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IDENTIFICATION OF COBALT PROTOPORPHYRIN IX FORMATION *IN VIVO* FOLLOWING COBALT ADMINISTRATION TO RATS*

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Abstract—An electron paramagnetic resonance method for the identification and quantitation of cobalt protoporphyrin IX has been developed to provide definitive evidence for the formation of the cobalt chelate of protoporphyrin IX *in vivo*. Both authentic and enzymatically generated cobalt protoporphyrin exhibited a characteristic primary resonance at $g = 2.32$ at 100° Kelvin (K) in the reduced state. At low concentrations, signal averaging was found to improve considerably the signal to noise ratio and thereby allow for the measurement of low concentrations of cobalt protoporphyrin. It was possible to determine concentrations of cobalt protoporphyrin IX as low as 0.5 μM in biologic preparations, and the signal height of the e.p.r. resonance was linear with cobalt protoporphyrin concentrations up to at least 8.0 μM. Following the administration of cobaltous chloride to rats, cobalt protoporphyrin IX was demonstrated in livers at times when alterations in heme biosynthesis are known to occur. Cobalt protoporphyrin IX at the level of 4.8 nmoles/g liver was detected 120 min after the administration of cobaltous chloride at a dose of 60 mg/kg of body weight. This finding supports the concept that cobalt protoporphyrin IX is rapidly produced *in vivo* after the administration of cobaltous chloride and may be responsible for the observed inhibition of hepatic heme biosynthesis.

The administration of cobaltous chloride to rats leads to marked effects on hepatic heme synthesis, particularly hepatic microsomal cytochrome P-450 synthesis, and on mixed-function oxidase reactions dependent on this hemoprotein [1, 2]. The inhibition of heme biosynthesis results from a rapid reduction of the rate-limiting enzyme δ-aminolevulinic acid synthetase [3–8], and inhibition of the activity of ferrochelatase [9, 10], the catalyst for the last step in heme biosynthesis. In addition, hepatic heme catabolism has been reported by Maines and Kappas [11], DeMatteis and Unseld [12] and Tephly *et al.* [8] to increase after cobalt administration; this is probably related to an enhanced hepatic heme oxygenase activity [11, 12]. Guzelian and Bissell [13] have suggested that cobalt administration also interferes with heme-apocytochrome P-450 interaction.

The question of whether cobalt acts directly or as the protoporphyrin chelate has been a source of some controversy [14]. Igarashi *et al.* [15] have identified a substance with spectral properties similar to cobalt protoporphyrin IX in livers of rats treated with allylisopropylacetamide and cobaltous chloride. However, neither the specific optical absorption properties nor the concentrations of the cobalt protoporphyrin complex in liver were reported. Sinclair *et al.* [16] have also provided evidence for the formation of cobalt protoporphyrin IX *in vitro* by comparing the absorption spectra of authentic cobalt protoporphyrin IX and solubilized protein pellets isolated from hepatic homogenates that had been incubated previously with cobalt and protoporphyrin. In both of these studies, the spectroscopic evidence for the presence of cobalt protoporphyrin IX was limited by its non-specific visible absorption characteristics and by the necessity for efficient separation of the cobalt protoporphyrin IX from iron protoporphyrin IX in the homogenate. In addition, in neither of the studies was the concentration of the cobalt protoporphyrin IX in the homogenates reported.

The present investigation, using electron paramagnetic resonance (e.p.r.) spectroscopy, provides a definitive method for the quantitative determination of cobalt protoporphyrin IX in liver homogenates of rats treated with cobaltous chloride under conditions that have been shown to provoke alterations in heme biosynthesis. A major advantage of the use of e.p.r. spectroscopy for the detection of cobalt protoporphyrin IX is that in the reduced state the cobalt complex exhibits a characteristic resonance, while reduced iron protoporphyrin is not paramagnetic and gives no resonance. In addition, e.p.r. spectroscopy can be used to detect paramagnetic species in homogenates or subcellular particles without the necessity of prior extraction. Yonetani *et al.* [17, 18] have prepared cobalt heme proteins and have characterized them by visible and e.p.r. spectroscopic methods. We have taken advantage of the e.p.r. technique and can demonstrate conclusively that cobalt protoporphyrin IX is synthesized in liver preparations *in vitro* and that this substance

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is also present in livers of rats that have been treated with cobaltous chloride. Furthermore, our studies allow for an estimate of the concentration of the cobalt protoporphyrin IX complex present in liver following cobalt administration at a time when the biological effects of cobalt are manifest.

MATERIALS AND METHODS

Materials. Cobalt (III) protoporphyrin IX was prepared by the method of Yonetani *et al.* [17]. Free protoporphyrin IX used for the preparation was obtained from Porphyrin Products (Logan, UT) and was sealed and stored at 4°. Sodium dithionite, purified grade, was obtained from the J. T. Baker Chemical Co. (Phillipsburg, NJ) and stored in a desiccator. All other chemicals and biochemicals employed were of the highest purity available.

Analytical procedures. Chemically synthesized cobalt protoporphyrin IX was analyzed by visible absorption and atomic absorption spectroscopy. Samples of cobalt protoporphyrin were prepared in an alkaline pyridine solution (100 ml pyridine, 195 ml water, and 3 ml of 1 M sodium hydroxide). Reduced minus oxidized difference spectra were recorded using an Aminco DW2 UV/Vis spectrophotometer. The reference cuvette contained 0.04 mM potassium ferricyanide while the sample cuvette contained 1 mg sodium dithionite/3 ml. Absolute spectra were obtained against the alkaline pyridine solution.

Cobalt was determined by atomic absorption spectroscopy using a Perkin–Elmer model 360 spectrometer in the flame mode. Measurements were made at 240.7 nm with a spectral slit width of 0.2 nm. Cobalt standards were prepared in alkaline pyridine solution and used on the day of preparation.

Electron paramagnetic resonance spectra were obtained using a Varian E-104A EPR spectrometer with a variable temperature accessory. Samples were frozen in matched, quartz capillary tubes by immersion in liquid nitrogen. The appropriate instrument parameters were: modulation amplitude, 20 G; modulation frequency, 100 kHz; power, 50 mW; and temperature 100 Kelvin (K). A Nicolet model 535-4 signal averager was used to obtain signal height values.

Tissue preparation. Adult male albino Holtzman rats weighing 200–350 g were used. Rats were injected subcutaneously with $CoCl_2 \cdot 6H_2O$ (60 mg/kg body wt) and decapitated. Livers were homogenized [20 per cent or 50 per cent (w/v)] in ice-cold 0.25 M sucrose.

Homogenates were solubilized for e.p.r. analysis by adding 1 ml of pyridine and 0.2 ml of 10 M sodium hydroxide to 4 ml of the homogenate. In certain studies, homogenates (20 per cent, w/v) were centrifuged for 20 min at 18,000 *g* in a Sorvall RC2-B refrigerated centrifuge, and the protein pellet and supernatant fraction were each solubilized in pyridine and 10 M sodium hydroxide. The samples were vortexed at room temperature and, after a homogeneous gel was obtained, the samples were centrifuged gently to remove trapped air. The mixture was diluted with distilled water to a known volume. Final concentrations of pyridine and sodium hydroxide were 2–2.5 M and 0.3–0.4 M, respectively. Sodium dithionite was added to the sample, and the reduction was monitored potentiometrically using an apparatus similar to that described by Dutton [19]. The mixture was transferred to a 50-ml standard taper two-necked flask equipped with a calomel reference electrode and a platinum indicating electrode. Each electrode was mounted in a rubber stopper and extended to the bottom of the flask. Sixteen-gauge needles were mounted in the stoppers to provide an inlet and outlet for purging gas. A polyethylene tube extending from the bottom of the flask through one of the rubber stoppers was used to transfer the reduced sample to the e.p.r. tubes. A Gilmont 2.0 ml microburet was used for the addition of the sodium dithionite solution (approximately 40 mM in 25 mM sodium borate). Prior to and during reduction, the flask was purged with oxygen-free nitrogen. Reduction with sodium dithionite was initiated after purging the apparatus for 10–15 min. Potential measurements were made using a pH meter operated in the minus mV range. Titration was terminated when the potential stabilized between −0.65 and −0.85 V. At this point, the sample was transferred by positive pressure from the flask to the e.p.r. tube which had been purged previously with oxygen-free nitrogen. The tube was then stoppered and immediately immersed in liquid nitrogen.

When cobalt protoporphyrin IX was generated enzymatically in liver homogenates, the preparation was diluted with a half volume of the alkaline pyridine solution and reduced with sodium dithionite to between −0.65 and −0.85 V.

Liver samples (1.6–1.8 g) were prepared for atomic absorption spectroscopy by homogenization in distilled water. The mixture was digested with 4.0 ml of concentrated nitric acid at 109° until solubilization was achieved. The samples were cooled to room temperature, and 2.0 ml of 30 per cent hydrogen peroxide was added. The mixture was heated slowly for about 1 hr, transferred quantitatively, and diluted to volume in 10-ml volumetric flasks. Cobalt standards were prepared in 1.0 per cent nitric acid.

RESULTS

Spectroscopic properties of chemically synthesized cobalt protoporphyrin IX. Chemically synthesized cobalt protoporphyrin IX was characterized initially by visible spectroscopy. All concentrations of cobalt protoporphyrin IX were based on cobalt content as determined by atomic absorption spectroscopy. A molecular weight of 778 for the molecular complex $[Co(HP)Py_2]$ was used, where HP represents the monoprotonated form of protoporphyrin, Py represents pyridine and cobalt exists in the +3 oxidation state. Purity of about 99 per cent was estimated.

The reduced minus oxidized difference spectrum of the pyridine complex of cobalt protoporphyrin IX yielded absorption band maxima and minimum (Table 1) comparable to those reported by Johnson and Jones [20], where cobalt was inserted enzymatically into protoporphyrin IX. The $\Delta\varepsilon_{mM}$ value, 555 nm minus 535 nm, is also comparable to the value reported by Johnson and Jones. The absolute absorption spectrum has three characteristic absorp-

Table 1. Difference spectra of the reduced and oxidized pyridine complex of cobalt protoporphyrin IX*

Absorption maxima and minimum (nm)			$\Delta\varepsilon_{mM}$
α_{max}	Minimum	β_{max}	($\varepsilon\alpha$-ε minimum)
555	535	513	8.2 mM^{-1} cm^{-1}
†554	533	512	9.1 mM^{-1} cm^{-1}

* Cobalt protoporphyrin IX was dissolved in alkaline pyridine solution, reduced with sodium dithionite and compared to a solution oxidized with potassium ferricyanide.
† Johnson and Jones [20].

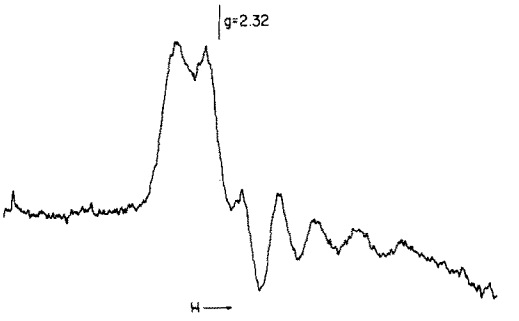

Fig. 1. Electron paramagnetic resonance spectrum of authentic cobalt protoporphyrin IX (15 μM) in alkaline pyridine solution and reduced with sodium dithionite.

tion bands (Table 2) that are similar to those reported by Yonetani *et al.* [17]. Molar absorptivities, however, differ somewhat from those reported by Yonetani *et al.* [17]. This discrepancy cannot be explained at this time, although a commercial source of cobalt protoporphyrin IX, obtained from Porphyrin Products, yielded values similar to those seen in Table 2.

Cobalt protoporphyrin IX, dissolved in alkaline pyridine solution and reduced with sodium dithionite, exhibited an e.p.r. spectrum at 100 K with a primary resonance at $g = 2.32$. In this spectrum, seven of the eight expected resonances produced by nuclear hyperfine coupling were observed (Fig. 1). The oxidized form of cobalt protoporphyrin IX was not paramagnetic and gave no resonance. When chemically synthesized cobalt protoporphyrin IX was added to a liver preparation in alkaline pyridine and reduced with sodium dithionite, a resonance at $g = 2.32$ was observed (Fig. 2), although the hyperfine structure was not apparent. The addition of sodium dithionite to a liver preparation in the absence of cobalt protoporphyrin IX did not result in a resonance at $g = 2.32$ (Fig. 3).

Synthesis of cobalt protoporphyrin IX in vitro. When cobalt and protoporphyrin IX were incubated together *in vitro* with liver homogenates for 60 min, an e.p.r. spectrum (Fig. 4) similar to that obtained upon the addition of authentic cobalt protoporphyrin IX to a liver preparation, as described in Fig. 2, was observed. In addition to the resonance at $g = 2.32$, there is evidence of some hyperfine structure reminiscent of that observed using chemically synthesized cobalt protoporphyrin IX. When e.p.r. spectra were determined using homogenates incubated with either cobaltous chloride or protoporphyrin IX alone, a resonance was not observed at $g = 2.32$. Also, zero-time samples yielded no evidence of the cobalt protoporphyrin IX complex.

Synthesis of cobalt protoporphyrin IX in vivo. Evidence for the synthesis *in vivo* of cobalt protoporphyrin IX was obtained by the e.p.r. spectroscopic method. The e.p.r. spectrum obtained using pellets from 20 per cent homogenates prepared from livers of rats treated with cobaltous chloride (60 mg/kg) 30 min prior to being killed was similar to those obtained with chemically and enzymatically produced cobalt protoporphyrin IX (Fig. 5).

Experiments were designed to determine the concentration of cobalt protoporphyrin IX in rat liver at times during which the biological effects of cobalt are known to occur. Thus, rats injected with 60 mg/kg of cobaltous chloride were killed 120 min after cobalt administration, livers were removed, and 50 per cent (w/v) homogenates were prepared and analyzed for cobalt protoporphyrin IX by the e.p.r. spectroscopic method. About 4.8 nmoles of cobalt protoporphyrin IX/g of liver was observed at this time period. Further studies are in progress to determine the localization of cobalt protoporphyrin IX in subcellular fractions.

Table 2. Absolute absorption spectra of the pyridine complex of cobalt protoporphyrin IX*

	Absorption maxima and minimum			
	α_{max}	Minimum	β_{max}	Soret
λ (nm)	569	554	536	425
λ (nm)†	569		535	424
ε_{mM}	12.0		11.5	132

* Cobalt protoporphyrin IX was dissolved in alkaline pyridine solution and oxidized with potassium ferricyanide. The millimolar absorptivity values were determined from the ratio of the measured absorbance to the cobalt concentration determined by atomic absorption spectroscopy.
† Yonetani *et al.* [17].

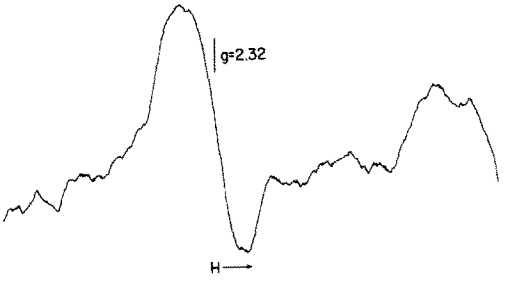

Fig. 2. Electron paramagnetic resonance spectrum of authentic cobalt protoporphyrin IX added to a pellet of a liver homogenate which had been obtained by centrifugation of a 20 per cent (w/v) homogenate at 18,000 *g* for 20 min and processed as described in Materials and Methods. The final concentration of cobalt protoporphyrin IX was 2.18 μM. The spectrum represents the average of four 2-min scans.

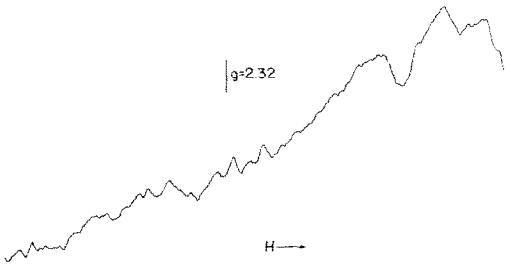

Fig. 3. Electron paramagnetic resonance spectrum of a liver homogenate processed as described in Fig. 2 but without cobalt protoporphyrin IX added. The spectrum represents the average of four 2-min scans.

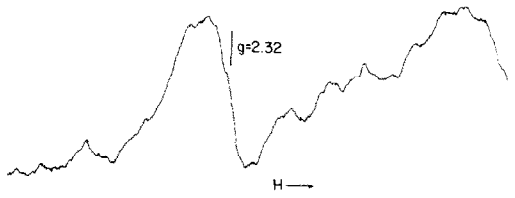

Fig. 5. Electron paramagnetic resonance spectrum of cobalt protoporphyrin IX obtained from livers of rats treated with $CoCl_2 \cdot 6H_2O$ (60 mg/kg of body wt) 30 min prior to killing. Liver homogenates (20 per cent, w/v) were centrifuged at 18,000 *g* for 20 min and the pellets were treated as described in Materials and Methods. The spectrum represents the average of four 2-min scans. A similar spectrum is observed using a 50 per cent (w/v) liver homogenate.

DISCUSSION

The feasibility of detecting cobalt protoporphyrin IX in hepatic tissue by e.p.r. spectroscopy is based on the characteristic spectrum at 100 K of reduced cobalt protoporphyrin IX. A characteristic resonance at $g = 2.32$ was observed for both chemically and enzymatically synthesized cobalt protoporphyrin IX. This resonance is similar to that reported for cobalt porphyrin proteins by Yonetani *et al.* [18]. Previous e.p.r. studies dealing with cobalt porphyrins involved model compounds such as octaethylporphyrin or tetraphenylporphyrins with emphasis on the effect of adduct, such as molecular oxygen, on the e.p.r. parameters of the cobalt porphyrin complex [21]. A major advantage of the use of e.p.r. spectroscopy for the demonstration of the formation of cobalt proporphyrin IX is that iron porphyrin complexes do not interfere with the cobalt protoporphyrin IX e.p.r. spectrum. The e.p.r. technique allows for the detection of the complex in a matrix of cell fragments and components without having to perform separating procedures. The work of Sinclair *et al.* [16] suggests that the cobalt protoporphyrin complex is tightly bound to hepatic proteins. These workers were unable to separate the cobalt complex using the usual solvent extraction techniques employed for heme. Igarashi *et al.* [15] were able to isolate the cobalt protoporphyrin IX complex using chloroform extraction procedures. The current study avoids extraction procedures. The alkaline pyridine solution employed in our studies disrupts membrane structure and converts iron protoporphyrins to diamagnetic forms. Further studies are now underway in our laboratory to compare solvent extraction methods with the solubilization technique described in the current work.

The current studies also took advantage of signal averaging technology. Using a signal averager, it was possible to increase the signal to noise ratio and thereby increase the sensitivity of the method. Thus, using the method described in the text, it was possible to determine with reliability an amount of cobalt protoporphyrin IX equal to 0.5 μM in liver preparations. Furthermore, the peak–peak signal height of the resonance at $g = 2.32$ was linear up to at least 8.0 μM. Using this technique, it was possible to estimate the concentration that was present in rat liver 120 min after the injection of cobalt: about 4.8 nmoles/g liver was observed.

The cobalt protoporphyrin IX complex may be a very powerful inhibitory substance toward δ-aminolevulinic acid synthetase activity. The activity of δ-aminolevulinic acid synthetase is reduced to 10 per cent of control values within 1 hr after the treatment of rats with cobaltous chloride [3–8]. Sedman and Tephly [22] have shown that cobalt does not directly inhibit cardiac δ-aminolevulinic acid synthetase activity. Studies on the hepatic enzyme yield similar data. Igarashi *et al.* [15] have shown that cobalt protoporphyrin IX injected into allylisopropylacetamide-treated rats leads to marked decreases in δ-aminolevulinic acid synthetase activity similar to that observed when cobaltous chloride is administered. The current studies, however, were performed in animals that had not received allylisopropylacetamide, and the results obtained demonstrate the formation of cobalt protoporphyrin IX in the livers of rats treated only with cobaltous chloride. Thus, the results presented in this report show that low but measurable amounts of cobalt protoporphyrin IX are produced in liver at a time when marked biological effects are observed.

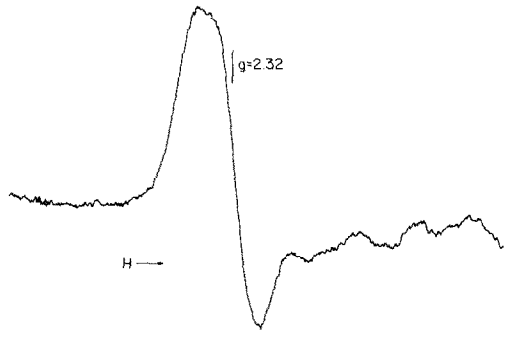

Fig. 4. Electron paramagnetic resonance spectrum of cobalt protoporphyrin IX enzymatically generated *in vitro*. Five milliliters of a 20 per cent (w/v) liver homogenate was incubated with 3.2 μM $CoCl_2$ and 66 μM protoporphyrin IX for 60 min at 37°. The reaction mixture was then mixed with a half volume of alkaline pyridine solution and reduced with sodium dithionite.

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