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Studies of Trace Metal Metabolism: Electron Paramagnetic Resonance of Manganese in Ribonucleic Acids.* (30384)

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In previous investigations(1-4), attention has been focused upon the subcellular metabolism of nickel and other trace metals, with especial emphasis upon the binding of trace metals to ribonucleic acids. These studies have confirmed the findings of Wacker and Vallee(5) that preparations of RNA from diverse biologic sources contain trace metals, including chromium, manganese, iron, nickel, copper and zinc. The investigations of Wacker and associates(5-10) have indicated that intramolecular metal bonds constitute a tertiary structure of RNA which stabilizes the helical secondary structure in a manner similar to the stabilization of proteins by disulfide bridges. The ligand interactions of the trace metals in RNA have not been established, although it has been suggested that the metals may be bound either to phosphate groups or to nitrogen atoms of the purine and pyrimidine bases(5,9-12).

In the present investigation, measurements have been made of the electron paramagnetic resonance (EPR) spectrums of RNA isolated from human and rat tissues. Principal consideration has been directed to the ligand interactions of manganese, since the EPR spectrum of manganese has been identified in samples of RNA(12,13), and since information is available regarding the interpretation of paramagnetic resonance patterns of manganese in a wide variety of compounds(14, 15).

The biological applications of magnetic resonance spectroscopy have been reviewed by Ingram(16), and by Jardetzky and Jardetzky(17). Determinations of the electron paramagnetic resonance of an atom are based upon changes in the absorption of microwave radiation which are induced by variations in the strength of a magnetic field. The EPR spectrum is sensitive to the electronic environment of the atom, *e.g.*, the way in which an atom is attached within a particular molecule, or the relationship of an atom to its surroundings within a crystal. Because of this sensitivity, measurements of electron paramagnetic resonance provide a means for the investigation of ligand interactions.

Methods. Preparation and characterization of RNA. Samples of RNA were prepared from human and rat tissues by a phenol extraction procedure(1,18). The samples of RNA were placed in cellophane sacs and dialyzed for 48 hours at 4°C against a volume of 5 liters of water, which was changed 6 times. RNA was precipitated with cold ethanol and dried in a vacuum desiccator over phosphorus pentoxide for 48 hours at 25°C. Immediately prior to spectrographic analyses or measurements of electron paramagnetic resonance, the samples were again dried *in vacuo* for 48 hours. Spectrographic analyses of trace metals were performed by the arc technique as previously described(1). Ultra-

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violet absorption measurements of RNA dissolved in water (25 μg per ml) were obtained with a ratio-recording spectrophotometer. The extinctions of the RNA preparations per mole of phosphorus (ϵ $P_{260_{m\mu}}$) ranged from 8,800 to 10,600.

Measurements of electron paramagnetic resonance. Measurements of electron paramagnetic resonance were performed with a microwave spectrometer which was designed and constructed in the Thomas J. Watson Research Center. A 5 mg sample of RNA was packed into a polystyrene sample holder 1.5 mm in diameter. The sample holder was positioned in the center of a cylindrical resonant cavity which formed the terminus of one arm of a microwave bridge circuit. The resonant cavity was inserted into a Dewar flask containing liquid nitrogen or helium. The Dewar flask was placed between the poles of an electromagnet which produced magnetic fields ranging from 0 to 18 $\times$ 10^3 gauss. While the magnetic field was slowly swept through this range, the first derivative of the microwave absorption in the sample was inscribed by a chart recorder.

The majority of the EPR measurements were performed at a microwave frequency of 24.43 gigacycles per second (Gc per sec). In order to observe the intensities of the resonances at different magnetic field strengths, some measurements were also made at frequencies of 9, 19, 21 and 35 Gc per sec. Measurements were made with the samples at temperatures of 4.2°, 20° and 77°K. No significant differences were observed between the EPR patterns of RNA at these temperatures. Measurements could not be performed at room temperature owing to non-resonant absorption of microwaves by RNA. This non-resonant absorption decreased at temperatures below 273°K (0°C) and was essentially absent below 230°K.

Results. EPR spectrums were obtained with 36 samples of RNA prepared from human lung and spleen, and from rat lung, spleen, liver and kidney. The concentrations of manganese in these samples ranged from 5 to 61 μg of manganese per gram of RNA. Concentrations of manganese and of other trace metals in these preparations of RNA have previously been reported(1).

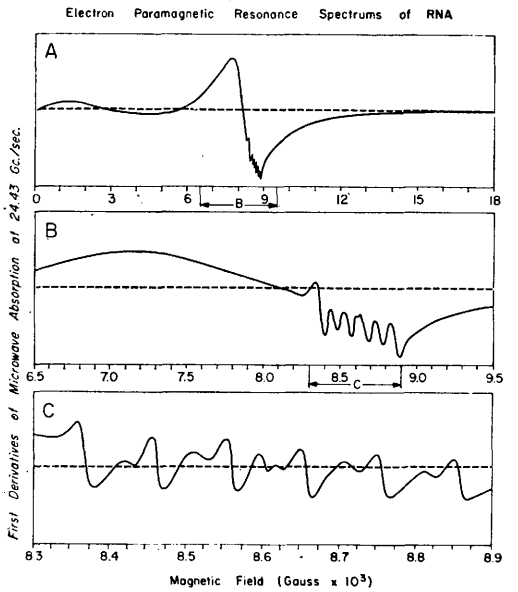

FIG. 1. EPR spectrums* of RNA from human lung.
FIG. 1-A. Spectrum from 0 to 18 $\times$ 10^3 gauss.
FIG. 1-B. Spectrum from 6.5 to 9.5 $\times$ 10^3 gauss.
FIG. 1-C. Spectrum from 8.3 to 8.9 $\times$ 10^3 gauss.

* The first derivatives of microwave absorption are plotted as the ordinates *vs* the strengths of the magnetic fields as the abscissae. Inasmuch as absorption is plotted as the first derivative, the midpoint of each biphasic deflection represents a resonance peak.

The EPR spectrums illustrated in Fig. 1 were characteristic of all of the samples of RNA which were examined. As illustrated in Fig. 1-A, a broad EPR resonance peak was detected at a magnetic field strength of approximately 7.8 $\times$ 10^3 gauss. This resonance peak is consistent with the presence in the RNA of precipitated ferromagnetic particles, such as have been observed in DNA by Walsh and coworkers(19).

The spin Hamiltonian appropriate for manganese ions has the form(20)

$$H = g\beta H \cdot S + D\{S_z^2 - \tfrac{1}{3}S(S+1)\} + AS \cdot I - g_1\beta H \cdot I \qquad (1)$$

The first (Zeeman) term is the largest and represents the interaction of the electronic spin with the external magnetic field. The second term is a "crystal field" term repre-

senting the influence of the surroundings on the paramagnetic ion. The parameter, "D," approximately represents the complicated, indirect effects of these electrostatic forces on the lowest energy levels of the manganese ion. The third term is the hyperfine interaction between the electronic and nuclear spins of the manganese ion. The magnitude of the parameter, "A," is influenced by the ligands of the manganese ion and a comparison of the value of "A" with the values obtained in other materials where the surroundings (ligands) of the manganese ion are known provides information about the bonding of the manganese to the RNA. The fourth term is very small and represents the direct interaction of the nuclear spin with the external field.

In Fig. 1-B, the 6 resonances of approximately equal intensity (located at magnetic field strengths of 8.3 to 8.9 $\times$ 10^3 gauss), represent the "hyperfine" splitting of the electron spin transition between the levels $M_s = +1/2$ and $M_s = -1/2$ for divalent manganese ($3d^5$) in a $^6S_{5/2}$ ground state. The observed hyperfine splitting results from the interaction between electronic and nuclear spins of the manganese atom. From the separation of these resonances, the magnitude of the hyperfine splitting parameter "A" was computed to be 92 $\times$ 10^{-4}cm^{-1}.

Inasmuch as the samples of RNA were powders, each EPR spectrum represented a superposition of spectrums produced by all possible orientations of the molecules within the magnetic field. The 4 additional allowed transitions of the $^6S_{5/2}$ ground state of divalent manganese were not observed, since the corresponding resonances depend upon the orientation of the external magnetic field.

The 5 intervening resonance peaks in Fig. 1-C represent partially resolved doublets, attributable to transitions of manganese involving a simultaneous "flip" of the nuclear and electronic spins(20). When the magnetic field is applied in a direction other than a symmetry axis of the crystalline field, the zero order wave functions of the manganese ion $|M_s, m_I>$, appropriate for a very large magnetic field, are admixed by off-diagonal terms in the spin Hamiltonian (Eq. (1)). These terms are important because they connect states with the same value of M_s but different values of m_I which differ in energy only by the small amount AM_s. Thus small admixtures of the wave functions $|M_s, m_I \pm 1>$ into the zero order functions $|M_s, m_I>$ give rise to appreciable intensities for transitions $\Delta M_s = \pm 1$, $\Delta m_I = \pm 1$ as compared to the allowed transitions $\Delta M_s = \pm 1$, $\Delta m_I = 0$. For the central electronic transition $M_s = +1/2 \leftrightarrow -1/2$, these forbidden lines occur as doublets, with a splitting $2g_I\beta H$, centered between the main lines. The calculated splitting of these forbidden doublets is approximately 15 gauss which is too small to be resolved in the spectrum, as given in Fig. 1-C.

The calculated intensities of these forbidden doublets is surprisingly large. Using equation 3 of Ref. 20, suitably averaged over all orientations for a powder sample, we obtain for the intensity of the "forbidden" doublet, $|1/2, m_I> \leftrightarrow |-1/2, m_I - 1>$ and $|1/2, m_I - 1> \leftrightarrow |-1/2, m_I>$, compared to the allowed transition $|1/2, m_I> \leftrightarrow |-1/2, m_I>$ the expression

$$\frac{1024}{15}\left\{\frac{D}{g\beta H}\right\}^2 \{I(I+1) - m_I(m_I-1)\} \quad (2)$$

where, for Mn^{55}, $I = 5/2$ and m_I takes values from $-3/2$ to $+5/2$. The evaluation of the last bracket yields the relative intensities of these resonances, 5:8:9:8:5. The spectrum in Fig. 1-C is clearly in qualitative accord with this expectation.

The values of $(D/g\beta H)^2$ and $|D|$ at 4 frequencies from 19.47 to 24.43 Gc per sec are given in Table I. Each tabulated value is the average of 3 to 7 individual intensity determinations which have a scatter of approximately $\pm$ 30% from the tabulated values. The apparent decrease of $|D|$ with increasing magnetic field (frequency) is prob-

TABLE I. Crystalline Field Splitting Parameter.

Frequency (Gc/sec)	$(D/g\beta H)^2$ (Eq. (2))	$\|D\|$ cm^{-1}
19.47	2.3×10^{-4}	.023
21.40	2.6×10^{-4}	.022
24.33	1.7×10^{-4}	.016
24.43	1.0×10^{-4}	.012

ably due to two effects. The "forbidden" lines are actually doublets whose splitting increases with increasing field. The use of derivative peaks can cause a significant underestimate of the intensity of partially resolved lines. In addition, the polycrystalline nature of the samples causes a distortion of the allowed lines at lower magnetic fields. This effect is not apparent at 19.47 Gc per sec but is very evident at lower frequencies and prevents estimations of "D" from measurements at 9 Gc per sec. These measurements cannot be extended to higher frequencies because the forbidden transitions become too weak. The best value for $|D|$ is 0.02 cm^{-1} with an estimated error of $\pm$ .01, considering both random and possible systematic errors.

Measurements of electron paramagnetic resonance were also undertaken upon commercial samples† of yeast and *E. coli* RNA and of calf thymus and herring sperm DNA. The commercial samples of RNA yielded manganese spectrums which did not differ significantly from those obtained with our preparations of mammalian RNA. The manganese spectrum was not found with the samples of DNA.

Discussion. The hyperfine splitting parameter "A" and the crystalline field splitting parameter "D" of manganese in RNA are similar to those which have been reported for manganese in a variety of hydrated sulfates and fluosilicates(14,15). In these compounds, manganese is known to be surrounded by a distorted octahedron of oxygen atoms present in water molecules. The similarities of the spectrums suggest that manganese in RNA is likewise located at the center of a distorted octahedron of oxygen. The "D" parameter provides a measure of the distortion of the environment of the manganese ion from a regular octahedron. The oxygen atoms might be present in phosphate groups, hydroxyl groups and/or water molecules.

Maling and coworkers(12) measured the EPR spectrums of manganese in yeast RNA at a microwave frequency of approximately 9 Gc per sec. Their spectrums are essentially identical with the spectrums observed at 9 Gc per sec in the present investigation. Maling and associates(12) observed similarities between the EPR spectrums of manganese in RNA, ATP and ADP, and inferred that the ligand interactions of manganese are similar in the 3 compounds. They concluded that their data are compatible with bonding of manganese to 2 phosphate groups.

Patten and Gordy(13) observed that the detection in RNA of the 6 components of the manganese hyperfine structure was dependent upon the presence of water of hydration. Patten and Gordy suggested that water molecules may cluster about the manganese atoms so as to alter significantly their electronic environments.

Walsh and associates(21) investigated the EPR spectrums of RNA to which Mn^{++} had been added in solution. These workers obtained the same value for the hyperfine splitting parameter ("A") as was observed in our studies, but they found a substantially lower value for the crystalline field splitting parameter ("D" less than .01 cm^{-1}).

Our observations, together with those of Maling and coworkers(12) are consistent with the bonding of manganese in RNA to oxygen atoms in 2 phosphate groups. The bonding of manganese to hydroxyl groups of ribose or of the nitrogenous bases cannot be excluded, but the spectrums do not support direct bonding of manganese to nitrogen atoms of the purine or pyrimidine bases.

Summary. In an attempt to elucidate the intramolecular bonding of trace metals in ribonucleic acids, measurements have been made of the electron paramagnetic resonance (EPR) spectrums of 36 samples of RNA isolated from human and rat tissues. The EPR pattern of manganese was readily detected in all of the samples of RNA. The resonance patterns indicate that manganese is present in RNA in a divalent state and suggest that manganese is located at the center of a distorted octahedron of oxygen atoms. From the intensity of nominally forbidden resonances, the crystal field splitting parameter D was estimated to be 0.02 cm^{-1}.

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Antagonistic and Antidotal Action of Vitamins K_1 and K_3 *Versus* Dicumarol in Dogs.* (30385)

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Clinically, it has been found that vit. K_3 is not an antidote to the indirect anticoagulant drugs, only K_1 being effective(1). In contrast to this is the observation by Johnson (2) of the equal effectiveness of K_1 and K_3 in preventing hemorrhage from dicumarol in rats and the observation of the antagonist action of K_3 as well as K_1 to dicumarol in dogs by Jaques and Dunlop(3). The differences between the antagonistic and antidotal effects of vit. K_1 and K_3 against dicumarol may be partially explained in terms of the marked difference in rates of absorption and metabolism of vit. K_1 and vit. K_3(4,5). On the other hand, the K-vitamins may have a hemostatic action independent of effects on the coagulation system, possibly through the effects of quinones on capillary resistance(6). To assist in clarification of this problem, a study has been made of the relative effectiveness of vit. K_1 and K_3 given orally, or intravenously, before, after and together with dicumarol, on the prothrombopenic effect of dicumarol in dogs.

Subjects, materials and methods. Mongrel dogs (mean weight 10.9 kg, range 5.2 to 20.4 kg) were fed a commercial dog ration (Early's Sunlight Fox Cubes). Direct spectrophotometric analysis of chromatographed extracts of the ration by Dr. R. Losito in our laboratory showed this ration contained $<1:3 \times 10^6$ parts of vitamins-K or an average daily intake of <30 μg of K-vitamins.

We are indebted to Abbott Laboratories Ltd., Montreal, for a supply of dicumarol. Vit. K_1 (2 methyl, 3 phytyl 1:4 naphthoquinone) and vit. K (2 methyl 1:4 naphthoquinone) were purchased from Nutritional Biochemicals Corp., Cleveland. The drug and vit. K_1 and K_3 were administered in single doses of 5.0, 13.05 and 5.0 mg/kg respectively. For oral administration of dicumarol and vit. K_3 the required amount was prepared in individual gelatin capsules. For intravenous and oral administration of vit. K_1, 1 ampoule

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