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Phosphate Metabolism in Intact Human Erythrocytes: Determination by Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy

(phosphorylated compounds/cold storage/blood)

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ABSTRACT Whole human blood was examined by ^{31}P nuclear magnetic resonance spectroscopy. Individual phosphates (α,β,γ) of ATP were identifiable, and two microenvironments appeared to be present for this molecule. When sequential recordings of freshly collected blood were made, 2,3-diphosphoglycerate was observed to decrease in association with a concomitant increase in inorganic orthophosphate. When aged cells containing little 2,3-diphosphoglycerate were incubated in the presence of inosine and pyruvate, 2,3-diphosphoglycerate formation could be demonstrated. These results show that cellular metabolism can be recorded directly in intact cells by ^{31}P nuclear magnetic resonance.

In this study, we have used ^{31}P nuclear magnetic resonance spectroscopy (^{31}P NMR) to identify phosphorus compounds in intact human erythrocytes and to record metabolic changes directly in intact erythrocytes incubated *in vitro*. We have detected the individual phosphate groupings (α,β,γ) of ATP in intact erythrocytes and have determined that there may be at least two microenvironments for this molecule. In addition, changes in 2,3-diphosphoglycerate were followed immediately after withdrawal of blood, as well as during incubation of aged cells in the presence of added inosine and pyruvate at 25°.

Recently, Moon and Richards (1) applied the technique of ^{31}P NMR to the study of rabbit whole blood and hemolysates, and were able to estimate the intracellular pH of carbon monoxide-treated rabbit erythrocytes by measuring the pH-induced changes in chemical shifts of the 2- and 3-phosphates of 2,3-diphosphoglycerate.

MATERIALS AND METHODS

The spectrometer used was a Bruker HFX-5 operating at 36.43 MHz for ^{31}P (21 KGauss) (2–4) and incorporating facilities for Fourier transform signal-averaging and broad-band heteronuclear ^1H decoupling.

Spectra were recorded in the presence and absence of ^1H broad-band decoupling in order to identify esterified phosphates, i.e., P—O—C—H coupling. The samples routinely contained 10% D_2O, which served as the reference signal. Spinning the sample led to significantly increased resolution and not to a decrease as observed by Moon and Richards (1).

Area measurements were determined by computerized integration of the transformed spectra, and the observed areas were adjusted for Overhauser enhancements by comparison with the areas obtained with a solution of purified nucleotides examined under conditions simulating the physiological state.

Blood was drawn from the antecubital vein of normal, fasted, adult males into standard acid–citrate–dextrose (ACD) medium (National Institutes of Health Formula A).

RESULTS AND DISCUSSION

Most of the prominent ^{31}P NMR signals observed in this study are shown in the signal-averaged, proton-decoupled spectrum of whole blood presented in Fig. 1. Localization of these signals to the major blood compartments was facilitated by obtaining spectra from packed erythrocytes (Fig. 2*A*) and homologous plasma (Fig. 2*B*).

The prominent singlet at −1.5 ppm in Fig. 1 arises from inorganic orthophosphate (P_i) inside the intact erythrocytes

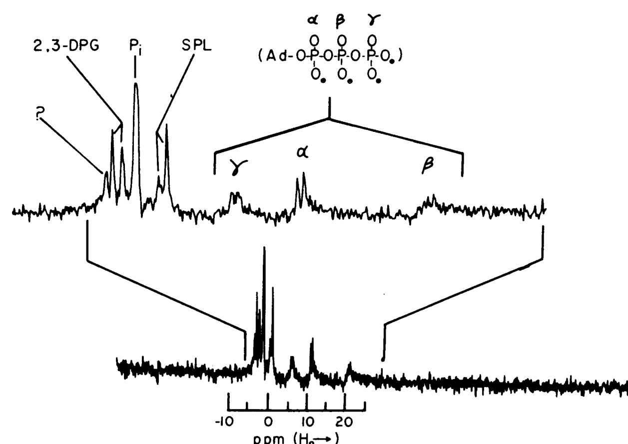

FIG. 1. Phosphorus-31 nuclear magnetic resonance spectrum of whole human blood. This sample had been stored in ACD medium at 4° for 24 hr, then subjected to about 8 hr (65,800 pulses) of Fourier transform ^{31}P NMR analysis. The *top spectrum* is an expanded scale readout of the lower spectrum. The major resonance bands are indicated by appropriate abbreviations: *2,3-DPG*, 2,3-diphosphoglyceric acid (3-P, −3.3 ppm; 2-P, −2.6 ppm); P_i, inorganic orthophosphate (−1.5 ppm); *SPL*, serum phospholipids (phosphatidylcholine, +0.9 ppm; sphingomyelin and other phospholipids, +0.3 ppm); *ATP* (α-P, +10.7 ppm; β-P, +20.2 ppm; γ-P, +5.9 ppm); *?* indicates the resonance from an unidentified monoesterified phosphate and is possibly a hexose or triose phosphate. H_3PO_4 (85%, sealed capillary) served as the external reference (0 ppm), and D_2O (10%, v/v) served as the locking reference in all spectra in this study.

Abbreviation: NMR, nuclear magnetic resonance.

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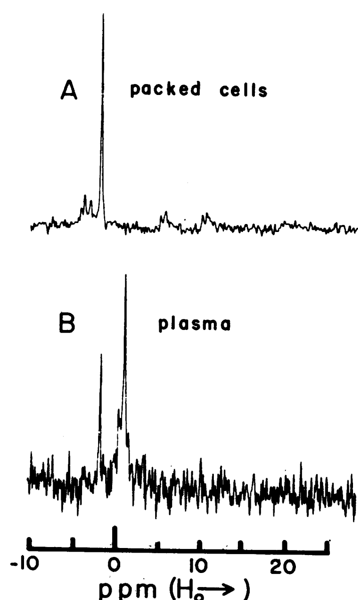

FIG. 2. Spectrum *A* is the ^{31}P NMR spectrum obtained from packed human erythrocytes after 2 hr (about 17,000 pulses) of NMR analysis. Note the decreased peak amplitude of the resonances attributable to 2,3-diphosphoglycerate (−2.6 and −3.3 ppm). This is due to the fact that the whole blood used for this study had been stored at 4° for 30 days. Spectrum *B* is the ^{31}P NMR spectrum of the homologous plasma after about 17,000 pulses. The prominent peak at 0.9 ppm, absent in spectrum *A*, arises from the phospholipids of the serum lipoproteins (compare refs. 4–6). The other resonances of these spectra are those indicated in Fig. 1 and described in *Results*.

(1). The plasma P_i signal (−1.3 ppm, Fig. 2*B*) appears as an upfield shoulder on the signal from erythrocyte P_i when whole blood is examined under certain conditions (compare Figs. 4*D* and 5*A*, and ref. 1). The two orthophosphate resonances at −3.3 and −2.6 ppm in Figs. 1 and 2*A* arise from the 3- and 2-positions, respectively, of 2,3-diphosphoglycerate (1). These two resonances are the major signals observed in freshly drawn blood (see Fig. 4*A*). The other orthophosphate signals observed in Fig. 1 that can be assigned with certainty are those related to the serum lipoprotein phospholipids (phosphatidylcholine, 0.9 ppm; sphingomyelin and other phospholipids, 0.3 ppm). These signals are present in the spectrum of plasma (Fig. 2*B*), but are absent in the spectrum obtained from packed erythrocytes (Fig. 2*A*). The well-established resonance positions of these plasma components (4–6) were erroneously ascribed to erythrocyte membrane phospholipids by Moon and Richards (1).

In the orthophosphate region of the spectrum (Figs. 1 and 2*A*), there are also a group of minor resonances centered at −3.7 ppm which, judging from their shift position, must arise from orthophosphate monoesters. Although it is not yet possible to assign these resonances to specific compounds, it is likely that they arise from triose and hexose phosphates since the prominent peaks observed in the presence of added inosine and pyruvate (Figs. 3*B* and 5*B*) correspond with the known elevation of these substances under these conditions (7, 8).

The resonances centered at 5.9 and 10.7 ppm (Figs. 1, 2*A*, and 3) correspond, respectively, to unesterified and esterified end phosphates of polyphosphate chains. The signal at 20.2 ppm corresponds to the unesterified middle phosphates of

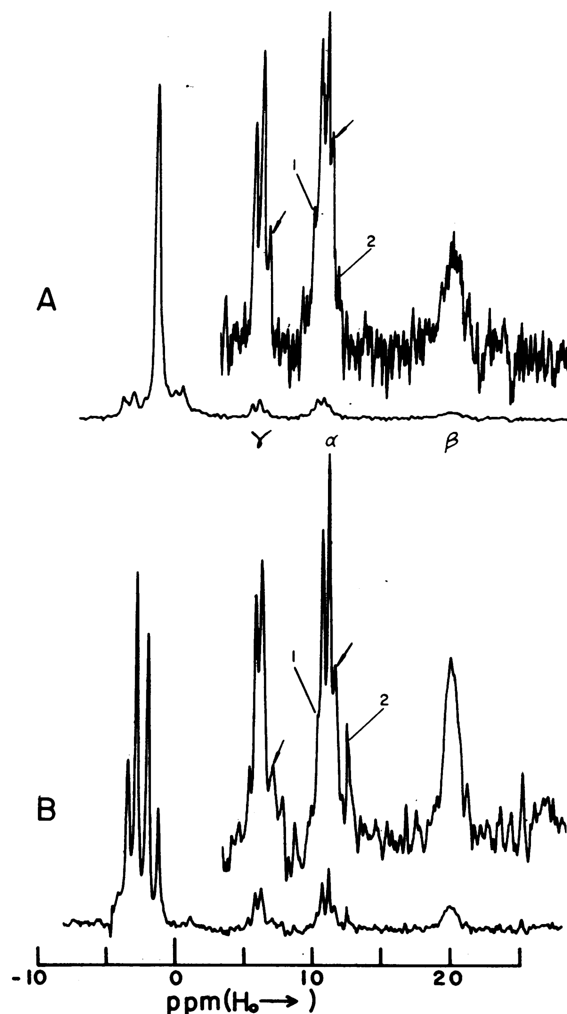

FIG. 3. Spectrum *A* is the ^{31}P NMR spectrum of packed human erythrocytes from blood that had been stored at 4° for 5 days, then depleted of 2,3-diphosphoglycerate by incubation at 25° for 16 hr. Spectrum *B* was obtained from packed erythrocytes isolated from an aliquot of the blood used for spectrum *A* that had been incubated for 4 hr at 25° with 10 mM inosine and 10 mM pyruvate to increase the 2,3-diphosphoglycerate content. The *insets* in both *A* and *B* are readouts of the ATP region of the spectra. Resonance *1* arises from an unidentified esterified end phosphate; resonance *2* most likely arises from NAD^+ and/or NADH. Note the differences in the amplitudes of the upfield shoulders of the α- and γ-P of ATP (indicated by the *unlabeled arrows*) between *A* and *B*. The slight differences in chemical shifts of the orthophosphate ester region between *A* and *B* result from differences in the intracellular pH of the two preparations (see ref. 1).

these chains. Fig. 3*A* is the ^{31}P spectrum of erythrocytes that had been depleted of 2,3-diphosphoglycerate by aging ACD blood overnight at 25°, whereas Fig. 3*B* is the spectrum of an aliquot of the same erythrocytes enriched in 2,3-diphosphoglycerate by incubation with 10 mM inosine and 10 mM pyruvate. In Fig. 3*B*, the proton-coupled areas of the three resonance bands of the polyphosphate chains approximate a 1:1:1 ratio; the measured coupling constant of 17.1 Hz for both end-group multiplets is consistent with P—O—P coupling. The middle group resonance has not been fully resolved, but the data indicate that the signal consists of at

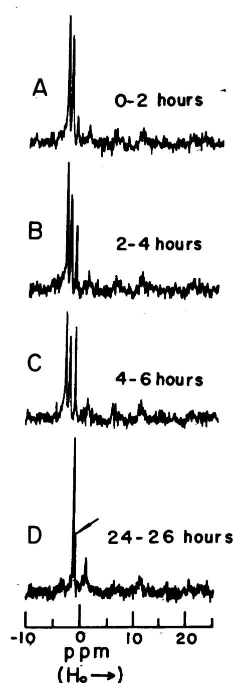

FIG. 4. Changes in the erythrocyte content of 2,3-diphosphoglycerate and P_i, as determined by ^{31}P NMR analysis of whole human blood in ACD medium, as a function of incubation time at 25°. Accumulation of ^{31}P NMR data for spectrum *A* was begun within 5 min after the blood was drawn and continued for 2 hr; spectrum *B* represents the data accumulated during the second 2-hr period after withdrawal; spectrum *C*, during the third 2-hr period after withdrawal; spectrum *D*, during the 2-hr period following 24 hr after withdrawal. Each spectrum represents about 17,100 pulses. The *arrow* in spectrum *D* refers to the P_i which is associated with the plasma (see Fig. 2*B* and ref. 1).

least two overlapping sets of triplets (one major and one minor) exhibiting the 17.1 Hz coupling constant. These results are consistent with a monoesterified tripolyphosphate and since ATP predominates in this system (7), the bulk of these resonances are ascribed to ATP. (See ref. 9, p. 13, for a well-resolved ^{31}P spectrum of this molecule; also ref. 10.)

The numbered arrows in Fig. 3 identify minor resonances for which no rigorous interpretation can be given at this time. Their shift positions and the fact that they are proton-coupled, however, identifies them as arising from esterified end-group phosphates. With respect to signal 2, the relative amount (11), multiplicity, and shift position (1) are consistent with the interpretation that it arises from NAD^+ and/or NADH.

It has been suggested from studies with hemoglobin solutions (12) that ATP exists predominantly in the intact erythrocyte as the Mg^{++}-bound and hemoglobin-bound (Hb·ATP) forms. Moreover, it has been proposed that the amount of Hb·ATP depends on the level of 2,3-diphosphoglycerate, with elevated amounts of the latter causing a lowering of the Hb·ATP levels. The magnified condensed phosphate regions of the ^{31}P spectra of Fig. 3*A* and *B* are consistent with this hypothesis. Both end-group resonance multiplets show a minor doublet on their high-field sides centered at 6.4 and 10.9 ppm. These signals, which account for about 20% of the total multiplet area in erythrocytes with high 2,3-diphosphoglycerate (Fig. 3*B*), exhibit the same

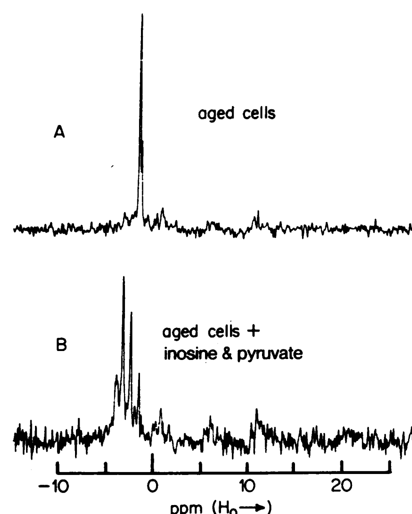

FIG. 5. Spectrum *A* was obtained from whole human blood that had been stored in ACD medium for 5 weeks at 4°. Under these conditions, erythrocytes contain barely detectable levels of 2,3-diphosphoglycerate. Spectrum *B* was obtained from an aliquot of the same blood used to obtain spectrum *A*, which had been incubated at 25° for 4 hr with 10 mM inosine and 10 mM pyruvate. Note the marked increase in the resonances attributable to 2,3-diphosphoglycerate and the appearance of increased amounts of an unidentified phosphate monoester indicated by the resonance at −3.7 ppm. Both spectra required 2 hr (about 17,000 pulses) of ^{31}P NMR signal accumulation.

spectral parameters as ATP and are thus assigned to this molecule. These minor doublets are enhanced under conditions of low 2,3-diphosphoglycerate concentration (Fig. 3*A*), in agreement with the relative affinities of ATP and 2,3-diphosphoglycerate for hemoglobin (12). Since these samples were treated in identical fashion, it seems unlikely that significant pO_2 differences were present to influence these results.

Fig. 4 presents spectra of whole human blood incubated aseptically at 25° for different periods of time following the collection of the blood. Each spectrum was obtained during 2 hr of ^{31}P analysis (about 17,000 repetitions). Comparison of the relative areas of the 2,3-diphosphoglycerate resonance bands at different time intervals demonstrates that the 2,3-diphosphoglycerate in the erythrocytes decreased rapidly at 25°, with only a small amount remaining after 26 hr. During the same interval, there was a concomitant increase in the P_i concentration but little, if any, change in the plasma phospholipid concentration. These observations are in general agreement with those seen in cells stored in ACD medium at 4° (13) as well as in cells incubated *in vitro* at 37° (14), both of which show 2,3-diphosphoglycerate decreases and P_i increases with time.

Fig. 5 presents ^{31}P spectra of blood that had been previously cold-stored in ACD medium for 5 weeks. Spectrum A was obtained from a sample of blood to which no additions had been made and the cells contained barely detectable levels of 2,3-diphosphoglycerate, in agreement with earlier observations on cold-stored whole blood (see ref. 8). Spectrum B was obtained after an aliquot of the same blood sample had been incubated with 10 mM inosine and 10 mM pyruvate for 4 hr at 25°. The 2,3-diphosphoglycerate content of the treated erythrocytes increased, as might have been predicted from

previous results (8) in which measurements were made on erythrocyte extracts with conventional biochemical techniques (8). In addition, the phosphate monoester resonance, which was barely detectable at −3.7 ppm in either the control (Fig. 5*A*) or freshly drawn blood (Fig. 4*A*), was markedly enhanced. The relative areas of the condensed phosphate signals, however, were not measurably affected by this treatment.

When the areas of the end and middle phosphate groups in the condensed phosphate regions of the spectra were compared, it was observed that these showed the expected 1:1:1 ratios in cases when the level of 2,3-diphosphoglycerate was elevated, i.e., fresh blood samples (Figs. 1 and 4*A*) or aged samples treated with inosine and pyruvate (Figs. 3*B* and 5*B*). In contrast, in aged samples with low 2,3-diphosphoglycerate concentration, the areas of the end groups relative to the middle group were considerably enhanced (compare Figs. 3*A* and 5*A*). It is suggested that this result is due to an increased proportion of ADP, relative to ATP. The ADP level is decreased in cold-stored erythrocytes but is, nevertheless, present to the extent of about 50% of the depressed ATP level, which is 0.05 mM after 6 weeks of storage (7). Further, the shifts and coupling constants of the β group of ADP and γ group of ATP are virtually indistinguishable at physiological pH (10). The α groups of these two nucleotides also have essentially the same coupling constant, but in this case the shift of the α phosphorus of ADP lies about 0.6 Hz downfield to the α phosphorus of ATP. These differences are too small to be resolved in the spectra of this study, and it would appear that the presence of ADP in experiments of this kind may only be detected by comparing the relative areas of the end and middle phosphate group resonances.

The two end-group multiplets were proportionately enhanced, indicating the presence of equal increments of phosphorus in each of the groups, as would also have been expected if ADP were contributing equally to the respective areas. In Fig. 3*B*, this relative enhancement amounts to from 30% to 40% of the area of each end-group multiplet which, if attributable to ADP, would indicate that this amount of the original ATP in fresh erythrocytes had been converted to ADP during cold storage for 5 days and the overnight incubation at 25°.

The ultimate goal in investigating cellular metabolism is to follow biochemical changes as they occur in intact cells under physiological conditions. This study demonstrates that, at least with human erythrocytes, this goal can be achieved through the use of ^{31}P NMR.

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1. Moon, R. B. & Richards, J. H. (1973) *J. Biol. Chem.* **248,** 7276–7278.
2. Glonek, T., Henderson, T. O., Hilderbrand, R. L. & Myers, T. C. (1970) *Science* **169,** 192–194.
3. Henderson, T. O., Glonek, T., Hilderbrand, R. L. & Myers, T. C. (1972) *Arch. Biochem. Biophys.* **149,** 484–497.
4. Glonek, T., Henderson, T. O., Kruski, A. & Scanu, A. M. (1974) *Biochim. Biophys. Acta,* in press.
5. Glonek, T., Henderson, T. O., Kruski, A. & Scanu, A. M. (1973) *Abstracts of the 166th National Meeting of the American Chemical Society,* Abstract 209 (Biochemistry Section).
6. Henderson, T. O., Glonek, T. & Myers, T. C. (1974) *Biochemistry* **13,** 623–628.
7. Yoshikawa, H. & Minakami, S. (1968) *Folia Haemat.* **89,** 357–373.
8. Gerlach, E. & Duhm, J. (1972) in *Oxygen Affinity of Hemoglobin and Red Cell Acid-Base Status,* eds. Rørth, M. & Astrup, P. (Munksgaard, Copenhagen), pp. 552–569.
9. Crutchfield, M. M., Dungan, C. H. & Van Wazer, J. R. (1967) in *Topics in Phosphorus Chemistry,* eds. Grayson, M. & Griffith, E. J. (Interscience, New York), Vol. 5, pp. 1–74.
10. Cohn, M. & Hughes, T. R. (1960) *J. Biol. Chem.* **235,** 3250–3253.
11. Omachi, A., Scott, C. B. & Ford, D. L. (1972) *Clin. Chim. Acta* **37,** 351–358.
12. Gerber, G., Berger, H., Jänig, G. & Rapoport, S. M. (1973) *Eur. J. Biochem.* **38,** 563–571.
13. Bartlett, G. R. & Barnet, H. N. (1966) *J. Clin. Invest.* **39,** 56–61.
14. Gerlach, E., Duhm, J. & Deuticke, B. (1970) in *Red Cell Metabolism and Function,* ed. Brewer, G. J. (Plenum Press, New York), pp. 155–174.