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Solubilization of the Carcinogen Nickel Subsulfide and Its Interaction with Deoxyribonucleic Acid and Protein†

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ABSTRACT: Significant concentrations (1–10 mM) of nickel(II) were found in solution after incubation of the potent carcinogen nickel subsulfide in 0.05 M Tris-HCl, pH 7.4, solutions containing DNA, rat liver microsomes, and NADPH. The presence of NADPH decreased the rate of solubilization of nickel subsulfide. The solubilized nickel exhibited electronic absorption spectra and magnetic moments characteristic of octahedral nickel(II). The solubilized nickel(II) bound to DNA with an apparent equilibrium constant of 730 M^{-1} and with a saturation binding value of one nickel per 2.4 nucleotides. Microsomes lowered the saturation binding of nickel to DNA but dramatically increased the amount of nickel–DNA complex stable to precipitation with salt and poly(ethylene glycol). The amount of protein associated with DNA precipitated from protein-extracted solutions correlated with the amount of nickel bound to DNA. These results suggest that microsomes mediate the binding of nickel to DNA by forming a stable ternary protein–nickel(II)–DNA complex.

Epidemiological evidence has implicated nickel compounds as the causative agents in human respiratory and renal cancers in nickel refinery workers worldwide (Doll et al., 1977; Pedersen et al., 1973; Lessard et al., 1978). Inhalation of nickel refinery dust appears to be the major route of exposure to particulate nickel compounds, which include nickel subsulfide (Ni_3S_2) (Sunderman, 1976). Nickel subsulfide induced renal carcinomas (Jasmin & Riopelle, 1976; Sunderman et al., 1979) following intrarenal (ir) injection in rats, and primary rhabdomyosarcomas following intramuscular (im) injection (Sunderman et al., 1976; Yamashiro et al., 1980). Ocular tumors were induced in rats following a single intraocular injection of nickel subsulfide (Albert et al., 1981).

Cellular uptake and solubilization of particulate nickel compounds are important to the mechanism of nickel-induced carcinogenesis. Crystalline nickel subsulfide, but not amorphous nickel sulfide, was actively phagocytized by cultured mammalian cells (Costa & Mollenhauer, 1980), resulting in an increased frequency of morphological transformation. Phagocytized nickel particles were observed in the cytoplasm where they were solubilized to a form capable of entering the nucleus and interacting with nuclear macromolecules (Costa et al., 1981). The rate of nickel subsulfide solubilization in vitro in rat serum was found to be dependent upon molecular oxygen and was enhanced by the presence of albumins and amino acids (Kasprzak & Sunderman, 1977). Solubilization of nickel compounds in vivo in the rat occurred after intraperitoneal (ip) injection of nickel carbonate, which was shown to interact with cellular macromolecules in kidney, inducing DNA single-strand breaks and DNA–protein cross-links (Ciccarelli et al., 1981).

The present investigation is concerned with the solubilization of the carcinogen nickel subsulfide in vitro in the presence of calf thymus DNA, rat liver microsomes, and reduced nicotinamide adenine dinucleotide phosphate (NADPH).[1] The

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[1] Abbreviations: BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DMG, dimethylglyoxime; DPA, diphenylamine; PEG, poly(ethylene glycol); NADPH, reduced form of nicotinamide adenine dinucleotide phosphate.

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rat liver microsome system in vitro has been used previously for investigating the metabolism of the carcinogen chromate (Gruber & Jennette, 1978). Our results show that nickel subsulfide undergoes gradual solubilization in this system. Rat liver microsomes and NADPH were observed to affect the rate of nickel subsulfide solubilization. The resulting nickel species in solution has been identified as octahedral nickel(II). DNA has been identified as a nickel(II) ligand, and equilibrium binding constants have been calculated. Our evidence suggests that microsomes mediate the binding of nickel(II) to DNA by forming a stable ternary protein–nickel(II)–DNA complex.

Experimental Procedures

Chemicals. Bovine serum albumin (BSA), diphenylamine (purified crystals), calf thymus DNA (type I sodium salt), and NADPH were obtained from Sigma Chemical Co. Nickel subsulfide was a gift of Dr. E. Mastromatteo, Inco Ltd. Analysis of nickel subsulfide (α form) provided by Dr. Mastromatteo was 73.3% nickel and 26.3% sulfur by weight. Average particle size was 3–5 μm. All other chemicals were of reagent grade. *Caution*: Nickel subsulfide is a potential carcinogen and should be handled with care.

Preparation of Rat Liver Microsomes. Male Sprague-Dawley rats (150–200 g) were obtained from Charles River Breeding Labs, Wilmington, MA. The rats were given water but no food for a period of 16–19 h. The rats were then killed, and their livers were removed. Subsequent operations were carried out at 0–4 °C. Microsomes were isolated from the rat livers by using the differential centrifugation method of Pietropaolo & Weinstein (1975). The microsomal pellets were suspended in 0.15 M KCl, centrifuged at $(2.3 \times 10^5)g$ for 1 h, resuspended in cold 0.25 M sucrose–0.05 M Tris-HCl, pH 7.4 (1.4 mL per g of liver), and stored at −80 °C. Microsomal protein concentration was determined according to the procedure of Peterson (1977). Absorbance values were corrected for nonlinearity (Peterson, 1977; Lowry et al., 1951) by using the log–log method of Stauffer (1975) and Bates & McAllister (1974). BSA was used as the standard. The concentration of BSA stock was determined by using $\epsilon_{280} = 0.66$ mL mg^{-1} cm^{-1} (Wetlaufer, 1962).

Preparation of Stock Solutions. All solutions were buffered at pH 7.4 with 0.05 M Tris-HCl and stored at 0–4 °C. A 24.0-mg sample of nickel subsulfide was weighed out to within ±0.1 mg directly into tared vials, and the vials were capped under a nitrogen atmosphere.

NADPH stock solutions were freshly prepared for each incubation. NADPH concentration was determined by using $\epsilon_{340} = 6.2 \times 10^3$ M^{-1} cm^{-1} (Horecker & Kornberg, 1948). DNA concentration of stock solutions was determined by using $\epsilon_{260} = 6.6 \times 10^3$ M^{-1} cm^{-1} for calf thymus DNA (Reichmann et al., 1954).

DNA was prepelleted twice to obtain a stock solution containing DNA of a molecular weight of $\geq 2 \times 10^6$ (Freifelder, 1976). A 6×10^{-3} M DNA stock solution was centrifuged at $(1.8 \times 10^5)g$ for 5 h and 55 min, 0 °C, with a Sorvall T-865 fixed-angle rotor. Following centrifugation, the supernatant was decanted, and the pellets were resuspended in 0.05 M Tris-HCl, pH 7.4.

Inactivated microsomes were prepared by heating microsome solutions of approximately 3.0 mg/mL in a boiling water bath for 10 min, followed by immediate chilling in ice.

Incubation of Nickel Subsulfide with Microsomes. Stock incubation solutions were prepared immediately preceding the incubation period and kept on ice. Stock incubation solutions contained all components with the exception of nickel subsulfide. The incubation was started by adding 5.0-mL aliquots of the appropriate stock incubation solution to numbered vials containing preweighed amounts of nickel subsulfide. Vials were recapped loosely and incubated at 37 °C for the indicated time period with shaking.

Following the incubation, solutions were transferred to ice and centrifuged for 5 min at 1000g to pellet undissolved nickel subsulfide. A 50-μL aliquot of the resulting supernatant was diluted in 0.95 mL of 0.05 M Tris-HCl, pH 7.4, for determination of NADPH concentration. Another 50-μL aliquot of the supernatant was diluted in 3.95 mL of 0.05 M Tris-HCl–0.1 M Na_2EDTA for total sulfite analysis by using the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) procedure (Humphrey et al., 1970). A third 50-μL aliquot of the supernatant was diluted in 0.95 mL of water for total nickel analysis by using the dimethylglyoxime (DMG) procedure. The incubation was stopped by extracting the microsomal protein from the aqueous solution with 0.5 volume of 24:1 chloroform:isoamyl alcohol and centrifuging 15 min at 1500g (Borgen et al., 1973). Following the first protein extraction, the upper aqueous layer was removed, extracted with 24:1 chloroform:isoamyl alcohol, and centrifuged. This protein extraction procedure was repeated 10 times.

DNA Pelleting. Binding isotherms were obtained by using the pelleting procedure of Barton & Lippard (1979). Following protein extraction, an aliquot (approximately 0.65 mL) was removed from the solutions and centrifuged 3 h and 20 min at $(2.4 \times 10^5)g$, 0 °C, with a Sorvall AH-650 swinging bucket rotor fitted with adaptors for cellulose nitrate tubes (5 × 42 mm). Immediately following centrifugation, five 50-μL aliquots were removed from the top of the centrifuged samples for determination of nickel concentration by using the DMG method. Correction for a nickel gradient in the centrifuged tubes was made as follows. The first two 50-μL aliquots were discarded. The concentration of nickel in the remaining three 50-μL aliquots was determined by using the DMG assay. These three values were averaged and taken as the free nickel concentration ($[Ni^{2+}]_f$).

DNA Precipitation. Following protein extraction, the DNA in incubation solutions was precipitated 3 times in 12% poly(ethylene glycol) and 0.35 M NaCl (Lis & Schleif, 1975). Final resuspension was in 0.05 M Tris-HCl, pH 7.4. Nickel to DNA ratios were found to be constant after three precipitations.

Sulfite Concentration. Sulfite concentration was determined by using the procedure of Humphrey et al. (1970). DTNB reagent was freshly prepared in a 10% solution of 95% ethanol by volume and diluted with 0.05 M Tris-HCl, pH 7.4. Sodium sulfite standards were freshly prepared in 0.01 M Na_2EDTA–0.05 M Tris-HCl, pH 7.4. A mean extinction coefficient at 412 nm of 1.38×10^4 M^{-1} cm^{-1} was obtained [literature value 1.39×10^4 M^{-1} cm^{-1} (Humphrey et al., 1970)].

Nickel Concentration. Nickel concentration was determined either by atomic absorption or by a modification of the DMG assay described by Marczenko (1976).

A Perkin-Elmer 503 atomic absorption spectrophotometer fitted with an HGA-2100 graphite furnace assembly was used to measure the concentration of nickel in the precipitated DNA samples for the purpose of obtaining nickel to DNA ratios.

The DMG procedure was used to obtain all other nickel concentration values. Modifications to the assay were increasing the final concentration of oxidant (potassium persulfate) to 1.85%, acidifying the test solution with HCl before the addition of the dimethylglyoxime reagent, waiting 5 min between the addition of oxidant and the addition of ammonium

hydroxide, diluting the stock ammonium hydroxide solution to 20%, and waiting 2 h for full color development. Samples were read against a water blank, and the absorbance of the appropriate reagent blank was subtracted. The color was found to be stable at room temperature for at least 12 h. A mean extinction coefficient at 445 nm of 1.33×10^4 M^{-1} cm^{-1} was obtained [literature value 1.5×10^4 M^{-1} cm^{-1} (Marczenko, 1976)].

DNA Concentration. The diphenylamine (DPA) assay was used to obtain DNA concentration values for determining pelleted and precipitated nickel to DNA ratios. The procedure used is a modification of the procedure of Burton (1956, 1968), described by Hopkins et al. (1973). The DPA reagent was freshly prepared in a foil-wrapped flask, and the incubation was carried out in the dark for 18–20 h at 30 °C. Sulfite in the protein-extracted incubation solutions was found to interfere with the diphenylamine assay. Sulfite was eliminated as an interfering agent by oxidation to sulfate. Sulfate was found not to affect the diphenylamine assay. The incubation sample (50 μL) was preincubated for 4.0 h at 30 °C with 0.5 mL of 20% ammonium hydroxide and 100 μL of 70% perchloric acid. A mean extinction coefficient at 600 nm of 1.1×10^4 M^{-1} cm^{-1} was obtained [literature value 1.1×10^4 M^{-1} cm^{-1} (Burton, 1956)].

Protein Concentration. Protein concentration in the DNA-precipitated incubation solutions was determined by using a modification of the procedure of Peterson (1977). Precipitation of protein from the samples was necessary to eliminate Tris-HCl as an interfering agent. Final trichloroacetic acid concentration in the precipitation step was increased to 4%, deoxycholate was eliminated, the assay solutions were incubated at 50 °C for 30 min, and centrifuging was carried out at $(1 \times 10^4)g$ for 15 min. BSA was used to determine the concentration of a microsome stock; these microsomes were then used as the protein standard. An average protein yield of 96% for BSA and 83% for microsomes was obtained after precipitation and resuspension of the protein. For a final BSA protein concentration of 20 μg mL^{-1}, a mean extinction coefficient of 21.5 mL mg^{-1} cm^{-1} was obtained [literature value 21.6 mL mg^{-1} cm^{-1} (Peterson, 1977)].

Magnetic Susceptibility. The magnetic susceptibility of the solubilized nickel was determined by the chemical shift method of Evans (1959) (Deutsch & Poling, 1969) or by the T_1 relaxation method (Bloch et al., 1946; Farrar & Becker, 1971). The effect of the solubilized nickel on the chemical shift of *tert*-butyl alcohol (2%) was determined at several nickel concentrations, and the magnetic moment was calculated from a plot of ΔS vs. m according to the equation $\Delta S = 2\pi\nu_0 m\chi/3$ where ΔS is the difference in chemical shift in hertz, ν_0 is the applied field, and m is the mass of nickel per milliliter of solution. Alternatively, the effect of solubilized nickel on the T_1 relaxation of H_2O was determined and the magnetic moment calculated from a plot of $1/T_1$ vs. N according to the equation $1/T_1 = 12\pi^2\gamma^2\eta N\mu_{eff}^2/(5kT)$ where N is the ions per cubic centimeter and η in the viscosity. Experiments were performed at 25 °C on a JEOL FX-60Q Fourier-transform NMR spectrometer.

Electronic Spectra. Absorption of the solubilized nickel in the visible region of the spectrum was recorded with a Cary 14 spectrophotometer.

Results

Solubilization of Nickel Subsulfide. The effect of Tris, microsomes, denatured microsomes, and NADPH on the solubilization of Ni_3S_2 is shown in Figure 1. The concentration of nickel in solution generally increased with time of incubation up to 12–16 h and then remained farily constant through 24 h. Substantial amounts of nickel subsulfide dissolved in all cases, leading to soluble nickel concentrations of 1–9 mM depending upon the components present in solution. The rates of solubilization of Ni_3S_2 in water and in 0.05 M NaCl were virtually identical, with a maximum nickel concentration of 1–1.5 mM being reached at 8–24 h of incubation. A 5–8-fold increase in soluble nickel (7.5–8.8 mM) was observed after 12–24 h when 0.05 M Tris-HCl buffer, pH 7.4, was used in the incubation instead of pure water. This increase in solubility can be accounted for by the ability of Tris to coordinate nickel(II) ion and form soluble charged complexes (Dotson, 1972).

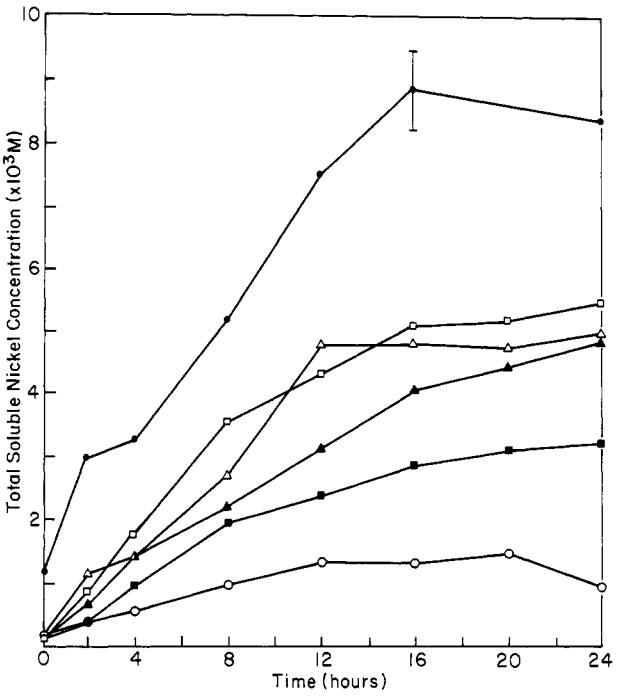

FIGURE 1: Effect of Tris buffer, calf thymus DNA, rat liver microsomes, and NADPH on the time course for the solubilization of nickel subsulfide. The total concentration of nickel in solution was determined after incubation of Ni_3S_2 in water (○), in 0.05 M Tris-HCl buffer, pH 7.4 (●), and in buffered solutions with DNA in the absence (□) and presence of NADPH (■), microsomes (△), and microsomes and NADPH (▲). Solutions were incubated at 37 °C with shaking and initially contained [Ni_3S_2] = 4.8 mg/mL, [DNA] = 2.0 mM, [NADPH] = 1.5 mM, and [protein] = 1.5 mg/mL. Maximum possible nickel concentration based on the initial amount of Ni_3S_2 was 60 mM. Mean variability was ±0.6 mM as indicated by the size of the error bar on the graph.

Addition of DNA to the Tris-buffered solution decreased the solubilized nickel concentration to 5–6.4 mM after 16–24 h. This may be due to interaction of DNA with the surface of the Ni_3S_2 particles which prevents uniform mixing and inhibits their dissolution. Addition of native or denatured rat liver microsomes to buffered solutions containing DNA caused a slight decrease in the amount of solubilized nickel (4.0–4.9 mM after 12–24 h) from that seen with DNA solutions in the absence of microsomes. The microsomes may inhibit the rate of Ni_3S_2 solubilization by forming a layer of microsomal protein over the surface of the particles in addition to the DNA layer and preventing complete mixing of the incubation components. The fact that the native and denatured microsomes acted in a similar manner suggests that this phenomenon is a physical effect. DNA and protein precipitated from solution after longer time periods (48–72 h) of incubation with Ni_3S_2. The concentration of soluble nickel was lower in the samples in which the macromolecules had precipitated, indicating that

Table I: Visible Spectral Characteristics of Protein-Extracted Ni_3S_2 Incubation Solutions and $NiSO_4$ Solutions in 0.05 M Tris-HCl, pH 7.4[a]

sample	incubation time (h)	λ_{max} (nm)[b]	ϵ (M^{-1} cm^{-1})[c]
Ni_3S_2	96	634	3.9
Ni_3S_2, DNA	4	650	2.7
	8	644	3.7
	48	636	4.3
Ni_3S_2, DNA, NADPH	8	649	3.9
	24	644	4.0
	72	642	4.2
Ni_3S_2, DNA, microsomes	24	636	3.4
	72	639	3.2
Ni_3S_2, DNA microsomes, NADPH	48	635	4.1
	72	636	3.8
$NiSO_4$		634	4.2
$NiSO_4$, DNA		644	2.9

[a] Initial incubation conditions were [Ni_3S_2] = 60 mM, [DNA] = 2.9 mM, [NADPH] = 1.5 mM, and [protein] = 1.5 mg/mL. Samples were incubated at 37 °C with shaking for the indicated period of time. Conditions for solutions containing $NiSO_4$ were [$NiSO_4$] = 10 mM and [DNA] = 2 mM. [b] Mean variability ±2 nm. [c] Mean variability ±0.2 M^{-1} cm^{-1}.

the precipitated protein and DNA contained bound nickel.

Addition of NADPH to buffered solutions containing DNA resulted in a 2-fold decrease in the amount of solubilized nickel (2.9–3.2 mM after 16–24 h). The inhibitory effect of NADPH, a reducing agent, on the rate of Ni_3S_2 solubilization is reasonable since spectroscopic and magnetic evidence identified nickel(II) as the species in solution (vide infra). A net oxidation must take place to convert nickel subsulfide (Kasprzak & Sunderman, 1977), where nickel exists in mixed oxidation states of O, I, and II, to nickel (II), and a reducing agent like NADPH would be expected to inhibit this net oxidation. NADPH also slowed the initial rate of solubilization of Ni_3S_2 in buffered solutions containing rat liver microsomes as well as DNA. However, the final concentration of soluble nickel after 24 h (4.8 mM) was unaffected by the presence of NADPH but was significantly higher (3.2 mM) than a similar solution without microsomes. This effect was due to the depletion of NADPH in the presence of microsomes. In the absence of microsomes, significant amounts of the initial NADPH (32%) remained in the incubation solutions after 24 h, whereas in the presence of microsomes only 15% of the initial NADPH remained. Microsomes incubated aerobically with NADPH have been shown to consume oxygen and NADPH by catalyzing lipid peroxidation (Buege & Aust, 1978).

Extraction of the solutions with 24:1 chloroform:isoamyl alcohol lowered the concentration of nickel 18% when incubation solutions contained native microsomes. The nickel concentrations were unchanged after protein extraction of solutions containing denatured microsomes. Since the extraction procedure removes microsomal protein from solution, this result suggests that nickel(II) binds to native protein and that removal of microsomal protein from solution leads to a concurrent loss of nickel from solution.

In addition to solubilization of nickel, the sulfide was also solubilized as demonstrated by the appearance of sulfate and sulfite in all incubation solutions. Sulfite was present at low concentrations [$(1.5 \pm 0.2) \times 10^{-4}$ M] after a 24-h incubation. The presence of sulfate was confirmed in protein-extracted solutions by the presence of a copious white precipitate upon addition of barium ion. Kasprzak & Sunderman (1977) had previously found nickel sulfate in sediments of α-Ni_3S_2 after incubation in O_2-saturated water.

Table II: Magnetic Properties of Protein-Extracted Ni_3S_2 Incubation Solutions and $NiSO_4$ in 0.05 M Tris-HCl, pH 7.4[a]

sample	μ_{eff} (μ_B)[b]
$NiSO_4$	3.4[c]
Ni_3S_2	3.2[c,d]
Ni_3S_2, NADPH	3.2[d]
Ni_3S_2, microsomes	3.1[d]
Ni_3S_2, microsomes, NADPH	3.0[d]

[a] Initial incubation conditions were [Ni_3S_2] = 13.3 ± 1.0 mM, [NADPH] = 1.22 mM, and [protein] = 1.5 mg/mL. Samples were incubated at 23 °C for 72 h with shaking. [b] Mean variability ±0.4 μ_B. [c] Evans method. [d] T_1 relaxation method.

Nickel Species in Solution. The nickel species in solution after solubilization of Ni_3S_2 were identified as octahedral nickel(II) complexes coordinated to oxygen and nitrogen ligands since their spectroscopic and magnetic properties (Tables I and II) were similar to those of known octahedral nickel(II) complexes (Figgis, 1966). The spectra (Figure 2 and Table I) of protein-extracted incubation solutions showed that the dissolved nickel species had wavelengths of maximum absorbance (634–650 nm) and extinction coefficients (2.7–4.3) intermediate between the two extremes of octahedral nickel(II) having all oxygen ligands, e.g., $[Ni(H_2O)_6]^{2+}$ (λ_{max} = 722 nm, ϵ = 2.3 M^{-1} cm^{-1}), or all nitrogen ligands, e.g., $[Ni(en)_3]^{2+}$ (λ_{max} = 540 nm, ϵ = 7.4 M^{-1} cm^{-1}). The energy of the $^3T_{1g}(F) \leftarrow {}^3A_{2g}(F)$ transition for the various complexes suggests that a maximum of two nitrogen ligands are coordinated to the nickel (Lever et al., 1979). The major species in the incubation solutions after long periods of incubation (48–72 h) appeared to be a complex with the Tris buffer. Essentially all the DNA had precipitated from these solutions, leaving nickel(II), Tris, and other products of Ni_3S_2 solubilization, e.g., sulfate and sulfite, in solution. The λ_{max} and ϵ values of these solutions were similar to those found for Ni_3S_2 incubated in Tris buffer for 96 h and $NiSO_4$ incubated with Tris buffer. Dotson (1972) isolated a Tris–nickel(II) complex from nonaqueous solution which contained octahedral nickel(II) coordinated to the nitrogen and oxygens of two Tris molecules.

Spectral data also provided evidence for the existence of a nickel(II) complex with DNA involving oxygen as a ligand. A solution containing $NiSO_4$, Tris, and DNA had a longer wavelength absorption maximum (644 nm) and lower extinction coefficient (2.9 M^{-1} cm^{-1}) than a solution of $NiSO_4$ in Tris buffer. These values of λ_{max} and ϵ were similar to those obtained from incubation of Ni_3S_2 with DNA in Tris buffer (Table I), for periods of time (4 and 8 h) before precipitation of DNA occurred.

Some of the incubation solutions contained potential nickel(II) ligands other than Tris and DNA, i.e., microsomal protein and NADPH. The λ_{max} and ϵ values of these solutions were generally between those of the buffered nickel(II) solution and the buffered nickel(II)–DNA. The number of possible ligands present in these solutions complicates interpretation; however, the data are consistent with the occurrence of octahedral nickel(II) complexes containing mainly oxygen and some nitrogen ligands.

The magnetic moments of the nickel complexes resulting from solubilization of Ni_3S_2 had values of 3.0–3.2 μ_B (Table II), similar to that of $NiSO_4$ in buffer, μ = 3.4 μ_B. These magnetic moments are in the typical range of values (2.9–3.3 μ_B; Kettle, 1969) for octahedral nickel(II) complexes, e.g., μ_{eff}^{300K} = 3.23 for $(NH_4)_2Ni(SO_4)_2 \cdot 6H_2O$ (Figgis, 1966).

Equilibrium Binding of Solubilized Nickel to DNA. The amount of nickel bound to DNA in protein-extracted solutions,

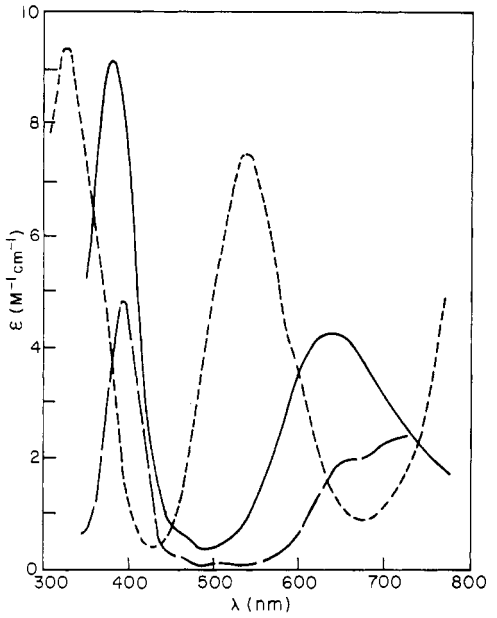

FIGURE 2: Visible absorption spectra of the Tris(ethylenediamine)-nickel(II) complex (---) and the hexaquonickel(II) complex (--) and representative spectrum obtained after incubation and protein extraction of a solution of Ni_3S_2 and calf thymus DNA (—). The incubation solution initially contained [Ni_3S_2] = 4.8 mg/mL, [DNA] = 2.0×10^{-3} M, and 0.05 M Tris-HCl, pH 7.4, and was incubated at 37 °C with shaking for 72 h.

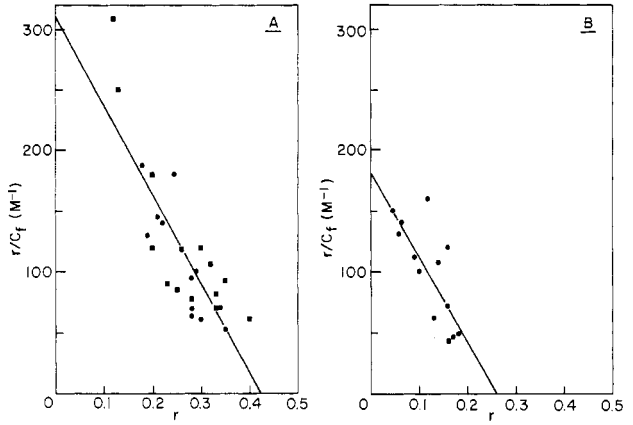

FIGURE 3: Scatchard plots of nickel bound to DNA determined by the pelleting method after incubation of Ni_3S_2 (4.8 mg/mL) with calf thymus DNA (2.0 mM) in 0.05 M Tris-HCl, pH 7.4. (A) In the presence (■) and absence (●) of denatured microsomes (1.5 mg/mL). Least-squares analysis yielded K = 730 M^{-1} and n = 0.43. (B) In the presence of microsomes (1.5 mg/mL). Least-squares analysis yielded K = 700 M^{-1} and n = 0.26. r is the ratio of bound nickel to DNA nucleotide concentrations, and C_F is the concentration of free nickel in solution.

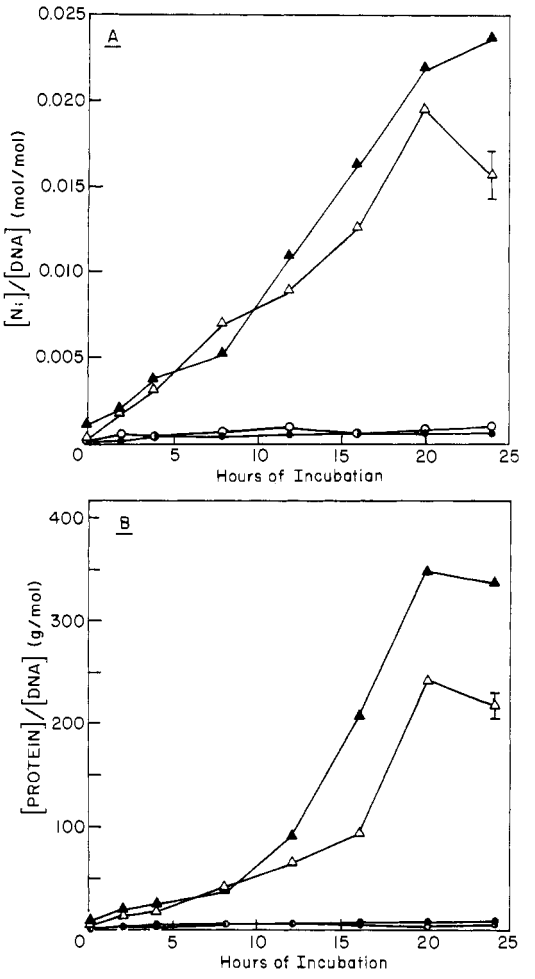

FIGURE 4: Time course for the binding of (A) nickel to DNA and (B) protein to DNA determined by precipitation of DNA with PEG and salt after incubation of Ni_3S_2 with calf thymus DNA in the absence (●) and presence of microsomes (▲), microsomes and NADPH (▲), and denatured microsomes and NADPH (○). Samples containing no Ni_3S_2 gave background readings for [Ni]/[DNA] and [protein]/[DNA] at all time points. Conditions were the same as those described in the Figure 1 legend.

as determined by DNA pelleting, increased as the amount of solubilized nickel subsulfide increased. Analysis of nickel binding to DNA by use of Scatchard plots (Figure 3A) showed that nickel bound to DNA with an apparent equilibrium association constant, K_{app}, of 730 M^{-1}. The maximum number of binding sites, n, was 0.425, which corresponds to saturation binding of one Ni per 2.4 nucleotides. The presence of denatured microsomes had little effect on K_{app} or n (Figure 3A). The value of n corresponds closely to that predicted on the basis of charge neutralization which would occur at a nickel to DNA ratio of 0.5. Phosphate binding is also consistent with the fact that a light green colored DNA precipitate formed in protein-extracted incubation solutions where dissolved nickel concentrations were equal to or greater than 8 mM and that high salt decreased the amount of nickel bound to DNA (vide infra). The presence of microsomes in the original incubation solution caused a decrease in the maximum number of nickel binding sites on DNA, n = 0.26, but had no effect on the apparent equilibrium constant, K_{app} = 700 M^{-1} (Figure 3B).

The presence of NADPH complicated the equilibrium binding of solubilized Ni_3S_2 to DNA in the presence or absence of microsomes, and Scatchard plots of the data were not linear. The assumption that all nickel sites on DNA are equivalent and independent did not hold when NADPH/$NADP^+$ was present. This may be due to equilibrium binding of the solubilized nickel to the oxidized and reduced forms of the cofactor.

Binding of Nickel to DNA after PEG–NaCl Precipitation. The amount of nickel bound to DNA (r < 0.025) decreased dramatically from the equilibrium values (r = 0.05–0.43) upon precipitation of DNA from protein-extracted incubation solutions with PEG and high salt concentration. This procedure removed the weakly bound nickel which was shown by equilibrium studies to be bound to the phosphates. Significant amounts of nickel bound to DNA were found only in samples which had been incubated in the presence of native microsomes (Figure 4A). The presence of NADPH slightly enhanced the amount of nickel bound to DNA in these samples. The amount of nickel bound to DNA increased with time of incubation. No significant binding of nickel to DNA occurred

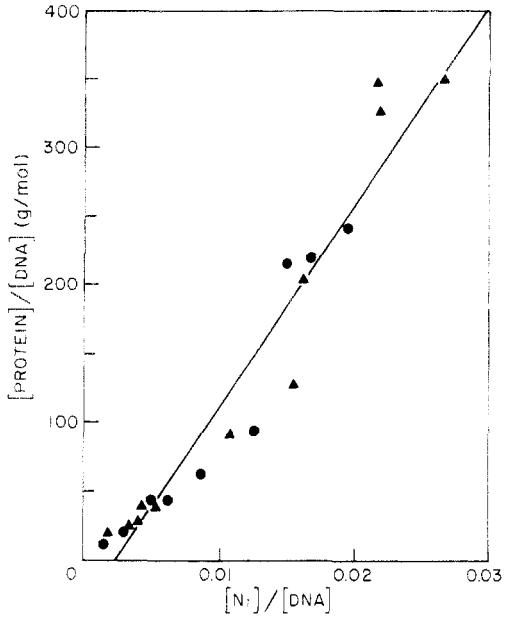

FIGURE 5: Relation between protein to DNA ratio and nickel to DNA ratio determined by precipitation of DNA with PEG and salt after incubation of Ni_3S_2 with calf thymus DNA and microsomes in the absence (●) and presence (▲) of NADPH.

in the presence or absence of NADPH or denatured microsomes.

The amount of protein associated with DNA increased (Figure 4B) with the time of incubation in protein-extracted samples originally containing Ni_3S_2 and microsomes. NADPH enhanced the amount of protein associated with DNA. No protein was found associated with DNA in the absence of Ni_3S_2 in the original incubation mixture. A linear relationship was found between the amount of nickel bound to DNA and the amount of protein associated with DNA (Figure 5). From the slope of this graph, the ratio of protein to nickel in the precipitated DNA samples was found to be 14600 g/mol. Assuming one nickel is bound per protein molecule gives a minimum average molecular weight for the protein of 14600. These results suggest that microsomes affect the interaction of nickel with DNA by allowing formation of a stable microsomal protein–nickel(II)–DNA complex.

Discussion

Nickel subsulfide was readily solubilized (millimolar concentrations) in aqueous solutions containing potential complexing ligands. Up to 15% of the Ni_3S_2 dust dissolved within 24 h. The solubilized nickel complexed the Tris buffer, NADPH/$NADP^+$, DNA, and protein present in solution. Kasprzak & Sunderman (1977) and Sunderman et al. (1976) found that the rate of solubilization of Ni_3S_2 in water was enhanced by the presence of rat serum or rat serum ultrafiltrate. Andersen et al. (1980) found that whole blood and iron citrate enhanced the solubilization of Ni_3S_2 in aqueous solutions. These results suggest that cells exposed to Ni_3S_2 particles will also be exposed to substantial concentrations of dissolved nickel. ^{63}Ni and ^{35}S were gradually solubilized from the injection site of mice administered Ni_3S_2 intramuscularly or subcutaneously (Oskarsson et al., 1979). Mammalian cells in tissue culture actively phagocytized nickel subsulfide particles (≤ 5 μm) into the cytoplasm, where they were solubilized to a form capable of entering the nucleus (Costa & Mollenhauer, 1980; Costa et al., 1981).

The reducing agent NADPH inhibited the rate of solubilization of Ni_3S_2. Sunderman et al. (1976) also found that manganese(0) decreased the solubility of Ni_3S_2 in water, rat serum, and serum ultrafiltrate. Kasprzak & Sunderman (1977) have shown that solubilization of Ni_3S_2 requires oxygen for nickel and sulfur oxidation. Nickel compounds have been associated with cancer of the lung and nasal cavity, highly aerobic organs. Yarita & Nettesheim (1978) observed gradual solubilization of Ni_3S_2 in vivo in respiratory tract epithelium following transplant of rat trachea implanted with nickel subsulfide. The sulfide from Ni_3S_2 injected in mice has been found to show distribution patterns characteristic of sulfate (Oskarsson et al., 1979). The low levels of sulfite seen in incubation solutions of Ni_3S_2 may represent its formation as an intermediate in the oxidation of sulfide to sulfate. The production of sulfite by Ni_3S_2 may account for its unusually high carcinogenicity since bisulfite is a known mutagen (Shapiro et al., 1973).

Spectroscopic and equilibrium binding studies of the interaction of nickel with DNA were consistent with the association of nickel(II) with the phosphate groups of DNA. DNA melting temperature experiments performed on solutions containing DNA and nickel(II) showed that nickel(II) binds to both phosphate and base groups on DNA (Eichhorn & Shin, 1968) but that nickel(II) had a much stronger affinity for DNA phosphate groups as compared to its affinity for DNA base groups with double-stranded structure. X-ray crystal structures of the complexes between nickel(II) and unhindered nucleotides, inosine 5′-phosphate (IMP) (Clark & Orbell, 1974), guanosine 5′-phosphate (GMP) (DeMeester et al., 1974), and adenosine 5′-phosphate (AMP) (Collins et al., 1975), showed that nickel(II) was bound directly to the N7 position on the base and indirectly to two phosphate oxygen atoms through hydrogen bonding of nickel-liganded water molecules. However, in aqueous solution, nickel(II) has been found to bind directly to the phosphate groups of AMP and CMP (Thomas et al., 1980a,b).

Microsomal protein decreased the equilibrium binding of nickel to DNA and markedly increased the stability of a DNA–nickel complex to precipitation by PEG and salt. The formation of a protein–nickel(II)–DNA complex is the most likely explanation for these results. Many studies have shown that nickel(II) is capable of binding to proteins as well as DNA. Injection of nickel chloride into rabbits has resulted in the formation of soluble complexes between nickel(II), rabbit serum albumin, and serum ultrafiltrates (Decsy & Sunderman, 1974; van Soestbergen & Sunderman, 1972; Asato et al., 1975). Purified serum albumins from several mammalian species, including rabbit, rat, and man, have been found to bind nickel(II) with varying levels of affinity (Callan & Sunderman, 1973). Oskarsson (1979) observed a nickel binding component of M_r 30000 in mouse kidney 24 h after ip injection of nickel chloride. Costa et al. (1981) observed that solubilized nickel coprecipitated with nuclear trichloroacetic acid insoluble material after nickel subsulfide particles were phagocytized by mammalian cells.

Association between solubilized nickel and macromolecules has also been demonstrated in other in vitro systems. Using the pM indicator method, equilibrium dialysis, and electrophoresis mobility data, Rao (1962) has found evidence for a strong interaction between nickel(II) and the imidazole groups of bovine serum albumin histidine residues and a weak interaction with carboxylate groups. Tsangaris et al. (1969) have used circular dichroism to interpret the binding of nickel(II) to lysine-vasopressin, conalbumin, α-chymotrypsin, and bovine serum albumin. Evidence was presented for a strong interaction between nickel(II) and amino-terminal residues and the

imidazole group of histidine residues, and for a weak interaction between nickel(II) and the sulfhydryl groups of cysteine residues.

Since the solubilized nickel was found to be in the form of octahedral nickel(II), it is reasonable that the nickel ion coordinates to both protein and DNA. We have recently demonstrated the ability of nickel carbonate to induce protein–DNA cross-links in rat kidney in vivo (Ciccarelli et al., 1981). The induction of protein–nucleic acid adducts by nickel (form not stated) in vitro has been reported (Kubinski et al., 1977). Other carcinogenic and mutagenic metal complexes have been found to induce protein–DNA cross-links. Chromate produced DNA–protein and DNA interstrand cross-links in rat liver and kidney (Tsapakos et al., 1981). DNA–protein and DNA interstrand cross-links have been found in L1210 cells treated with *cis*- and *trans*-diamminedichloroplatinum(II) (Zwelling et al., 1979). It is possible that the carcinogenic and mutagenic properties of inorganic species are related to their ability to form stable protein–DNA complexes.

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Hyperproduction of *araC* Protein from *Escherichia coli*†

Robert Ferber Schleif* and Margaret Anne Favreau

ABSTRACT: Hypersynthesis of *araC* protein from *Escherichia coli* has been accomplished. The *araC* gene was cloned on plasmid pBR322, and some of the noncoding DNA preceding the *araC* gene was removed by exonuclease digestion. Finally, a DNA fragment containing the *lac* promoter and ribosome binding site was placed in front the *araC* gene. By these means the level of *araC* protein was increased about 5000-fold above the levels found in wild-type cells. This level of protein permits straightforward purification of sizeable quantities of *araC* protein.

The arabinose operon of *Escherichia coli* is positively and negatively regulated by the protein product of the *araC* gene (Greenblatt & Schleif, 1971; Wilcox et al., 1974; Sheppard & Englesberg, 1967; Englesberg et al., 1969). *araC* protein can bind to DNA and act as a repressor; however, when arabinose is present, *araC* protein can also act as in inducer by binding to a different DNA site (Ogden et al., 1980). As a consequence of the latter binding, RNA polymerase is able to bind to DNA, and transcription of the *araBAD* genes proceeds at a high rate.

In the past, the study of *araC* protein has been hampered by the low numbers of molecules which are present in cells, about 10 per cell, the difficulty of assaying, and the instability of *araC* protein (Greenblatt & Schleif, 1971; Steffen & Schleif, 1977a,b). The problems have been partly alleviated by the fusion of *araC* to the late gene promoter of phage λ or to a copy of the *lac* promoter carried on a plasmid. Both of these fusion products yield about a 50-fold overproduction of C protein (Steffen & Schleif, 1977a). Such overproduction has permitted development of techniques which yield up to 1 mg of *araC* protein which is about 20% pure (Steffen & Schleif, 1977a). While these quantities have been sufficient for determination of the binding sites of *araC* protein (Ogden et al., 1980), they are insufficient for study of the means by which *araC* protein recognizes the repression and induction regions on the DNA.

Recently methods have been described for increasing the synthesis of proteins from cloned genes (Guarente et al., 1980; Roberts et al., 1979). Sequences immediately in front of the gene are trimmed away and in their place is fused a sequence containing a highly active promoter and a particularly good ribosome binding site. This approach seemed appropriate to apply to increase synthesis of *E. coli araC* protein. The *araC* promoter in wild-type cells is only about $^1/_{100}$ as active as the derepressed *lac* promoter (Casadaban, 1976), and the translation efficiency of *araC* messenger must be much lower than that of *lac* messenger since the level of *araC* protein, 20 monomers per cell (Kolodrubetz & Schleif, 1981), is far below 1% of the level of derepressed β-galactosidase.

Three findings are presented in this paper. First, removal of some noncoding DNA lying in front of the *araC* gene and the insertion of a DNA fragment containing the *lac* promoter and *lac* ribosome binding site lead to about 5×10^3 greater synthesis of *araC* protein than is found in wild-type cells. Second, this great hypersynthesis is not sensitive to the exact amount of the noncoding DNA preceding the *araC* gene which is removed before insertion of the *lac* fragment, and the hypersynthesis does not require the *lac* ribosome binding site. Third, application of only early steps in previously devised purification procedures (Steffen & Schleif, 1977a) applied to cell extracts containing the elevated levels of *araC* protein yields essentially pure *araC* protein.

Experimental Procedures

Miscellaneous Techniques. Except as noted, enzymes were from New England Biolabs and were used as directed by the supplier. *Bal*31 was from Bethesda Research Labs and was used as directed. T4 polymerase was a gift of William McClure, Harvard University.

DNA sequencing was done by the method of Maxam & Gilbert (1980). Plasmid DNA was cut and labeled at the *Eco*R1 site, and the *Eco*R1-*Hha*I fragment was purified and sequenced.

Constructing the Plasmid Carrying the araC Gene. Approximately 200 ng each of *Bam*H1 cut pRB322 (Bolivar et al., 1977) and λ *para*C116 DNA (Lis & Schleif, 1975) were mixed, ethanol precipitated, and resuspended in 9 μL of 0.05 M Tris-HCl,[1] pH 7.8, and 0.01 M $MgCl_2$. This was incubated 10 min at 65 °C and chilled, and 2 μL of the same buffer but containing 10 mM ATP and 0.1 mM dithiothreitol was added. This was incubated for 2 h at 1 °C with 10 units of Bethesda Research Labs T4 DNA ligase, then 190 μL of the buffer with

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; IPTG, isopropyl thio-β-D-galactoside; Tris, tris(hydroxymethyl)aminomethane.

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