STUDIES OF HUMAN PLATELETS BY ^{19}F and ^{31}P NMR

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Received 8 January 1979

1. Introduction

Nuclear magnetic resonance spectroscopy (NMR) has been shown to be a valuable method for studying the contents of intact cells [1,2]. Most studies have involved ^{31}P NMR, but other nuclei such as ^{1}H, ^{13}C and ^{15}N have also been investigated [3–5]. Resolvable NMR signals are generally observed only from molecules which are present in relatively high concentrations, and which give rise to narrow lines. This generally restricts observation to small molecules which are not strongly bound to large proteins or to membranes.

Here we describe ^{19}F and ^{31}P NMR studies of human platelets. These cells possess two well defined regions, the cell cytoplasm and membrane-enclosed storage vesicles (dense bodies), each containing molecules of low molecular weight in concentrations sufficiently high to be observable by NMR. Platelet cytoplasm contains inorganic phosphate (P_i), sugar phosphates, and a metabolic pool of adenine nucleotides believed to serve as the energy source for most cellular functions [6,7]. The vesicles contain high concentrations of serotonin (5-hydroxytryptamine), ADP, ATP, pyrophosphate and Ca^{2+} [8–11]. The purpose of the work described here is to identify resonances from molecules in the different cellular regions, and to obtain information about the freedom of motion of molecules in the different regions. This is done to provide the basis for future studies of platelet physiology and the factors governing the transport of molecules between the different compartments.

To our knowledge ^{19}F NMR has not yet been applied to the study of intact cells. However, ^{19}F is an attractive nucleus for this purpose because:

(i) Its sensitivity is high;
(ii) It is possible to introduce specific ^{19}F-labelled molecules into specific compartments of the cell;
(iii) Spectra can be obtained without the complications of overlapping resonances.

We have used two methods to introduce fluorinated molecules into platelets.

1. To allow uptake of a specific fluorinated molecule, here 4,6-difluoroserotonin, $\geqslant 90\%$ of which is incorporated into the vesicular region [11].
2. To rely on biosynthesis of a molecule of interest from a precursor fluorinated molecule which is taken up by the cell.

Thus, incubation of the platelets with 2-fluoroadenosine results in the accumulation of 2-fluoro-ATP in the cytoplasmic region [12]. There is essentially no exchange of ATP (or 2-fluoro-ATP) between the cytoplasmic and the vesicular regions [12,13]. Thus ^{19}F probes can be incorporated specifically into the different regions of the cell.

2. Materials and methods

4,6-difluoroserotonin and 2-fluoroadenosine were synthesized by the procedures in [14,15]. Antimycin A, 2-deoxy-D-glucose and nucleotides were obtained from Sigma. The ionophore X537A was the generous gift of Dr J. Berger of Hoffman-LaRoche. Other chemicals were all reagent grade.

For each experiment, 4 units of platelet-rich plasma (PRP) were obtained from a single donor by collection in ACD-A anticoagulant and differential centrifuga-

tion [16]. Donors had no history of hematologic disorders or drug ingestion for at least 10 days prior to donation. For ^{31}P NMR studies, PRP was incubated overnight at 22°C with 2×10^{-5} M adenine (to raise intracellular nucleotide levels [9,17]). For ^{19}F NMR studies of cells containing 4,6-difluoroserotonin, PRP was incubated overnight at 22°C with 2×10^{-5} M adenine, and subsequently for 60 min at 37°C with 2×10^{-5} M 2,6-difluoroserotonin. For ^{19}F NMR studies of cells containing 2-fluoro-ATP, PRP was incubated overnight with 5×10^{-5} M 2-fluoroadenosine. All PRP was then diluted 1:1 (v/v) with buffer similar to that in [18] but containing 2 mM EDTA, 0.35% human serum albumin and no glucose, cooled to 4°C, and spun at 4500 × *g* for 5 min. Platelets were resuspended in 50 ml total vol. of the same buffer; cells intended for ^{19}F NMR studies of 2-fluoro-ATP were further incubated for 60 min at 37°C with 10^{-4} M 2-fluoroadenosine. Cells were pelleted (1000 × *g* for 20 min), and resuspended (in glucose-free buffer made up with D_2O) so that the final volume contained ~50% cells and 50% buffer. Resuspended platelets were maintained at 0°C. Aliquots were treated:

(i) With 2-deoxy-D-glucose (20 mM final conc.) and antimycin A (8.2 μg/ml final conc. [19]), incubated at 37°C for 60 min, then cooled to 0°C;

(ii) with X537A (25 μM final conc. [20]), incubated at 37°C for 1 min, then cooled to 0°C. Cell extracts were prepared by the addition of perchloric acid (final conc. 0.4 N), incubation for 5 min at room temperature, followed by addition of EDTA and neutralization to pH 7.5 by the addition of NaOH.

^{19}F NMR spectra were recorded at 254 MHz using a Bruker spectrometer with 5 mm diam. sample tubes, and at 94 MHz using a Varian XL-100 spectrometer with 12 mm diam. sample tubes. ^{31}P NMR spectra were recorded at 109.3 MHz using the Bruker spectrometer with 15 mm diam. sample tubes. All spectra were recorded at 4°C, in the Fourier transform mode without ^{1}H noise decoupling.

3. Results

Figure 1(c) shows a 254 MHz ^{19}F NMR spectrum of platelets incubated with 4,6-difluoroserotonin.

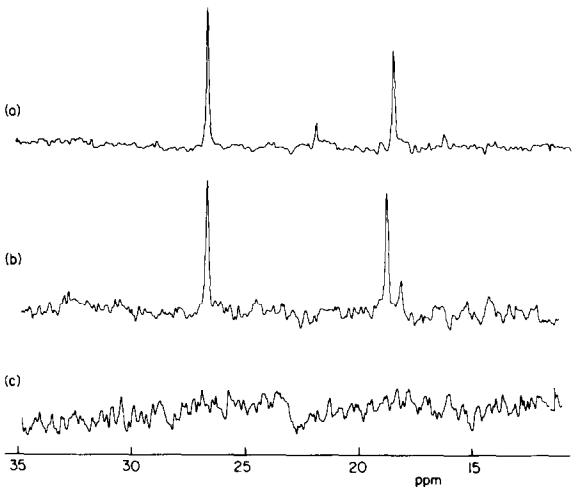

Fig.1. 254 MHz ^{19}F NMR spectra of: (a) 1 mM 4,6-difluoroserotonin, 3000 scans; (b) perchloric acid extract of platelets incubated with 4,6-difluoroserotonin, 5000 scans; (c) intact platelets incubated with 4,6-difluoroserotonin, 39 000 scans. Chemical shifts are in ppm from C_6F_6.

Despite a long accumulation time, no resonances could be detected either at this frequency or at 94 MHz. However, lysis of a portion of these cells with perchloric acid showed clearly the expected ^{19}F resonances (fig.1(b)). Similarly, treatment of other portions of these cells with X537A or with 2-deoxy-D-glucose and antimycin A resulted in the appearance of well-resolved resonances, following release of 4,6-difluoroserotonin to the extracellular medium [21,22]. Thus, not only do the intact cells contain sufficient 4,6-difluoroserotonin to be readily observed by NMR if the resonances are sharp, but also the presence of intact cells does not interfere with observation of resonances from extracellular material. We therefore conclude that the ^{19}F resonances of 4,6-difluoroserotonin contained in the vesicles are extremely broad.

Figure 2(b) shows the 254 MHz ^{19}F NMR spectrum of platelets incubated with 2-fluoroadenosine. The observed peak has a linewidth of 240 Hz at this frequency, but at 94 MHz the linewidth is only 100 Hz. The spectrum of the perchloric acid extract contained a sharp resonance with a chemical shift different by 0.09 ppm from that of 2-fluoroadenosine. The chemical shift difference was clearly

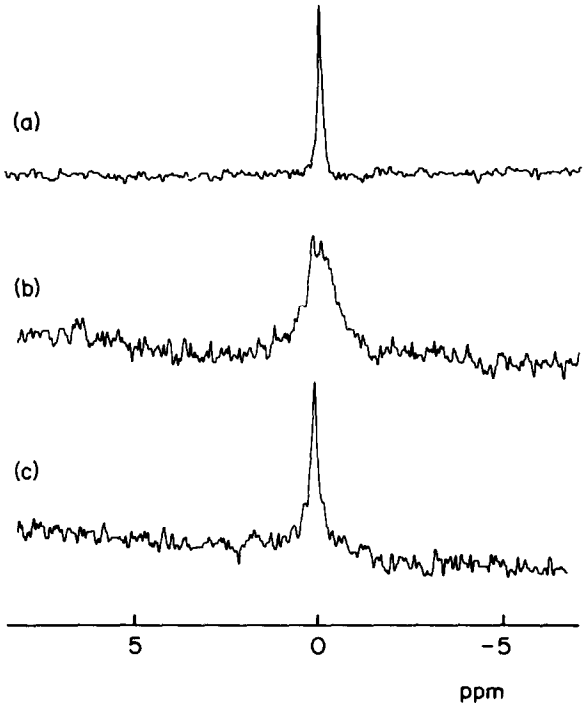

Fig.2. 254 MHz ^{19}F NMR spectra of: (a) 5 mM 2-fluoro-adenosine, 500 scans; (b) platelets incubated with 2-fluoro-adenosine, 5000 scans; (c) platelets incubated with 2-fluoro-adenosine, then treated with 2-deoxy-D-glucose and anti-mycin A, 3000 scans. Chemical shifts are in ppm from 2-fluoroadenosine.

seen by addition of 2-fluoroadenosine to the extract. 2-fluoro-ATP has been shown [13] to accumulate in cells under the conditions used here, and we therefore attribute the observed resonance in both the extract and the intact cells to this compound. (In the spectrum of the extract another resonance with an intensity of $< 10\%$ of that of the above resonance was also observed. This had a chemical shift very close to that of 2-fluoroadenosine, and pre-sumably arose from this compound or from 2-fluoro-AMP.) Treatment of the cells with the metabolic poisons 2-deoxy-D-glucose and antimycin A results in a spectrum (fig.2(c)) containing a peak having a line-width much narrower than that of the untreated platelets. The spectrum of the perchloric acid extract of these poisoned cells showed that the peak assigned to 2-fluoro-ATP had disappeared, and that a new peak with a chemical shift close to that of 2-fluoro-adenosine had appeared. As 2-fluoro-AMP is known [23] to accumulate in platelets treated in this way, it is likely that the observed signals in the extract and in the intact cells arise primarily from this compound.

Figure 3(a) shows the ^{31}P NMR spectrum of platelets. Resonances from sugar phosphate, inorganic phosphate (P_i) and ATP are well resolved. Treatment with 2-deoxy-D-glucose and antimycin A results in substantial changes in this spectrum, revealed clearly in the difference spectrum. Without treatment, the spectrum remains unchanged for many hours,

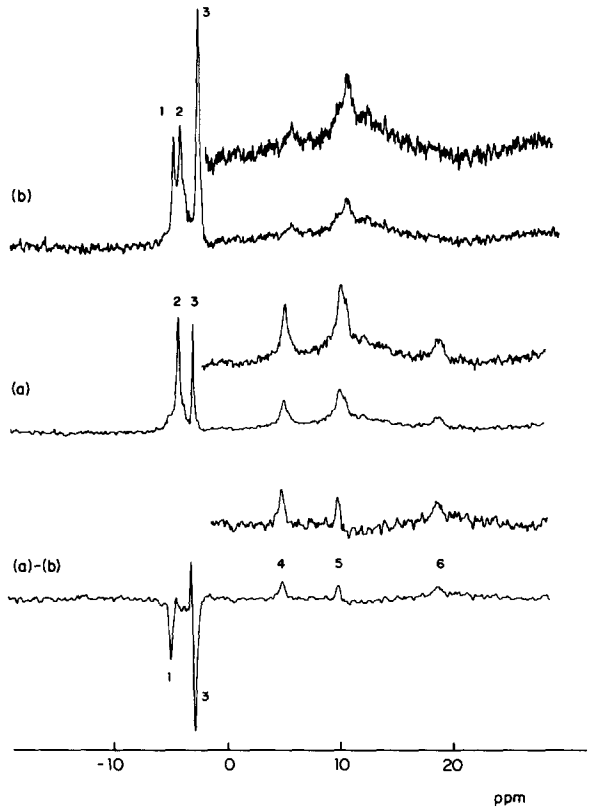

Fig.3. 109 MHz ^{31}P NMR spectra of: (a) untreated platelets; (b) platelets treated with 2-deoxy-D-glucose and antimycin A. The difference (a)–(b) is plotted, and the high field region of each spectrum is shown expanded by a factor of two in the vertical scale. Peak assignments: (1) 2-deoxy-D-glucose-6-phosphate; (2) sugar and nucleotide monophosphates; (3) P_i; (4) ATP γ- and ADP β- ; (5) ATP α- and ADP α- ; (6) ATP β-, α-, β- and γ- refer to positions of phosphates, α- being adjacent to the CH_2 groups of ribose. Chemical shifts are from external phosphoric acid.

provided that the cells are maintained at 4°C. Treatment with metabolic poisons has resulted in an increase in the intensity of the P_i peak (and a slight shifts due to a pH change), and appearance of a new peak in the sugar phosphate region (with a chemical shift identical to that of 2-deoxy-D-glucose-6-phosphate), and a decrease in the intensity of the ATP resonances. The difference spectrum reveals clearly the three ATP resonances which have different linewidths, that of the β-phosphate being broader by a factor of about two than those of the α- and γ-phosphates. Treatment in this way is expected to cause a total breakdown of cytoplasmic ATP [19], hence increasing the peak intensity of P_i and producing a peak of phosphorylated 2-deoxy-D-glucose, without significant change in the vesicular contents [22]. Spectrum 3(b), therefore, is expected to contain resonances from vesicular ATP, ADP and pyrophosphate. However the only resonances which can possibly be attributed to these compounds are two very broad ones, in the positions expected for ATP (α- and γ-phosphates) or ADP (α- and β-phosphates). No resonances attributable to the β-phosphate of ATP or to pyrophosphate can be detected. However, perchloric acid extracts of the cells (fig.4) show that indeed the treated cells do contain ADP, ATP and pyrophosphate, and that the effects of 2-deoxy-D-glucose and antimycin A on the platelets are to decrease the total concentration of ATP, and to increase the concentrations of P_i and the new sugar phosphate. We therefore conclude that in the intact cells the ^{31}P resonances of all the vesicular contents are very broad (in agreement with [24]), although the broadening appears to be different for different resonances. It is possible that the broad resonances in fig.3(b) arise from vesicular ADP, whilst the resonances of ATP and pyrophosphate are yet broader. The spectra of both untreated and treated cells in fig.3 contain a broad component in the baseline, which may be attributed to phospholipids of cell membranes.

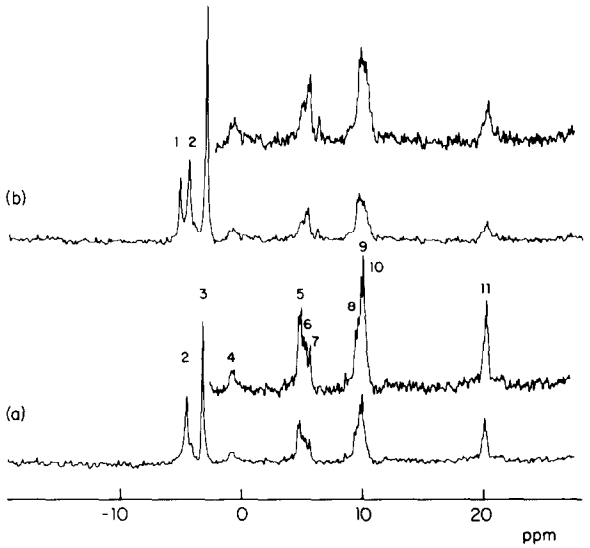

Fig.4. 109 MHz ^{31}P NMR spectra of perchloric acid extracts of: (a) untreated platelets; (b) platelets treated with 2-deoxy-D-glucose and antimycin A. Peak assignments; (1) 2-deoxy-D-glucose-6-phosphate; (2) sugar and nucleotide monophosphates (3) P_i; (4) unassigned; (5) ATP γ-; (6) ADP β-; (7) pyrophosphate; (8) ADP α-; (9) ATP, α-; (10) unassigned; (11) ATP, β-.

4. Discussion

The ^{31}P resonances of molecules in the platelet cytoplasm are well resolved with linewidths comparable with those of the contents of other cells. Some differential broadening is observed, notably of the ATP resonances. The ^{19}F resonance of 2-fluoro-AMP or 2-fluoroadenosine is sharp, and in separate work [25] we have observed sharp resonances from 2-fluoro-2-deoxy-D-glucose-6-phosphate in cells. However, the ^{19}F resonance of 2-fluoro-ATP is very broad, and the linewidth is field dependent. In red blood cells, we have found similar characteristics of the 2-fluoro-ATP ^{19}F resonance [25]. There are several possible reasons for the linebroadening associated with the cytoplasmic ATP, including varying magnetic susceptibility across the cells and effects of paramagnetic ions. However, the former cannot explain the large difference in linewidths for the different nuclei, or the different linewidths of fluorinated molecules in the cytoplasm. The effects of paramagnetic ions would be more pronounced on ^{31}P resonances of ATP, to which they bind, than on the ^{19}F resonance of the nucleotide base. This is the opposite of the observed effects. The field dependence of the ^{19}F linewidths of 2-fluoro-ATP therefore indicates that the broadening arises either from chemical exchange or from chemical shifts anisotropy. If chemical exchange effects are responsible, then considerable chemical shift perturbation must

arise presumably from relatively strong binding of the ATP to another species. An alternative mechanism for relaxation, however, is chemical shift anisotropy [26], which has been observed previously in ^{19}F spectra of large molecules [27] and in ^{31}P spectra of, e.g., phospholipids [28]. If this is the correct mechanism, the dipolar contribution to the ^{19}F linewidth of 2-fluoro-ATP is < 80 Hz. The ^{19}F chemical shift tensor of 2-fluoro-ATP is not known although the tensors for some substituted benzenes have been determined [29]. These vary in an unpredictable manner, but a calculation shows that for isotropic tumbling, the correlation time (τ_c) for the motion of the fluorine atom of 2-fluoro-ATP would be about 10^{-7} s. This would not be inconsistent with the relatively well resolved ^{31}P spectra, because the smaller magnetogyric ratio of the ^{31}P nucleus would, even if the shift tensor were the same as for the ^{19}F nucleus, reduce the broadening of the ^{31}P resonances by a factor > 5. Regardless of the mechanism of relaxation, however, the data indicate that ATP in the cytoplasm does not exist as a freely tumbling, unbound species.

The linewidths of the ^{19}F and ^{31}P resonances of all the molecules in the platelet vesicles, unlike the cytoplasm, are so broad as to be effectively unobservable. This observation contrasts markedly with NMR studies of chromaffin granules, both inside adrenomedullary cells and following biochemical isolation, where well-resolved ^{1}H, ^{13}C and ^{31}P resonances are observed [3,30–32]. The linebroadening, likely to be dominated by chemical shift anisotropy, indicates that the contents of the platelet vesicles have very highly restricted motional freedom. A reasonable explanation is that the vesicular serotonin and nucleotides are bound in highly aggregated complexes with Ca^{2+}. It is to be noted that the chromaffin granules do not contain high concentrations of metal ions, and that their contents undergo relatively unrestricted molecular tumbling. Full details of the structural nature of the platelet vesicles remain to be established, as does the significance of the present observation in an understanding of their biological function.

It is clear from this study that ^{19}F NMR studies of cells employing fluorinated molecules constrained to specific cellular compartments complement studies of other nuclei, and that the degree of molecular motion prevailing for different molecules inside these compartments can be investigated.

Acknowledgement

The high field NMR experiments were performed at the NMR Facility for Biomolecular research located at the F. Bitter National Magnet Laboratory, MIT. The NMR Facility is supported by grant no. RR00995 from the Division of Research Resources of the NIH and by the National Science Foundation under contract no. C-670. The work was also supported by the US Navy through Naval Medical Research and Development Command Research Task no. 63706N-M0095 PN001-0040. The opinions or assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or naval service at large.

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