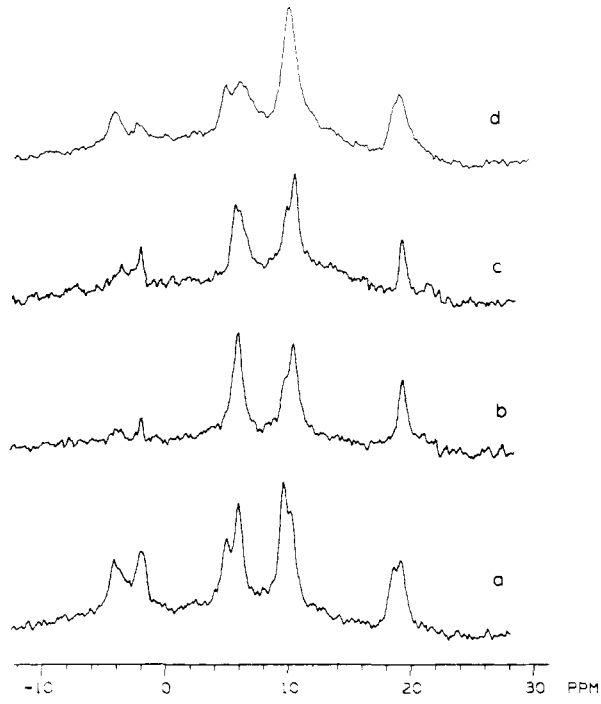

FIGURE 4: Isolated dense granules of bovine and porcine platelets. (a) ^{31}P NMR spectrum of intact pig platelets, 10-min scan. (b) NMR spectrum of dense granule fraction from pig platelets, 10-min scan. (c) Spectrum of dense granule fraction from cow platelets, 40-min scan. (d) ^{31}P NMR spectrum of whole cow platelets, 10-min scan.

enhance lysis of the platelet plasma membrane by disruption in a standard French pressure device at 800 psi. This technique works well to give partially purified dense granule preparations with a yield of 10–20% suitable for ^{31}P NMR. The spectrum of pig dense granules, shown in Figure 4b, remarkably gives nucleotide peaks that are virtually superimposable on the storage pool nucleotide peaks in a whole platelet spectrum (Figure 4a).

Using bovine platelets, we obtained a dense granule-enriched preparation whose ^{31}P NMR spectrum is shown in Figure 4c. The yield of dense granules was lower than with the pig platelets, so a series of four consecutively collected spectra were added to give the trace shown. Over the time course of 40 min, a peak at 21.5 ppm disappeared as did the downfield shoulders of the peaks at 6 and 10 ppm. At the same time, new peaks appeared at −2.0 and −3.6 ppm. This behavior apparently results from breakdown of ATP outside the granules to P_i and probably AMP. In contrast, the β-ATP signal at 19.5 ppm and the composite ATP–ADP peaks at 6 and 10 ppm were constant in amplitude over the time period. Since these peaks have approximately the same chemical shift as the whole platelet dense granule nucleotides, it appears that ATP and ADP inside the isolated granules do not leak out from these preparations. In the dense granules, the ATP/ADP concentration ratio is 1.5 ± 0.4, close to the ratio in a whole platelet spectrum following addition of metabolic inhibitor (1.9 ± 0.2). The adenine nucleotide spectrum of isolated bovine-dense granules has chemical shifts close to the granule position of the whole platelet tracing (Figure 4d), but the peaks at 6 and 19.5 ppm in the whole platelet spectrum are substantially broader. Changes in divalent metal ion concentrations, or loss of serotonin upon isolation, or selection of a subpopulation of granules are possibilities which might account for this behavior.

Thrombin-Induced Release. Thrombin is a potent agent for triggering aggregation, shape change, and the platelet release reaction, whereby the contents of dense granules and

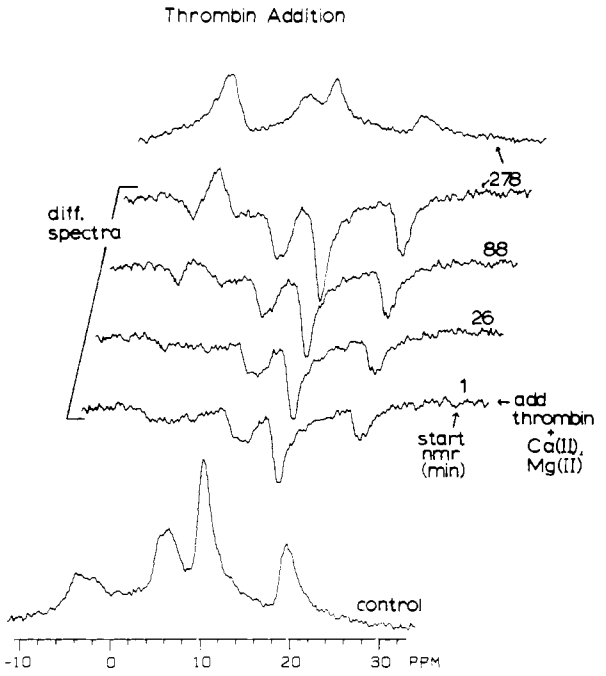

FIGURE 5: Thrombin-induced release of bovine platelets. After a 5-min control spectrum was obtained by using the same NMR parameters as in Figure 1B, bovine thrombin was added to a final concentration of 60 units/mL, along with 0.1 mM $MgSO_4$, 0.2 mM $MgCl_2$, and 0.25 mM $CaCl_2$. Five scans of 1-min duration each were collected and separately stored, and then several 5-min scans were collected at later times as indicated in the figure. The five initial spectra were examined, found to be equivalent within the noise level, and added together. From this sum and from the other 5-min scan spectra, the control spectrum was subtracted. The resulting difference spectra are displayed as the inner four traces in Figure 5. The top trace is the normal spectrum, 278 min after thrombin addition.

α granules are spewed to the outside. In the NMR spectra of bovine platelet suspensions, following addition of thrombin, we consistently observed a large decrease (up to 50%) in the area of peaks ascribed to the storage pool nucleotides, initially without a concomitant increase in the area of other peaks or appearance of new peaks (Figure 5). This decrease occurs within 2 min of thrombin addition, the shortest time period that we are able to check. After 10–20 min, the inorganic phosphate and monophosphate signals begin to increase in area, reflecting breakdown of di- and trinucleotides. Electron micrographs taken of samples 5 min after identical thrombin treatment show extensive loss of dense and α granules, morphological evidence of the release reaction. Though rare, unreacted platelets are still present even 25 min after thrombin addition. There is little further change in the extent of dense and α granule loss, comparing the 5-min sample to the 25-min thrombin-treated sample.

Evidently, the initial effect of thrombin-induced release is to produce a new environment for stored adenine nucleotides wherein their phosphorus nuclei are essentially "NMR silent". The lack of new signals in the spectrum indicates that breakdown of the released nucleotides is not the cause of loss of signals unless the breakdown products also yield no peaks. The most likely conditions to result in peak broadening beyond detectability are that the released nucleotides encounter a milieu containing paramagnetic ions (e.g., Cu^{2+} or Mn^{2+}) or else they become partially or wholly immobilized (Ugurbil et al., 1979). Loss of adenine nucleotide signals was seen for washed platelets as well as for crude fractions, making paramagnetic ion broadening less likely. When 5 mM ATP (an amount on the order of that secreted from the storage pool) was mixed into a fresh platelet sample ("crude" preparation), new peaks appeared in the platelet spectrum at 8.0, 10.3, and 20.8 ppm, corresponding to γ, α, and β of ATP outside the cells. During the first 10 min, approximately 30% of the added ATP was hydrolyzed to ADP, AMP, and P_i, judging from relative peak areas in the difference spectrum. After 40 min, 10% of the added ATP remained in the platelet spectrum. This result indicates the presence of an "ecto" ATPase. However, the action of this enzyme would certainly not prevent immediate expression of nucleotide resonances. In particular, the results show that paramagnetic ion concentrations are low *outside* these cells. If significant concentrations of paramagnetic ions were present *inside* bovine platelets, then granule or metabolic nucleotide peaks would be broadened beyond detection, which is not the case. Dipole–dipole interactions of the phosphorus nuclei with unpaired electron spins of paramagnetic metal ions would not appear to explain the loss of nucleotide peaks after thrombin addition.

When 5 mM ATP was added to platelets that had been preincubated with thrombin for 10 min, the result was strikingly different. At least 90% of the ATP was lost from the spectrum in the first 10 min. Most importantly, the lost trinucleotide peaks showed up immediately in the AMP and P_i regions of the spectrum. This is in marked contrast to the fate of nucleotides released by thrombin, which disappear from the spectrum with little or no increase in area in the monophosphate region. We conclude that released nucleotides encounter a special environment which is not simply the outside medium. They may precipitate as large molecular weight aggregates or may bind to immobilized surfaces. In any case, they are refractory toward the potent "ecto" ATPase activity of thrombin-treated platelets.

Acid Extracts. In order to follow changes in total levels of nucleotides and other platelet metabolites, we made $HClO_4$ extracts of bovine platelet samples at various time intervals in parallel with whole cell NMR spectra. After neutralization, the extracts were checked by ^{31}P NMR for relative concentrations of acid-soluble, phosphorus-containing metabolites. An assay of total phosphate then gave the absolute quantities. A typical extract spectrum is shown in Figure 6 where over 20 different compounds have been resolved. From the pH dependence of chemical shifts and from addition of known compounds to the solution, we have made assignments for more than half of the species seen (Table I). Of interest is the finding of the diphosphodiester CDP-choline in relatively high concentration (greater than NAD+, for example).

Nucleotide concentrations were determined by analytical high-pressure liquid chromatography (LC) as well as by NMR. A Varian Associates AX-10 column, 30 cm long, was used with a linear gradient from 0.01 M KH_2PO_4, pH 2.85, to 0.75 M KH_2PO_4, pH 4.4. For the extracts used in Table I, the relative concentrations from high-pressure LC were ADP 1.0, ATP 0.9, GTP 0.1, UTP 0.1, GDP 0.2, UDP 0.2, CDP 0.05, and AMP 0.4. CTP was detectable but not accurately measurable, and mononucleotides, other than AMP, were present at levels less than 0.1 relative to ADP. These high-pressure LC data confirm the finding from NMR spectra that adenosine nucleotides predominate in bovine platelet acid extract solutions. The data also showed that the peak assigned to AMP in the NMR spectra contained extra intensity not due to AMP. On this basis, an appropriate correction was made to the results of Table I and Figure 7.

Figure 7 is a plot of the concentration of several bovine platelet metabolites prior to and at intervals following thrombin addition. The gradual drop of ATP and ADP along with the

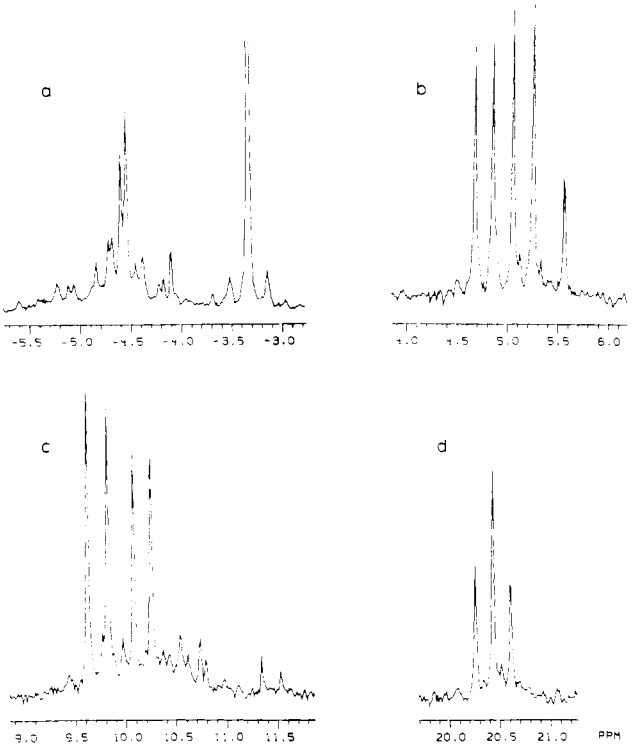

FIGURE 6: Perchloric acid extract of bovine platelets. A spectrum of an $HClO_4$ extract of bovine platelets was acquired under the following conditions: 50° pulse width, 10-s repetition period, 5000 scans, broad-band noise-modulated decoupling of protons, 30 °C. A line broadening of 1 Hz was used to improve the signal-to-noise ratio. The sample was in D_2O with 2.5% EDTA added, pH* 8.05. The region between −6 and −3 ppm is displayed at one-half the vertical gain of the other regions.

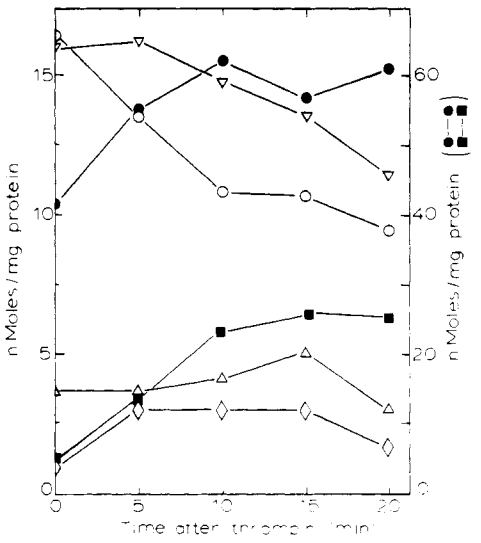

FIGURE 7: Selected nucleotide and metabolite levels following thrombin addition. $HClO_4$ extracts were taken of equivalent bovine platelet samples prior to and following thrombin addition, with the phosphate metabolite levels determined by ^{31}P NMR. Relative peak areas were calculated by digital integration or by the "cut and weigh" method and were converted to nmol/mg of protein from consideration of total phosphate analysis of the extract and protein content of the original platelet suspension. P_i (●) and AMP (■) are plotted with reference to the scale on the right side of the chart which is four times greater than the scale for ATP (○), ADP (▽), phosphocholine (△), and CDP-choline (◇). Indicated concentrations have an uncertainty of approximately ±10% for most species except CDP-choline where the uncertainty is approximately ±20%.

buildup of P_i and AMP is consistent with previous findings of Holmsen & Day (1971) for human platelets. These data

Table I: Perchloric Acid Extract of Bovine Platelets[a]

chemical shift[b] (ppm)	assignment	nmol/mg of protein[d]
−5.60	α-Fru-1,6-P	0.4
−5.22	Glc-6-P	2.4
−5.12	α-D-glycerol-P	1.2
−5.07	dihydroxyacetone-P	1.0
−4.85	β-Fru-1,6-P	1.7
−4.72	NA[c]	
−4.68	β-Fru-1,6-P	1.7
−4.59	Fru-6-P	8.2
−4.54	AMP	5.5[e]
−4.45	NA	
−4.38	NA	
−4.22	NA	
−4.18	NA	
−4.10	choline-P	3.6
−3.33	P_i	38.4
−1.06	glycero-P-Eth	2.5
−0.50	glycero-P-Cho	1.5
0	phospholipids	4.0
4.66	ATP-γ	13.4
4.84		
4.71	NTP-γ	1.2
4.89		
5.05	ADP-β	15.7
5.25		
5.12	NDP-β	1.0
5.32		
5.56	PP_i	2.8
9.62	ADP-α	17.0
9.82		
9.76	NDP-α	2.4
9.96		
10.08	ATP-α	13.2
10.26		
10.36	NA	
10.43	NAD^+	0.5
10.62		
10.80		
10.98		
10.55	CDP-choline	0.7
10.74		
11.34		
11.53		
20.25	ATP-β	12.5
20.43		
20.61		
20.34	NTP-β	0.7
20.51		
20.69		

[a] Freshly prepared crude platelet suspension (see text), extract in D_2O, pH 8.0. [b] Glycerophosphorylcholine = −0.50 ppm. [c] Not assigned. [d] Platelets were 85 mg of protein/mL of suspension = 1×10^{11} platelets/mL. [e] Based on liquid chromatography analysis.

bear on the finding, in whole cell spectra, that nucleotide peak areas immediately decrease by up to one-half after thrombin addition, without concomitant increases in monophosphate or P_i signals. The total extract data suggest that the adenine nucleotides are still there and support the conclusion that loss of signal in whole cell spectra reflects a transition of granular ATP and ADP into an environment where the peaks are broadened beyond detection.

Discussion

^{31}P NMR spectra of bovine platelets are dominated by dense granule nucleotide signals. The fact that these resonances are easily observable makes it feasible to probe the intragranular environment in situ. With bovine platelets, we rapidly produce samples of abundant volume, and using optimized NMR probe design, we have collected data at short intervals in order to follow the dynamics of platelet release.

A key finding of this paper is the immediate disappearance of dense granule nucleotide signals upon addition of thrombin to bovine platelet suspensions. We were quite surprised to observe this effect, but it has been reproduced in over a dozen experiments by using different bovine platelet preparations, both crude and washed. We cannot completely rule out paramagnetic ion induced broadening as a contributor, but experiments with ATP added to the samples suggest that paramagnetic effects are minimal.

In preliminary experiments with pig platelets, prepared in the same way as the cow cells, thrombin again produced a large decrease in the granule nucleotide peak areas, but simultaneously an equal area increase was observed in the monophosphate region due to breakdown products. Evidently, released adenine nucleotides of pig platelets are readily accessible to an ecto ATPase activity whereas in cow platelets they are not. The conditions responsible for transition of released nucleotides to an "NMR silent", and ATPase refractory, form are currently under investigation.

^{31}P NMR spectra of bovine platelets differ from those of the pig (Johnson et al., 1978a; Ugurbil et al., 1979) in that the cow dense granule nucleotide peaks are substantially broader than the corresponding signals in pig platelets, but both cow and pig are distinct from human cells where the dense granule peaks are broadened beyond detection (Ugurbil et al., 1979; Costa et al., 1979). In preliminary work, we have found significant differences in platelet dense granule peaks in whole cell spectra from five different species. There are known to be species differences in the ATP/ADP ratios concentrations (Dodds, 1978), serotonin levels (Drummond, 1976), and the magnesium/calcium concentration ratios in dense granules (Kinlough-Rathbone et al., 1973). For example, the difference in dense granule levels of Ca^{2+} and Mg^{2+} has been proposed by Ugurbil et al. (1979) to explain the difference in ^{31}P NMR results for pig and human platelets. Future work will be aimed at understanding granule adenine nucleotide line shapes in platelets of various species in terms of the above factors and possibly others.

Nucleotide chemical shifts are pH dependent and, in principle, offer an accurate measure of pH inside dense granules. However, the pK_a of β and γ and the chemical shift limits of the β phosphorus are strongly affected by the presence of divalent cations (Son et al., 1975). Until the granular contents can be modeled exactly, it is difficult to assign an internal pH based on the chemical shifts observed, but it is clear that the intragranular pH in bovine platelets is more acid than the cytosol since the granule ATP γ and β peaks appear upfield of the corresponding cytosolic resonances.

To our knowledge, this is the first report to include the isolation and ^{31}P NMR of intact dense granules from bovine platelets. The preparation contained α granules and a low percentage of mitochondria, but these organelles are not likely to contribute substantially to the ^{31}P NMR spectra, so no further purification was undertaken. Bovine dense granule spectra resemble closely spectra of the corresponding pig granule preparation; both are characterized by relatively narrow peaks, indicating a mobile environment for nucleotides. Both ATP and ADP contribute to the bovine dense granule spectra in the ratio ATP/ADP = 1.5 ± 0.1. Within the estimated error, the same ratio is found in whole platelet spectra after metabolic inhibition.

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