ments were carried out at the High Flux Isotope Reactor. We discuss here only the main results of our study (*3*). The neutron cross sections of the $^{144}NdCl_3$ solution (having the smallest scattering factor; see Table 1) were subtracted from those of the other three solutions to yield three difference curves, from which a structure function was extracted, namely

$$H_{Nd}(k) = 0.279h_{NdO}(k) + 0.642h_{NdD}(k) + 0.079h_{NdCl}(k) \quad (1)$$

where $k = (4\pi/\lambda)\sin\theta$, $\lambda = 0.89$ Å being the neutron wavelength and 2θ the scattering angle.

The numerical coefficients in Eq. 1 depend only on the neutron scattering factors and the concentrations of the nuclides in the solutions (*2*). The functions $h_{ij}(k)$ are the Fourier transforms of the atom pair correlation functions $h_{ij}(r) = g_{ij}(r) - 1$, where the atom pair distribution functions $g_{ij}(r)$ measure the probability of finding a j atom at a radial distance r from an i atom in the solution. Fourier transformation of the function $H_{Nd}(k)$ derived from experiment yields an average radial distribution function, namely

$$G_{Nd}(r) = 0.279g_{NdO}(r) + 0.642g_{NdD}(r) + 0.079g_{NdCl}(r) \quad (2)$$

which contains the desired information on the ion-water interactions in the functions $g_{NdO}(r)$ and $g_{NdD}(r)$. The function $G_{Nd}(r)$ is shown in Fig. 1.

The maxima in the function $G_{Nd}(r)$ derived from experiment correspond to the most frequent Nd–O, Nd–D, and Nd–Cl distances in the solution. The first two pronounced maxima at 2.48 and 3.13 Å must be ascribed, respectively, to Nd–O and Nd–D interactions. This is because the areas under these peaks, which are related to the number of atoms at these relative positions, have a ratio of ~ 1:2. The area under the peak at 2.48 Å corresponds to 8.6 oxygen atoms, and the area under the peak at 3.13 Å to 16.7 deuterium atoms around a neodymium ion. Taking the average (8.6 + 16.7/2)/2 as the best estimate of the number of water molecules around the ion, we obtain a coordination number of 8.5 ± 0.2. These numbers, together with the sharpness of the first two peaks in Fig. 1, show that the Nd^{3+} ion has a very well-defined first hydration sphere with the deuterium atoms pointing away from the ion at a tilt angle of 55°, as shown in Fig. 2. This picture is, of course, an average one; at any instant an average Nd^{3+} ion "sees" the water molecules in its primary hydration sphere as indicated in Fig. 2. The residence time of these molecules is finite but may be as long as 10^{-6} second (*4*).

Table 1. Neutron-scattering factors (f) of the Nd nuclei in the four 2.85 molal solutions studied by neutron diffraction.

Isotope	Abundance (%)	f (10^{-12} cm)
^{144}Nd	97.51	0.31
^{nat}Nd*		0.72
^{142}Nd	97.55	0.78
^{146}Nd	97.63	0.87

*Natural abundance.

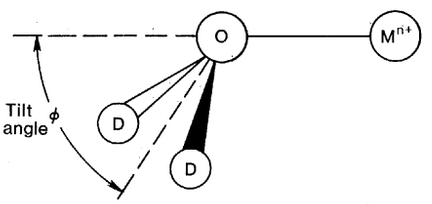

Fig. 2. Arrangement of a water molecule in the first hydration sphere of a cation, M^{n+}, in solution. There are 8.5 such molecules around Nd^{3+}, and the tilt angle is 55°.

We emphasize that these distances and coordination numbers should be regarded as direct measurements, no assumptions or models being used in the analysis of the neutron data. This method avoids the difficulties encountered in other experimental techniques (*5*); the primary hydration sphere is precisely defined in terms of the number of water molecules included and their average distance from the cation. The 8.5 water molecules are sufficiently firmly bound to be regarded as part of the Nd^{3+} ion in statistical mechanical calculations of the properties of these solutions (*6*).

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References and Notes

1. J. Burgess, *Metal Ions in Solution* (Horwood, Chichester, England, 1978).
2. J. E. Enderby and G. W. Neilson, in *Water: A Comprehensive Treatise*, F. Franks, Ed. (Plenum, New York, 1979), vol. 6, p. 1.
3. A full report by A. H. Narten and R. L. Hahn is in preparation.
4. J. E. Enderby, *Sci. Prog. (London)* **67**, 553 (1981).
5. In a recent x-ray diffraction study by A. Habenschuss and F. H. Spedding [*J. Chem. Phys.* **70**, 3758 (1979)] a coordination number of 8.9 water molecules at a distance of 2.51 Å from the neodymium ion was obtained by fitting the total radial distribution function with five Gaussian distributions.
6. R. Triolo, in *Advances in Solution Chemistry*, I. Bertini, L. Lunazzi, A. Dei, Eds. (Plenum, New York, 1981), p. 51.
7. Research sponsored by the Divisions of Materials Sciences and Chemical Sciences, Office of Basic Energy Sciences, U.S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation.

19 May 1982; revised 9 July 1982

Chemoprevention of Neonatal Jaundice: Potency of Tin-Protoporphyrin in an Animal Model

Abstract. *The substantial increases of hepatic, splenic, and renal heme oxygenase levels that occur shortly after birth in neonatal rats were prevented by a single administration of tin-protoporphyrin (10 micromoles per kilogram of body weight). With this treatment serum bilirubin levels declined within 24 hours to near-normal adult levels and remained low throughout the postnatal period. Zinc-protoporphyrin at doses up to 50-fold greater than the effective dose of tin-protoporphyrin did not prevent the immediate increases in tissue heme oxygenase activities and in serum bilirubin levels that occur postnatally. Studies in vitro with microsomal heme oxygenase in human spleen indicate that tin-protoporphyrin is a potent competitive inhibitor of the oxidation of heme to bile pigment in this tissue.*

Bilirubin is a potential central nervous system toxin for infants in the period immediately after birth when the blood-brain barrier is still permeable to many substances (*1*). In the human newborn, large amounts of this bile pigment are produced as a result of lysis of fetal red cells and enhanced degradation of the heme (iron-protoporphyrin) moiety of the fetal hemoglobin molecule. Since the capacity of the newborn liver to detoxify bilirubin by glucuronide conjugate formation is not fully developed, the unconjugated bile pigment accumulates in the bloodstream during the first week or more of neonatal life. Quite high plasma levels of bilirubin may occur during this period and the term "physiological" or neonatal jaundice has been applied to this phenomenon in humans (*2*).

Neonatal jaundice is common, and many clinical events such as prematurity, infection, and hypoxia can exaggerate the degree of hyperbilirubinemia to such an extent that serious risk of the development of subtle or overt neurotoxicity may occur (*2*). Treatments for excessive hyperbilirubinemia include phototherapy to degrade bilirubin to more hydrophilic isomers (*3*), drugs to induce

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prematurely the hepatic glucuronyl transferase system (*4*), and, in extreme instances, total exchange transfusions (*2*). Each mode of therapy currently available is successful to some extent in ameliorating the problem of excessive hyperbilirubinemia in the newborn, but each has certain complications. Moreover, the application of these treatments is arbitrary in the sense that, depending on particular clinical circumstances, a specific level of plasma bilirubin or rate of increase of this bile pigment in the circulation is judged to be potentially deleterious to the infant before a decision to commence therapy is made. These therapeutic modalities are all directed at enhancing the metabolic disposition of bilirubin after the bile pigment has been produced in the spleen, liver, and other tissues through the action of heme oxygenase.

In a new approach to the prevention of neonatal hyperbilirubinemia (*5*), we used the principle of competitive enzyme inhibition to block the degradation of heme to bile pigment by heme oxygenase. For this purpose, we used tin-protoporphyrin, a synthetic metalloporphyrin that has a much greater affinity for the catalytic site on the enzyme than does the substrate heme. Since tin-protoporphyrin does not bind molecular oxygen, it is not degraded by the enzyme. Bile pigment formation is therefore diminished and serum bilirubin levels drop promptly, effects that may last for several weeks, depending on the dose of the synthetic metalloporphyrin used. Other synthetic metalloporphyrins, such as cobalt-protoporphyrin and manganese-protoporphyrin can competitively inhibit heme oxidation in vitro. Manganese-protoporphyrin does not, however, even in very large doses, prevent neonatal hyperbilirubinemia (*5*), and cobalt-protoporphyrin is a potent inducer of heme oxygenase in vivo (*6*). Thus, whole animal studies are essential to establish whether a synthetic metalloporphyrin that competitively inhibits heme oxygenase in vitro will also prevent hyperbilirubinemia in the neonate.

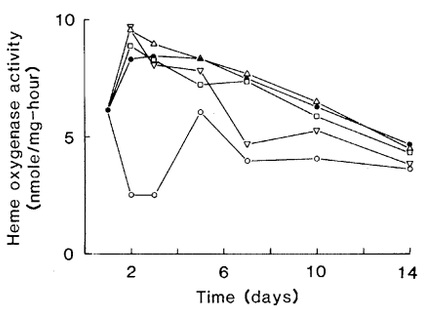

Fig. 1. Effects of tin-protoporphyrin and zinc-protoporphyrin on hepatic heme oxygenase activity in vivo in neonates. Metalloporphyrins were administered subcutaneously: (○) tin-protoporphyrin, 10 μmole per kilogram of body weight; zinc-protoporphyrin, (△) 20 μmole/kg or (□) 100 μmole/kg at birth, or (▽) 100 μmole/kg at birth and again 12, 24, 48, and 72 hours later; (●) control animals received equivalent volumes of saline. Animals were killed at the times indicated. Each time point represents the average of a minimum of three litters (approximately ten animals per litter). Microsomes were prepared and heme oxygenase activity was assayed as described (*5*). Heme oxygenase activity is expressed as nanomoles of bilirubin formed per milligram of protein per hour. Tin-protoporphyrin and zinc-protoporphyrin were purchased from Porphyrin Products, Logan, Utah. Metalloporphyrin solutions were prepared as described (*5*).

Table 1. The K_m of human spleen heme oxygenase and the K_i of tin-protoporphyrin are each shown for six different samples. The K_m was derived from Lineweaver-Burk plots. The K_i was calculated from double reciprocal plots of substrate concentration versus the velocity of heme oxygenase in the presence of tin-protoporphyrin.

Heme oxygenase K_m (μM)	Tin-protoporphyrin K_i (μM)
20.00	0.013
22.22	0.018
15.63	0.019
16.12	0.018
21.05	0.019
19.23	0.020
Mean: 19.04 ± 0.08	Mean: 0.018 ± 0.001

We have now established the lowest effective dose of tin-protoporphyrin that will block neonatal hyperbilirubinemia in the rat and have also explored the ability of zinc-protoporphyrin, which inhibits heme oxygenase activity in vitro (*5, 7*), to block the development of hyperbilirubinemia in the newborn animal. Zinc-protoporphyrin is found in high concentration in erythrocytes in disorders such as lead poisoning and iron-deficiency anemia (*8*).

Single doses of tin-protoporphyrin ranging from 5 to 100 μmole per kilogram of body weight were administered at birth to groups of newborn rats; hepatic, splenic, and renal heme oxygenase activities and plasma bilirubin levels were determined (*5*) sequentially for the next 14 days. When tin-protoporphyrin was administered at 10 μmole/kg, hepatic heme oxygenase activity promptly decreased, and low levels of enzyme activity were maintained throughout the 14-day study period (Fig. 1). A transient increase in hepatic enzyme activity occurred at day 5; however, heme oxygenase activity in the neonates treated with tin-protoporphyrin remained significantly lower than that in the control group throughout the period of study. Tin-protoporphyrin (10 μmole/kg) also produced an immediate lowering of renal heme oxygenase activity and a halt in the developmental increase of splenic heme oxygenase activity (data not shown). Within 24 hours after tin-protoporphyrin was administered at 10 μmole/kg, total serum bilirubin decreased to levels close to those found in adult animals (Fig. 2). This dose of tin-protoporphyrin proved to be the lowest single dose of the compound that was effective in preventing hyperbilirubinemia in the immediate postnatal period.

In contrast, even multiple administrations of zinc-protoporphyrin (five doses each of 100 μmole/kg) during the 72-hour period immediately after birth failed to prevent the normal postnatal rise in hepatic heme oxygenase (Fig. 1). At day 7 a significant lowering of this hepatic enzyme activity was observed in treated animals compared to controls, but only at the highest dose of zinc-protoporphyrin studied (total dose 500 μmole/kg) (Fig. 1). A comparable late decline in renal heme oxygenase activity was also observed. In spleen, the inhibition of heme oxygenase activity did not become manifest until day 7. Zinc-protoporphyrin administration also failed to prevent the prompt postnatal rise in plasma bilirubin that occurs in neonates (Fig. 2). Thus zinc-protoporphyrin, while producing a delayed (5 to 7 days after birth) decrease in heme oxygenase activity in several organs, was not capable of block-

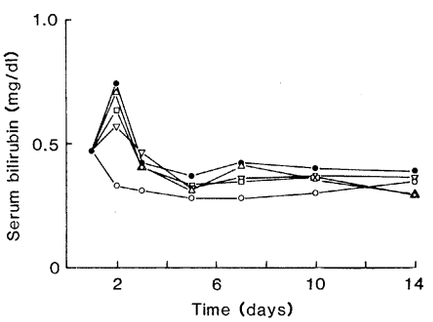

Fig. 2. Effects of tin-protoporphyrin and zinc-protoporphyrin on serum bilirubin levels in the neonate. The animals were treated as described in the legend to Fig. 1. Each time point represents the average of a minimum of three litters (approximately 10 animals per litter). Bilirubin was estimated by a fluorometric method (*15*). Metalloporphyrins were administered subcutaneously: (○) tin-protoporphyrin, 10 μmole per kilogram of body weight; zinc-protoporphyrin, (△) 20 μmole/kg or (□) 100 μmole/kg at birth, or (▽) 100 μmole/kg at birth and again 12, 24, 48, and 72 hours later; (●) control animals received equivalent volumes of saline.

ing the postnatal rise in plasma bilirubin in the newborn, even with a dose of the metalloporphyrin 50-fold greater than that of an effective dose of tin-protoporphyrin (Fig. 2). It is highly improbable, therefore, that the amounts of zinc-protoporphyrin that might be ultimately released from erythrocytes in individuals with plumbism or iron-deficiency anemia would significantly affect tissue heme oxidation activities or plasma bilirubin levels.

The apparent K_m (K_m, Michaelis constant) for human splenic heme oxygenase (*9*) was determined from six different samples to yield a mean ± standard deviation of 19.04 ± 0.08 μM (Table 1). This value is similar to that previously reported for the human spleen enzyme (*10*), but is three to four times greater than that reported for rat spleen microsomal heme oxygenase (*11*) and nearly 20 times greater than that for the heme oxygenase system reconstituted with its purified enzymic components (*12*). Tin-protoporphyrin was a potent competitive inhibitor of the human spleen enzyme (six different samples), with an average value of the inhibition constant (K_i) of 0.018 ± 0.001 μM (Table 1). This value is similar to the K_i of 0.011 μM previously reported for rat microsomal hepatic and splenic heme oxygenase (*5*). These findings indicate that the degradation of heme to bile pigment in human spleen is several times more sensitive to blockade by tin-protoporphyrin administration than is the comparable tissue in the rat. If tin-protoporphyrin can be safely employed to prevent the development of threatening levels of hyperbilirubinemia in the human newborn, it can probably be used at a much smaller dose than that required to prevent this form of jaundice in the rat. Pharmacokinetic studies with tin-protoporphyrin in humans (*13*) are consistent with this expectation, since significant blood levels of this metalloporphyrin can be achieved with doses of the compound at least two orders of magnitude smaller than those utilized to suppress postnatal jaundice in the rat neonate.

Temporary suppression of heme oxidation would presumably lead to transient heme sequestration in the circulation or in tissues. The consequences of extending the period during which the newborn is normally exposed to high concentrations of unmetabolized heme are not known; in adult humans very large amounts of heme, as hematin, administered therapeutically (100 to 400 mg per infusion intravenously one or two times daily) to patients with hereditary erythroid or hepatic porphyrias have been essentially without detriment (*14*). In neonatal rats, multiple administrations of tin-protoporphyrin in amounts totaling 500 μmole/kg have produced no evident acute or chronic toxicities, and treated animals have matured and reproduced normally (*5*).

The biological properties of tin-protoporphyrin and of related metalloporphyrins that may have a comparable activity should be intensively explored, since a control mechanism of this type may have therapeutic potential in humans.

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References and Notes

1. R. L. Levine, W. R. Fredericks, S. I. Rapoport, *Pediatrics* **69**, 255 (1982); D. A. Ritter, J. D. Kenny, H. J. Norton, A. J. Rudolph, *ibid.*, p. 260; S. B. Turkel, C. A. Miller, M. E. Guttenberg, D. R. Moynes, J. E. Hodgman, *ibid.*, p. 267.
2. L. M. Gartner, in *Pediatrics*, A. M. Rudolph, Ed. (Appleton-Century-Crofts, New York, ed. 16, 1977), p. 1079.
3. A. F. McDonagh, L. A. Palma, D. A. Lightner, *Science* **208**, 145 (1980).
4. S. J. Yaffe, G. Levy, T. Matsuzawa, T. Baliah, *N. Engl. J. Med.* **275**, 1461 (1966); H. M. Maurer, J. A. Wolff, M. Finster, P. J. Poppers, E. Pantuck, R. Kutzman, A. H. Conney, *Lancet* **1968-II**, 122 (1968).
5. G. S. Drummond and A. Kappas, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6466 (1981).
6. ———, *ibid.* **79**, 2384 (1982).
7. M. D. Maines, *Biochim. Biophys. Acta* **673**, 339 (1981).
8. A. A. Lamola and T. Yamane, *Science* **186**, 936 (1974).
9. Samples of normal human spleen, obtained during the course of operative procedures requiring spleen removal, were provided to us through the Tissue Procurement Service at the Sloan-Kettering Cancer Center.
10. B. A. Schacter, B. Yoda, L. G. Israels, *Ann. Clin. Res.* **8** (Suppl. 17), 28 (1976).
11. R. Tenhunen, H. S. Marver, R. Schmid, *J. Biol. Chem.* **244**, 6388 (1969).
12. T. Yoshinaga, S. Sassa, A. Kappas, *ibid.* **257**, 7778, 7786, 7794, 7803 (1982).
13. K. E. Anderson, G. S. Drummond, A. Kappas, unpublished data.
14. C. J. Watson, I. Bossenmaier, R. Cardinal, Z. J. Petryka, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 278 (1974); J. M. Lamon, M. M. Poh-Fitzpatrick, A. A. Lamala, *Gastroenterology* **79**, 115 (1980); H. L. Bonkowsky, D. P. Tschudy, A. Collins, J. Doherty, I. Bossenmaier, R. Cardinal, C. J. Watson, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2725 (1971); J. M. Lamon, B. C. Frykholm, R. A. Hess, D. P. Tschudy, *Medicine* **58**, 252 (1979); C. A. Pierach, I. Bossenmaier, R. Cardinal, M. Weimer, C. J. Watson, *Klin. Wochenschr.* **58**, 829 (1980).
15. M. Roth, *Clin. Chem. Acta* **17**, 487 (1967).
16. Supported by a grant from the Dyson Foundation, New York, grant 1-804 from the March of Dimes Birth Defects Foundation, and by PHS grant ES-01055. We thank H. Robinson for preparation of the manuscript and S. Spinner and B. Sheard for technical assistance.

26 April 1982; revised 6 July 1982

Fluorescence Microscopy: Reduced Photobleaching of Rhodamine and Fluorescein Protein Conjugates by *n*-Propyl Gallate

Abstract. *n-Propyl gallate (0.1 to 0.25 molar, in glycerol) reduces by a factor of 10 the rate of fading of fluorescence of cell structures labeled with tetramethylrhodamine or fluorescein-conjugated antibodies. Hence, prolonged photographic exposure of immunofluorescently labeled cells in the fluorescence microscope yields images with increased sensitivity, making feasible multiple data collection, as with serial optical sectioning.*

Fluorescence microscopy is rapidly becoming a powerful tool in cell biology. The method allows identification with high sensitivity of specific fluorescently labeled molecules in biological material. These molecules can be analyzed in their native conformation amidst their natural environment, without interference from neighboring substances or cell structures, even in whole multicellular organs. The main application of fluorescence microscopy is in immunocytology (*1*), but direct labeling of cellular organelles (mitochondria and nuclei) (*2*) and visualization of cell-to-cell interactions (*3*) are possible after uptake of fluorescent dye by cells. Microinjection into cells of fluorescent proteins or peptides has been used to trace their intracellular metabolism (*4*), distribution, organization, and function (*5*, *6*) and to elucidate cell lineages (*7*). Additional examples of the extreme specificity of fluorescence microscopy are the characterizations of cell constituents [their subcellular localizations (*8*) and roles in cell function (*9*)] with fluorescently labeled monoclonal antibodies and the analysis of three-dimensional chromosome structure with DNA-specific dyes (*10*).

Tetramethylrhodamine isothiocyanate (rhodamine) and fluorescein isothiocyanate (fluorescein) are the fluorescent dyes most commonly coupled to biological protein probes. A major problem accompanying the use of these dyes in microscopy is light-induced bleaching, apparent as fading of the emitted fluorescent light. Photobleaching is very rapid with microscopy in which epifluorescent illumination is used under conditions of high magnification and resolution because the excitation light beam is extremely intense (proportional to the

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