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# $^{31}$P NMR study of erythrocytes from a patient with hereditary pyrimidine-5′-nucleotidase deficiency

(intracellular pH/free $Mg^{2+}$/Mg-NTP level)

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**ABSTRACT** The composition of phosphate metabolites and the intracellular pH in erythrocytes from a patient with hereditary pyrimidine-5′-nucleotidase deficiency were examined using $^{31}$P NMR spectroscopy. Several resonances were identified in spectra from intact cells and from extracts. The 2,3-bisphosphoglycerate line intensities were normal but the NTP resonances were about twice normal due to the presence of millimolar quantities of pyrimidine phosphates. Several intense resonances were also observed in the diphosphodiester region of the spectrum. One compound contributing to these lines has been identified as cytidine diphosphocholine. The resonances of NTPs were in a position indicating that the additional triphosphates were also bound by $Mg^{2+}$. Direct measurement shows that there is a nearly proportional increase in total cell $Mg^{2+}$ in the patient's cells, in agreement with the interpretation of the spectra. The intracellular pH was about 0.2 unit lower in the patient's erythrocytes. This lower pH is due to the elevation in intracellular fixed negative charges and the shift in permeable anions consequent to the Donnan equilibrium. We suggest that the lower intracellular pH may explain the lower oxygen affinity of these cells in the presence of otherwise normal 2,3-bisphosphoglycerate levels and the increased $Mg^{2+}$ triphosphates level, because the $Mg^{2+}$ form of NTPs is known not to alter the oxygen affinity of hemoglobin under physiologic conditions. Furthermore, the lower intracellular pH can also explain the abnormalities in glycolytic intermediates observed for these cells.

In 1974, Valentine *et al.* (1) described a hereditary hemolytic anemia characterized by pronounced basophilic stippling with a 3- to 6-fold increase in total erythrocyte nucleotides, predominantly of the pyrimidine type. They then found a pyrimidine-specific 5′-nucleotidase in erythrocytes and showed that the activity of this enzyme was deficient in their patient's erythrocytes. Although it was initially assumed that pyrimidine nucleotide accumulation was derived from RNA degradation in maturing erythrocytes, Harley *et al.* (2) showed that the salvage pathway from uridine was the most likely major contributor to accumulation of erythrocyte nucleotides in pyrimidine-5′-nucleotidase deficiency (PND).

Torrance and Whittaker (3) have recently made a detailed analysis of the composition of the nucleotide pool in this disease. They showed large increases in UTP and CTP levels with little change in 2,3-bisphosphoglycerate ($P_2$-glycerate) or ATP concentrations. High levels of UDP-glucose and an unknown cytidine diphosphate compound were noted, among other differences. They also showed that the oxygen affinity of blood from these patients was lower than normal, despite the fact that $P_2$-glycerate levels were normal. It was suggested, although not shown, that one reason for the difference might be a lowered intracellular pH consequent to Donnan-related ion shifts that result as intracellular fixed negative charges accumulate (4, 5). The possiblity that the increased NTPs may act as allosteric cofactors much like $P_2$-glycerate was also considered in order to account for the lower blood oxygen affinity (3). However, Bunn *et al.* (6) showed that the $Mg^{2+}$-bound form of ATP does not affect the oxygen affinity of hemoglobin.

In this communication, we apply $^{31}$P NMR to the study of this hereditary erythrocyte enzyme deficiency to directly measure the intracellular pH and to further characterize the phosphate metabolites. The use of $^{31}$P NMR in the study of intracellular pH and metabolism in intact cells (7–9), perfused organs (10–13), and even whole organisms (14) is now a well-established and often preferred method.

## MATERIALS AND METHODS

Venous blood from healthy donors was drawn into heparin-containing tubes and washed several times in cold phosphate-buffered saline (pH 7.4). Blood was similarly drawn from the PND individual.

Metabolic phosphates were extracted by the method of Chen *et al.* (15) in which the erythrocytes are hemolyzed in distilled water and protein is precipitated with trichloroacetic acid. Removal of trichloroacetic acid was accomplished by using tri-*n*-octylamine. Neutralized extracts were made 5 mM in EDTA (pH 7.4). Metabolites, especially the unknown "CDP" peak identified previously (3), were isolated by using HPLC anion exchange columns (16) and desalted by gel filtration on Sephadex G-15. Erythrocyte magnesium was determined by $C_2H_2$ flame atomic absorption spectroscopy.

$^{31}$P NMR spectra were obtained as described (11, 13, 17) at 60.7 MHz using a Nicolet NTC 150 wide-bore superconducting spectrometer operating in the Fourier-transform mode. All spectra were broad-band proton decoupled and measurements were made at 25°C. Spectra are presented in ppm relative to phosphoric acid as an external standard. pH was determined by measuring the difference (in ppm) between the 3-phosphate peak of $P_2$-glycerate and the $\alpha$-P ATP peak of MgATP, which serves as an internal standard insensitive to pH over the range used. These shifts were compared with a simulated intraerythrocytic environment by titration of a hemolysate.

## RESULTS AND DISCUSSION

Typical 60.7-MHz $^{31}$P NMR spectra of normal and PND erythrocytes are shown in Fig. 1. The normal spectrum is typical of

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Abbreviations: PND, pyrimidine-5′-nucleotidase deficiency; $P_2$-glycerate, 2,3-bisphosphoglycerate.

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intact washed erythrocytes (7, 18, 19). The strong $P_2$-glycerate and MgATP lines indicate that our cells were in excellent metabolic condition. The spectrum of PND erythrocytes is highly unusual and reflects the large increase in pyrimidine phosphates (1–3). The $P_2$-glycerate concentration is the same in the normal and PND cells, in agreement with previous chemical analyses (3).

The other peaks present in the spectrum of the patient's cells are NTP-$\gamma$, -$\alpha$, and -$\beta$ peaks (the $Mg^{2+}$-bound form) and a collection of diphosphodiester peaks between about +10 and +13 ppm (see below). Comparison of the NTP-$\beta$ peaks shows an approximately 2-fold increase in the level of cellular triphosphates despite virtually identical $P_2$-glycerate signals. The increase agrees with the chemical analysis of Torrance and Whittaker (3), who showed that patient and normal levels of ATP and $P_2$-glycerate were about the same while the UTP/CTP levels equaled that of ATP in the patient. It is interesting that the position of the NTP-$\beta$ peak is the same as control. This indicates that the bulk of the extra NTPs are complexed with $Mg^{2+}$. However, the patient erythrocytes would have to contain a higher total $Mg^{2+}$ to account for the spectrum. We have measured the total erythrocyte $Mg^{2+}$ levels directly and found, in agreement with the NMR spectrum, that there was a compensatory increase in total $Mg^{2+}$ (Table 1). Also, we have calculated the concentrations of species present in normal and patient cells, using our computer-assisted multiequilibria methods as described (17) in conjunction with previously published metabolite concentrations and stability constants (3, 20) and found that,

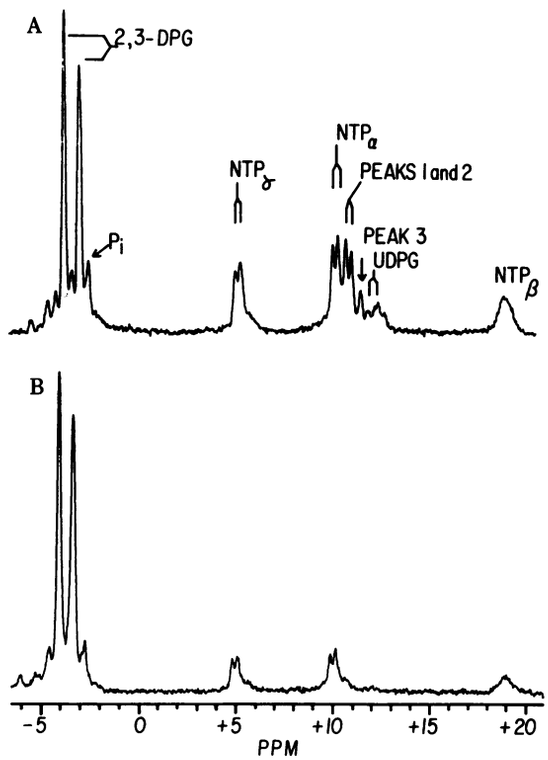


FIG. 1. $^{31}P$ NMR spectra at 60.7 MHz of normal (*B*) and PND (*A*) erythrocytes. The cells were washed in Tris-buffered saline (pH 7.4). Hematologic data from this subject showed a normal mean corpuscular hemoglobin concentration (34.5 g/dl) with 8.3% reticulocytes and polychromatophilia and basophilic stippling. Pyrimidine-5'-nucleotidase activity was 4.2 mol of uridine formed per hr per g of Hb (normal, 10.7–19.6). Three thousand pulses of 70° free-induction decays of 1.024 sec each at 25°C were accumulated for each spectrum. The spectra were collected with broad-band proton decoupling. 2,3-DPG, $P_2$-glycerate; UDPG, uridine-$P_2$-glycerate.

Table 1. Distribution of metabolites in normal and PND erythrocytes

| | Normal, mM | PND, mM |
|---|---|---|
| Total $Mg^{2+}$ | 2.3 | 3.2 |
| Total ATP | 1.2 | 1.06 |
| Total CTP/UTP | — | 1.05 |
| Total NTP | 1.2 | 2.11 |
| Total $P_2$-glycerate | 5.08 | 4.06 |
| Total $HbO_2$ | 6.7 | 6.7 |
| Free $Mg^{2+}$ | 0.61 | 0.68 |
| Free ATP | 0.11 | 0.09 |
| Free CTP/UTP | — | 0.09 |
| Free $P_2$-glycerate | 2.11 | 1.62 |
| Free $HbO_2$ | 4.2 | 4.4 |
| MgATP | 0.8 | 0.71 |
| MgCTP/MgUTP | — | 0.71 |
| $Mg^{2+}$ $P_2$-glycerate | 0.77 | 0.66 |
| NTP–$HbO_2$ | 0.17 | 0.28 |
| $P_2$-glycerate–$HbO_2$ | 2.21 | 1.78 |
| MgNTP–$HbO_2$ | 0.13 | 0.24 |

Total $Mg^{2+}$ was measured by atomic absorption spectroscopy. Other concentrations were calculated (17) based on total values given in ref. 3. Conditions were pH 7.2, 37°C, 20 mM NaCl/130 mM KCl.

70% of the total CTP/UTP exists in the $Mg^{2+}$-bound form as a consequence of the approximately equivalent increase in total $Mg^{2+}$.

The composition of the phosphate metabolites in PND cells was investigated further by studying the spectra of trichloroacetic acid extracts from patient cells. The spectra show several resolved resonances (Fig. 2). The NTP-$\beta$ region, now shifted to the $Mg^{2+}$-free position due to the presence of EDTA, shows two sets of triplet peaks. They are assigned to NTP-$\beta$ (purine) and NTP-$\beta$ (pyrimidine) compounds (21). The concentration of purine triphosphates equals the concentration of pyrimidine triphosphates (Table 1). Similar splittings are seen in the NTP-$\gamma$ resonances. The NTP-$\alpha$ resonances are more difficult to resolve in the extract spectra because they are shifted upfield due to the absence of $Mg^{2+}$ and now overlap peak 1 of the erythrocyte spectrum.

The positions of peaks, 1, 2, and 3 and the peaks in the region of UDP-glucose are not changed with extraction. The concentrations of the compounds in the cell giving peaks 1, 2, and 3 are 1 to 2 mM (Fig. 1). Torrance and Whittaker (3) have found, and we have confirmed, that an unknown cytidine diphosphate compound is present at about the 1 mM level. However, they were not able to completely identify the compound. We have attempted to identify the compounds that may account for the resonances in the diphosphodiester region. We have isolated one compound in the unknown "cytidine diphosphate" peak of Torrance and Whittaker (3) as described above and have collected $^1H$ NMR spectra at 360 MHz using the spectrometer in the Department of Chemistry at Lincoln. The spectrum (data not shown) was the same as the model compound spectrum for CDP-choline.

Although the $^1H$ NMR measurements show that CDP-choline is present, this compound alone cannot account for the signals in the diphosphodiester region of the $^{31}P$ NMR spectrum of the patient's cells when model compounds are used for comparison. CDP-choline showed a 1:3:3:1 quartet with resonances at +10.78, +11.12, +11.62, and +11.98 ppm. The last two peaks of CDP-choline would correspond to peak 3 and one of the peaks in the region of Figs. 1 and 2 labeled UDP-glucose, both with regard to chemical shift difference (0.35 ppm) and peak intensities. The chemical shift difference between peaks 2 and 3 in the PND spectrum (0.49 ppm) is very similar to that

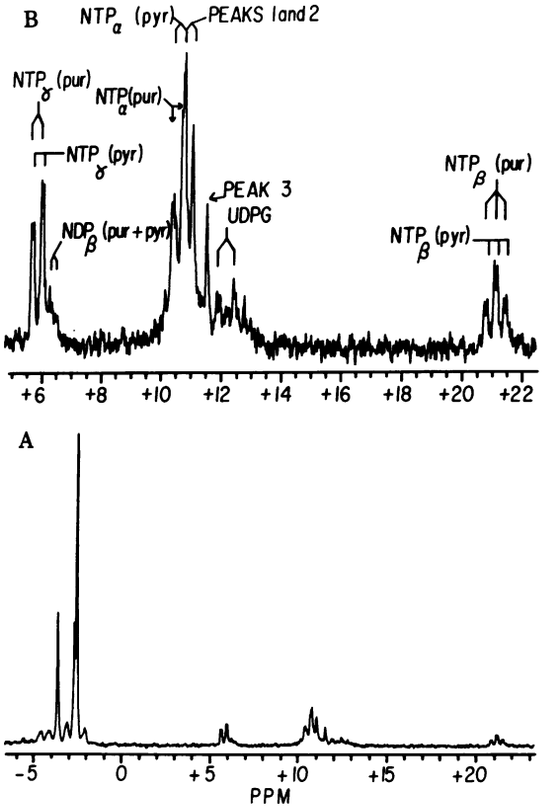


FIG. 2. $^{31}$P NMR spectra of trichloroacetic acid extracts from PND erythrocytes. The extracts were neutralized to pH 7.2. (*A*) Full-display spectrum. (*B*) Expanded version of *A* from about +6 to +22 ppm. Fifteen thousand pulses of 70° free-induction decays of 1.024 sec each at 25°C were accumulated. The spectrum was collected with broad-band proton decoupling. UDPG, uridine-$P_2$-glycerate; pyr, pyrimidine; pur, purine.

between the two central peaks of CDP-choline. Finally, the difference between peaks 1 and 2 in the PND spectrum (0.35 ppm) is also very similar to that between the first two peaks of CDP-choline. However, the peak-intensity ratios are not the same. We believe that the presence of other compounds in this region could explain this discrepancy. For example, CDP-ethanolamine has a single peak at 10.82 ppm, which, together with the CDP-choline peak in that region, would give a higher intensity to peak 1 in the PND spectrum at about 10.78 ppm. Peak 2 of the PND spectrum at 11.0 ppm may gain added intensity from the UDP-glucose peak at 11.0 ppm. Although it is at present difficult to positively identify all of the compounds contributing to the diphosphodiester region of the PND spectrum, we suggest that CDP-choline, CDP-ethanolamine, and UDP-glucose are present.

One of the most useful recent applications of $^{31}$P NMR is in measuring intracellular pH nondestructively (9, 14). The near doubling of the total nucleotide phosphate pool in PND erythrocytes should lower the intracellular pH consequent to the Donnan equilibrium. This phenomenon was shown several years ago by Salhany *et al.* (5) and Duhm (4), who correlated the fall in intracellular pH level with a rise in $P_2$-glycerate level. Plots of measured (pH meter) pH values vs. the difference in chemical shift (in ppm) between the $P_2$-glycerate 3-phosphate resonance and the NTP $\alpha$-phosphate resonance for a hemolysate and for intact control and PND erythrocytes are shown in Fig. 3. The hemolysate was titrated at physiologic ionic strength and serves as a calibration curve. Qualitatively, the fact that the control and PND curves are displaced to the right of the hemolysate

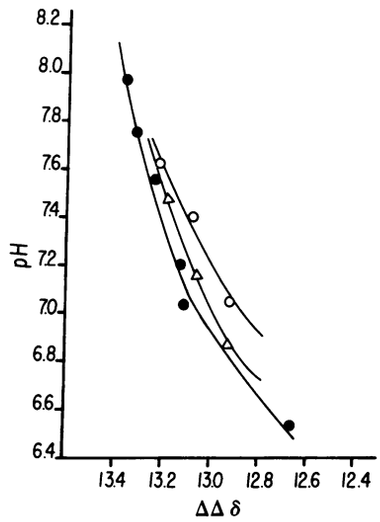


FIG. 3. Dependence of the chemical shift differences ($\Delta\Delta\delta$) between the $P_2$-glycerate 3-phosphate and the ATP $\alpha$-phosphate resonances on measured pH for hemolysate (●) at physiologic ionic strength and for control (△) and PND (○) erythrocytes. Spectral collection conditions were as in Fig. 1.

line shows that intracellular pH is lower in control cells over this range and even lower in PND cells. Quantitatively, we have measured intracellular pH for both sets of cells at an extracellular pH of 7.4 and found that the pH of control cells was 7.2, in agreement with other results (19), while the patient's cells had a pH of 7.0, fully 0.2 unit below the normal value. These pH values are accurate to ±0.03 pH unit based on an experimental error in measuring peak positions to within 0.01 ppm. Furthermore, since the ionic strength of the hemolysate titration curve was physiological, comparison with intracellular ionic strength should be valid.

Intracellular pH values as low as reported here have been observed in a patient with autoimmune hemolytic anemia by Lam *et al.* (19), who also showed that the pH of erythrocytes from patients with sickle cell disease was about 0.1 unit lower than normal. It is difficult to know how the lower intracellular pH found for PND erythrocytes might contribute to the hemolytic anemia. Several enzymes of the cell are sensitive to pH. It is well known that a decrease in pH reduces overall flux through the glycolytic pathway and so changes the levels of glycolytic intermediates. This has been studied by Minakami and Yoshikawa (22), who showed little change in ATP but a substantial increase in glucose 6-phosphate. This increase will inhibit hexokinase. Fructose 6-phosphate is also elevated and a crossover point occurs with phosphofructokinase, which is inhibited due to the effect of pH on the normally marked ATP inhibition of this enzyme. A second crossover point occurs with pyruvate kinase with an accumulation of pyruvate. Examination of the peaks in the sugar phosphate region of the $^{31}$P NMR spectra of normal and PND cells shows a significant difference in the intensity of the peak at −4.9 ppm (second peak from the left). This peak is twice as intense in PND cells as in normal, and its intensity and position in normal cells would be consistent with glucose 6-phosphate. Although positive identification was not made in our experiments, Paglia *et al.* (23) have recently reported a 2-fold increase in glucose 6-phosphate, consistent with the tentative interpretation of the PND spectrum (Fig. 1). Thus, we conclude that the 0.2-unit lowering of intracellular pH of PND cells can explain the 2-fold increase in a sugar phosphate tentatively identified as glucose 6-phosphate. The other peak intensities are the same as control (the peak in the PND spectra

between the 2- and 3-phosphate peaks of $P_2$-glycerate is also present in the control but is more evident in the PND spectrum due to the pH shift in the resonances).

Besides alterations in glycolysis, the lower intracellular pH will promote increased binding of cytosol proteins to the membrane (24). Eisinger *et al.* (25) have recently shown increased hemoglobin binding to or closer association with the membrane of intact erythrocytes at an intracellular pH of 7.2–7.0. The extent to which additional hemoglobin or enzyme binding to band 3 protein alters membrane integrity remains an open and speculative area in need of further research.

The lower pH observed for PND cells very probably also explains the lower oxygen affinity observed for PND blood (3). We have calculated that a 0.2-unit lowering of intracellular pH should decrease the oxygen affinity by about 25% over this pH range. Torrance and Whittaker (3) observed a 13% lower affinity. The possibility that these phosphates directly affect the oxygen affinity by binding to the $P_2$-glycerate site on hemoglobin seems less likely based on our finding that the extra NTP compounds exist in the $Mg^{2+}$-bound form (Fig. 1 and Table 1) and on the demonstration by Bunn *et al.* (6) that MgATP has no effect on the oxygen affinity of hemoglobin. Finally, although standard blood pH is usually taken as 7.4, acidosis caused by exercise or other reasons may have larger effects on the oxygen affinity and metabolism of PND blood because of the larger pH gradient across the erythrocyte membrane consequent to elevations in pyrimidine phosphates.

In summary, we have shown that the fixed pH gradient across the erythrocyte membrane of PND erythrocytes is very large. It very probably explains the lower oxygen affinity of the cell and its abnormal glycolysis. Other consequences of the lower intracellular pH and altered metabolic state to cell survival remain to be established.

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1. Valentine, W. N., Fink, K., Paglia, D. E., Harris, S. R. & Adams, W. S. (1974) *J. Clin. Invest.* **54**, 866–879.
2. Harley, E. H., Heaton, A. & Wicomb, W. (1978) *Metabolism* **27**, 1743–1754.
3. Torrance, J. D. & Whittaker, D. (1979) *Br. J. Haematol.* **43**, 423–434.
4. Duhm, J. (1971) *Pflügers Arch.* **326**, 341–356.
5. Salhany, J. M., Keitt, A. S. & Eliot, R. S. (1971) *FEBS Lett.* **16**, 257–261.
6. Bunn, H. F., Ransil, B. J. & Chao, A. (1971) *J. Biol. Chem.* **246**, 5273–5279.
7. Moon, R. B. & Richards, J. H. (1973) *J. Biol. Chem.* **248**, 7276–7278.
8. Salhany, J. M., Yamane, T., Shulman, R. G. & Ogawa, S. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4966–4970.
9. Shulman, R. G., Brown, T. R., Ugurbil, K., Ogawa, S., Cohen, S. M. & den Hollander, J. A. (1979) *Science* **205**, 160–166.
10. Hoult, D. I., Busby, S. J. W., Gadian, D. G., Radda, G. K., Richards, R. E. & Seeley, P. J. (1974) *Nature (London)* **252**, 285–287.
11. Salhany, J. M., Pieper, G. M., Wu, S. T., Todd, G. L., Clayton, F. C. & Eliot, R. S. (1979) *J. Mol. Cell. Cardiol.* **11**, 601–610.
12. Meyer, R. A., Kushmerick, M. J. & Brown, T. R. (1982) *Am. J. Physiol.* **242**, C1–C11.
13. Salhany, J. M., Stohs, S. J., Reinke, L. A., Pieper, G. M. & Hassing, J. M. (1979) *Biochem. Biophys. Res. Commun.* **86**, 1077–1083.
14. Gadian, D. G. & Radda, G. K. (1981) *Annu. Rev. Biochem.* **50**, 69–83.
15. Chen, S. C., Brown, P. R. & Rose, D. M. (1977) *J. Chromatogr. Sci.* **15**, 218–221.
16. Angle, C. R., McIntire, M. S., Swanson, M. S. & Stohs, S. J. (1982) *Pediatr. Res.* **16**, 331–334.
17. Wu, S. T., Pieper, G. M., Salhany, J. M. & Eliot, R. S. (1981) *Biochemistry* **20**, 7399–7403.
18. Henderson, T. O., Costello, A. J. R. & Omachi, A. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2487–2490.
19. Lam, Y. F., Lin, A. K.-L. C. & Ho, C. (1979) *Blood* **54**, 196–209.
20. Gerber, G., Berger, H., Janig, G. R. & Rapoport, S. M. (1973) *Eur. J. Biochem.* **38**, 563–571.
21. Ugurbil, K., Rottenberg, H., Glynn, P. & Shulman, R. G. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2244–2248.
22. Minakami, S. & Yoshikawa, H. (1966) *J. Biochem.* **59**, 145–150.
23. Paglia, D. E., Fink, K. & Valentine, W. N. (1980) *Acta Haematol.* **63**, 262–267.
24. Salhany, J. M. & Gaines, K. C. (1981) *Trends Biochem. Sci.* **6**, 13–15.
25. Eisinger, J., Flores, J. & Salhany, J. M. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 408–412.