Purification and Some Properties of a Protein Containing Bound Manganese (Avimanganin)*

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ABSTRACT: A protein of unknown function which is present in chicken liver mitochondria has been purified to homogeneity as judged by four independent criteria. This protein which has been assigned the trivial name, avimanganin, contains 1 g-atom of tightly bound manganese per mole (89,000 g). The absorption spectrum observed for avimanganin indicates that the bound manganese in this protein is probably present as high-spin Mn(III). Exclusion of water molecules from the first coordination sphere of the bound manganese is suggested by (i) the characteristics of the visible region of the absorption spectrum which most closely resembles that observed for certain model Tris–bidentate complexes of high-spin Mn(III); and (ii) by studies of the longitudinal ($1/T_1$) and transverse ($1/T_2$) nuclear magnetic relaxation rates of water protons under various conditions. Growth of chickens under conditions of extreme manganese deficiency causes depletion of the bound manganese in avimanganin. This depletion is accompanied by disappearance of the absorption bands in the visible region of the spectrum. No gross alterations in the structure of this protein appear to result from loss of the bound metal ion.

During previous studies which established the presence of bound manganese in pyruvate carboxylase from chicken liver mitochondria, administration of ^{54}Mn *in vivo* resulted in incorporation of radioactivity both into pyruvate carboxylase and also into another protein fraction which was not examined in detail at that time (Scrutton *et al.*, 1966). However, since few other proteins have been described which contain bound manganese as the native metal (Dieckert and Rozacky, 1969; Keele *et al.*, 1970) a more extensive investigation was undertaken to define the nature of this latter fraction. These studies have resulted in the isolation of a second protein containing bound manganese from chicken liver mitochondria. This protein, which is of unknown function, has been named avimanganin. Avimanganin has been purified to homogeneity as indicated by several criteria and some of its properties are described.

Materials and Methods

I. Experimental Methods. Chicken liver mitochondria were isolated and lyophilized as described previously (Scrutton *et al.*, 1969). Since only small amounts of avimanganin are obtained, up to 2 kg of chicken liver were processed at one time. Lyophilization was performed using a Virtis Large Port Unitrap freeze dryer. Chickens were raised under conditions of manganese deficiency to 6 weeks of age as described by Griminger and Scrutton (1970).

Metal contents were estimated by atomic absorption spectrophotometry using either a Varian Techtron AA-4 atomic absorption spectrophotometer equipped with an Aztec SX-2 scale expander and a Photovolt Varicord 43 linear–log recorder or more recently an Aztec AAA-3 Atomic analyzer equipped with a Honeywell Model 194 Electronik recorder. Metal contents were estimated by reference to an "internal standard" as described previously (Scrutton *et al.*, 1970). The absolute concentrations of manganese present in the samples used for the analyses were in the range 0.1–1.0 μg/ml, *i.e.*, 5- to 50-fold greater than the limit of detection for this metal ion under the conditions employed (0.02 μg/ml). The observed sensitivity (defined as the metal concentration required for $\Delta A = 0.044$) was in the range 0.06–0.07 μg of manganese/ml. Standard metal solutions were prepared from analytical grade reagents, or from spectrally pure reagents (Johnson-Matthey Chemicals Inc.) for manganese and iron. The conditions used for preparation of the fractions for metal analysis differed in the various studies and are described in the text. However, since similar manganese contents were observed before and after gel filtration in the presence of 0.05 M Tris·Cl (pH 7.0) containing 10 mM EDTA when purified preparations of avimanganin were analyzed, the gel filtration procedure was omitted for routine manganese analysis of purified fractions.

Protein concentrations were estimated by the method of Warburg and Christian (1941) or in some instances by a micromodification of the biuret procedure (Layne, 1957).

Sephadex G-200 columns were prepared and calibrated for molecular weight estimation as described by Andrews (1964, 1965) using proteins of known molecular weight. The standard proteins used are indicated in the figure legend. The Stokes' radius of avimanganin was estimated on the same Sephadex G-200 columns using the procedure described by Ackers (1964). The void volume (V_0) of the column was estimated from the elution volume for Blue Dextran 2000 and the included volume (V_i) from the elution volume for tritiated water.

Zone electrophoresis was performed on cellulose acetate strips. The strips after equilibration with buffer were mounted in a horizontal chamber and the protein preparations (5–10 μg) were applied using a thin wire applicator (Beckman Instruments Inc.). The buffers used (sodium acetate, pH 4.0–6.0; sodium phosphate, pH 5.5–7.5) were adjusted to an ionic strength of 0.1 μ. Current was applied from a constant voltage power supply at 250 V for 30 min.

Polyacrylamide gel electrophoresis was conducted in the absence of sodium dodecyl sulfate as described by Davis (1964) except that in some instances the stacking gel was omitted. The buffer system employed was 0.05 M Tris·PO_4 (pH 6.7) containing 0.01 M sodium citrate.

Polyacrylamide gel electrophoresis was performed in the

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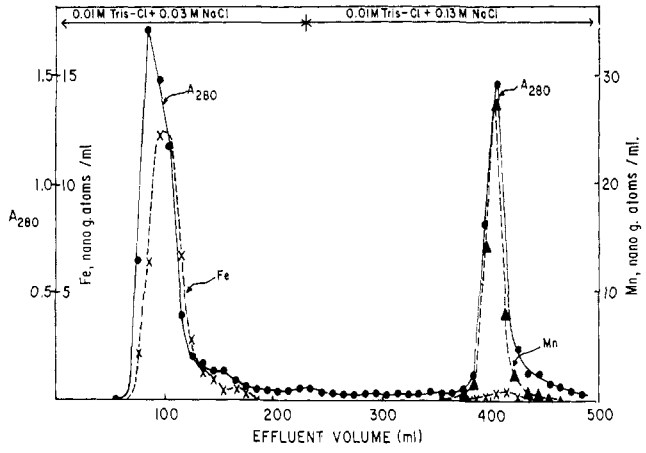

FIGURE 1: Protein (A_{280}), manganese (Mn), and iron (Fe) distributions observed for chromatography of stage 2 (72–95% ammonium sulfate precipitate) Avimanganin preparations on DEAE-Sephadex-Cl⁻. A 25 × 1.5 cm column of DEAE-Sephadex-A-50 (Cl^- form) was equilibrated with 0.01 M Tris-Cl (pH 7.1) containing 0.03 M NaCl. A dialyzed stage 2 preparation (94 mg; specific activity 3.5 mμg-atoms of Mn/mg) was placed on the column which was washed with the equilibrating buffer until the absorbance of the effluent at 280 mμ decreased to less than 0.03. The column was then eluted with 0.01 M Tris-Cl (pH 7.1) containing 0.13 M NaCl. Fractions (approximately 9 ml) were collected and the absorbance at 280 mμ was measured. The iron and manganese contents of these fractions were estimated by atomic absorption spectrophotometry as described in Methods. The small iron peak which appears at approximately 420 ml may be due to the presence of contaminating metal ion which is loosely associated with the protein under these conditions.

presence of 0.1% sodium dodecyl sulfate essentially as described by Dunker and Rueckert (1969). The avimanganin preparations were incubated with 5 mM 2-mercaptoacetate (2 hr at 37° under N_2) prior to introduction of the detergent. These conditions are adequate to ensure cleavage of disulfide bridges if present in this protein (Fish *et al.*, 1970).

Absorption spectra were determined in a Cary 14 double-beam recording spectrophotometer. The calibration of the instrument was checked with K_2CrO_4 solutions of known absorbance. The ultraviolet region of the absorption spectrum was examined at a full-scale deflection of 2.0 *A* units; the visible and near-ir regions at a full-scale deflection of 0.2 *A* unit.

Electron paramagnetic resonance (epr) spectra were measured at X band (9 GHz) in a Varian E-3 epr spectrometer equipped with a variable-temperature accessory.

The longitudinal relaxation rate ($1/T_1$) of water protons was measured at 24.3 MHz with a pulsed nuclear magnetic resonance (nmr) spectrometer as described previously (Mildvan and Cohn, 1963). In experiments where the relaxation rate was measured as a function of temperature, the sample temperature was maintained to ±0.5°. The transverse relaxation rate ($1/T_2$) of water protons was measured at 24.3 MHz utilizing the shape of the spin echo envelope (Carr and Purcell, 1954).

Stage 3 preparations of avimanganin were assayed for superoxide dismutase activity as described by McCord and Fridovich (1969); for pyruvate carboxylase activity as described by Scrutton *et al.* (1969); for oxalacetate decarboxylase activity as described by Scrutton and Mildvan (1968); and for arginase activity as described by Greenberg (1955).

II. Purification of Avimanganin from Lyophilized Chicken Liver Mitochondria. The initial steps in this procedure are similar to those used for purification of pyruvate carboxylase (Scrutton *et al.*, 1969). They are described briefly for the sake of completeness. All operations were performed at 25°.

STAGE 1. EXTRACTION OF THE MITOCHONDRIAL POWER. Approximately 100 g of lyophilized chicken liver mitochondria were stirred with 0.05 M Tris·acetate (pH 6.5) containing 0.1 mM dithioerythritol in a ratio of 10 ml of extracting medium/g of powder. The pH dropped during addition of the powder to the extracting medium and was maintained in the range pH 6.3–6.5 by addition of 1 M Tris base. After completion of the addition of the mitochondrial powder, the system was stirred for a further 15 min and was then centrifuged at 37,000*g* for 45 min. The precipitate was discarded.

STAGE 2. $(NH_4)_2SO_4$ FRACTIONATION. Solid $(NH_4)_2SO_4$ (19.6 g/100 ml) (to raise the $(NH_4)_2SO_4$ concentration to 33% saturation) was added to the supernatant fraction from stage 1. The pH was adjusted to 7.2 with 1 M Tris base prior to this addition of $(NH_4)_2SO_4$ and was maintained in the range 7.0–7.2 during the addition. Stirring was continued for 15 min and then the precipitate was collected by centrifugation at 37,000*g* for 15 min. This precipitate which contains the bulk of the pyruvate carboxylase activity present in the crude extract was retained for further purification of this enzyme.

Solid $(NH_4)_2SO_4$ (27.0 g/100 ml) was added to the supernatant fraction raising the concentration of this salt to 72% saturation and the pH maintained as before. After stirring for 15 min the red precipitate was removed by centrifugation at 37,000g for 15 min and was discarded.

Finally solid $(NH_4)_2SO_4$ (18.0 g/100 ml) was added to this final supernatant fraction with maintenance of pH as before and the system was stirred for 1 hr. Since this final addition of $(NH_4)_2SO_4$ raises the concentration of this salt to 95% saturation, incomplete solution was often observed even after prolonged stirring at 25°. The precipitate which formed was collected by centrifugation for 30 min at 37,000*g*.

The precipitate was dissolved in a minimal volume of 0.01 M Tris·Cl (pH 7.0) containing 0.03 M NaCl and was dialyzed against 4 l. of this buffer using a flow dialysis system. The precipitate which formed during dialysis was removed by centrifugation at 37,000*g* for 10 min. Examination of the resulting supernatant fraction by cellulose acetate strip electrophoresis in 0.08 M Tris·Cl (pH 7.5) showed the presence of two major protein bands. These two components were separated by chromatography on DEAE-Sephadex A-50.

STAGE 3. CHROMATOGRAPHY ON DEAE-SEPHADEX (Cl^-). A 25 × 2.0 cm column of DEAE-Sephadex A-50, Cl^- form, was prepared and equilibrated with 0.01 M Tris-Cl (pH 7.0) containing 0.03 M NaCl. The dialyzed supernatant fraction from stage 2 was placed on this column which was then washed with the equilibrating buffer. A large protein peak which emerges at the void volume of the column contains protein-bound iron (Figure 1). This fraction probably contains a hemoprotein since strong absorbance is observed in the Soret band region which parallels the distribution of iron. However, no manganese was present in this peak. Washing was continued with the equilibrating buffer (0.01 M Tris·Cl, pH 7.0, containing 0.03 M NaCl) until the absorbance of the effluent at 280 mμ decreased to less than 0.030 *A* unit. Elution was then performed with 0.01 M Tris·Cl (pH 7.0) containing 0.13 M NaCl. A sharp protein peak which was obtained after passage of a total of approximately 400 ml of effluent contained manganese in constant ratio to protein in the peak fractions. Fractions having an absorbance greater than 0.2 at 280 mμ were pooled and concentrated in a Schleicher & Schuell collodion bag apparatus to a final volume of 1–2 ml.

TABLE I: Purification of Avimanganin from Chicken Liver Mitochondria.

Stage No.	Vol (ml)	Total Protein[a] (mg)	Total Protein[b]-Bound Mn (μg)	Sp Act. (μg of Mn/mg)	Yield (%)
I. Mitochondrial extract	210	2070	51.2	0.025	100
II. $(NH_4)_2SO_4$ fractionation					
0–33% precipitate	22	460	26.6	0.058	
33–72% precipitate	20	1380	4.0	0.003	
72–95% precipitate	15	87	18.4	0.21	36
III. DEAE-Sephadex chromatography (pooled fractions)	18	14.5 (19.7)	14.0	0.97 (0.71)	27

[a] Determined as described by Warburg and Christian (1941) and not corrected to true protein concentration except for the figure in parentheses (stage 3). [b] Defined as manganese which remains associated with protein after gel filtration on Sephadex G-25 (20 × 1 cm) in the presence of buffer containing 5 mM EDTA. In all instances the manganese concentration in the samples used for analysis (0.2–1.2 μg of Mn/ml) was at least an order of magnitude greater than the limit of detection of the assay system (0.02 μg of Mn/ml).

A summary of the results obtained in a typical preparation is presented in Table I. On the basis of total manganese this procedure provides a 39-fold purification from the mitochondrial extract with an overall yield of 27%. However, these figures underestimate the efficiency of the procedure since approximately half the protein-bound manganese of the crude extract is present in association with pyruvate carboxylase. If we assume that the bound manganese present in the protein precipitated by addition of $(NH_4)_2SO_4$ to 33% saturation (stage 2) represents the bound manganese of pyruvate carboxylase, the corrected purification factor and overall yield become 82-fold and 57%, respectively. It is also apparent that in excess of 90% of the protein-bound manganese in the mitochondrial extract is present as pyruvate carboxylase and avimanganin. Previous studies in which $^{54}MnCl_2$ was administered to chickens by either intravenous or intraperitoneal injection have provided no evidence for the presence of any other fractions which contain bound manganese (Scrutton *et al.*, 1966). It therefore appears that these two proteins may account for all of the bound manganese present in chicken liver mitochondria. It should be noted that evidence has been presented previously demonstrating that authentic mitochondria are obtained from chicken liver by the procedure used in these studies (Utter and Keech, 1963; Keech and Utter, 1963).

III. Materials. Sephadex G-200 and DEAE-Sephadex A-50 (Cl^- form) were obtained from Pharmacia; $(NH_4)_2SO_4$ (enzyme grade) from Mann Research Laboratories; acrylamide, *N,N'*-methylenebisacrylamide from Eastman Organic Chemicals; myoglobin, ovalbumin, bovine serum albumin, and bovine γ-globulin from Pentex, Inc.; and lactate dehydrogenase and chymotrypsinogen, from Sigma Chemical Co. Crystalline preparations of these standard proteins were used in all cases and the purity of the preparations was checked by zone electrophoresis in most instances. All other chemicals employed were A. R. grade.

Results

I. Criteria of Purity. Four different approaches have been used to evaluate the purity of the stage 3 preparations of avimanganin obtained using the procedure described above.

First, when the pooled fractions from stage 3 are chromatographed again on DEAE-Sephadex (Cl^-) as described for Figure 1 a single protein peak is observed which is coincident with the distribution of manganese, *i.e.*, a constant Mn:protein ratio is observed across the peak. Similar results are obtained when stage 3 preparations are subjected to gel filtration on a 50 × 2 cm column of Sephadex G-200 equilibrated with 0.01 M Tris-Cl (pH 7.2) containing 0.1 M NaCl. In both cases the mean specific activity is 12.7 ± 1.0 mμg-atoms of Mn/mg of biuret protein, in good agreement with the specific activity of the pooled fractions from stage 3 (Table I).

Second, examination of stage 3 preparations of avimanganin in the analytical ultracentrifuge showed the presence of a single component which sedimented as a symmetrical peak with a sedimentation coefficient ($s_{20,w}$) of 5.45 S (2.5 mg of protein/ml; solvent, 0.01 M Tris·Cl (pH 7.2) containing 0.13 M NaCl).

Third, zone electrophoresis of stage 3 preparations on cellulose acetate strips demonstrates that the purified protein moves as a single band when electrophoresis is performed over the range from pH 4.0 to 9.2. From the relative mobilities at different pH values the isoelectric point of avimanganin was estimated as 5.65. Zone electrophoresis of bovine serum albumin in the same system permitted the estimation of the isoelectric point as 4.9 for this protein, in reasonable agreement with published values (Sober, 1968).

Finally, purified avimanganin was subjected to disc electrophoresis on polyacrylamide gel at pH 6.7 using the system of Davis (1964). As shown in Figure 2 a single protein band is observed when stage 3 preparations are analyzed by this procedure in either the presence (Figure 2, gel B) or absence (Figure 2, gel A) of a stacking gel and despite the application of a high protein concentration (90 μg) to gel B.

A single sharp band was also observed when this protein was subjected to polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate as described by Dunker and Rueckert (1969).

Hence, no evidence is obtained for the presence of signifi-

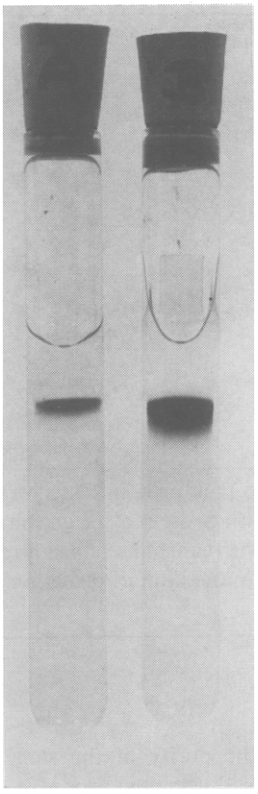

FIGURE 2: Polyacrylamide gel electrophoresis of stage 3 preparation of avimanganin. Polyacrylamide gel electrophoresis was performed essentially as described by Davis (1964) but using a Tris-phosphate–sodium citrate buffer, pH 6.7. The samples were subjected to electrophoresis for 60 min at an average current of 1 mA/tube. The protein was stained using buffalo black and excess stain was removed using 5% acetic acid. (A) 36 μg of avimanganin (12.5 mμg-atom of Mn/mg): no stacking gel employed: (B) 90 μg of avimanganin (12.9 mμg-atom of Mn/mg): with stacking gel.

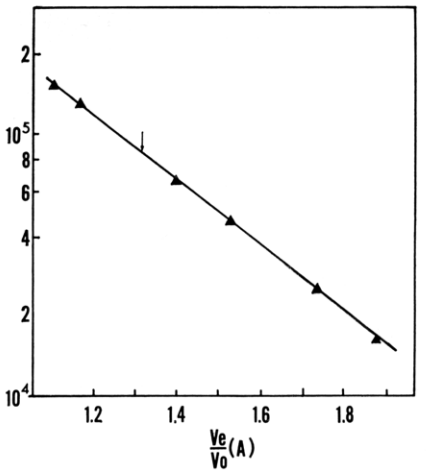

FIGURE 3: Determination of molecular weight of avimanganin by gel filtration on a calibrated column of Sephadex G-200. A 50 × 1.5 cm Sephadex G-200 column was prepared and equilibrated with 0.1 M Tris-Cl (pH 7.4). The column was calibrated as described by Andrews (1964) using myoglobin, chymotrypsinogen, ovalbumin, bovine serum albumin, lactate dehydrogenase (beef heart), and bovine γ-globulin as standard proteins. After layering of the standard proteins or of avimanganin on this column 1.2–1.3-ml fractions were collected in tared test tubes and the exact volume was determined from the increase in weight. The excluded (V_0) (= 31.9 ml) and included (V_i) (= 98.6 ml) volumes of the column were determined from the elution volumes (V_e) for the Blue Dextran 200 and ^3H_2O, respectively. The position of elution of avimanganin from this column in relation to the standard proteins is indicated by an arrow.

cant concentrations of any impurities in stage 3 preparations of avimanganin by any of the above procedures. These preparations appear therefore to be at least 95% pure (*cf.* section IV).

II. Molecular Weight. The elution volume observed when stage 3 preparations of avimanganin are subjected to gel filtration on a Sephadex G-200 column calibrated as described by Andrews (1964, 1965) permits estimation of the molecular weight of this protein as 89,000 ± 5000. The estimate of the molecular weight obtained in this way appears valid since no evidence has been obtained for the presence of significant concentrations of hexose in the stage 3 preparations. The V_e/V_0 value for avimanganin is indicated by an arrow in Figure 3. However, Ackers (1964) and Laurent and Killander (1964) have suggested that the position of elution of a globular protein from Sephadex G-200 correlates better with the Stokes' radius of the protein than with the molecular weight. Hence, the procedure described by Ackers (1964) was used to estimate the Stokes' radius of avimanganin as 36.1 ± 1.5 Å. Application of the Stokes–Einstein equation then permitted calculation of the diffusion coefficient ($D_{20,w}$) of this protein as 5.8×10^{-7} cm^2 sec^{-1}. This diffusion coefficient may be used together with the $s_{20,w}$ (2.5 mg/ml) obtained by analytical ultracentrifugation to obtain an independent estimation of the molecular weight of avimanganin (Svedberg, 1925). Although $s^0_{20,w}$ and $D^0_{20,w}$ should be employed for this calculation, the error introduced by using $s_{20,w}$ and $D_{20,w}$ obtained at finite and similar protein concentration is probably minimal. If the partial specific volume of this protein is taken as 0.75 ml^{-1} g^{-1}, this approach gives the molecular weight as 89,800, in agreement with the value obtained from the relationship between molecular weight and V_e/V_0 (Andrews, 1964). The molecular weight of avimanganin is, therefore, taken as 89,000 in subsequent calculations.

III. Manganese–Protein Stochiometry. Quantitative analysis of stage 3 avimanganin preparations for manganese content by three independent methods indicates the presence of 1.0–1.2 g-atoms of manganese/mole (89,000 g) of protein. As shown in Table II excellent agreement is obtained between the three analytical procedures employed.

Stage 3 preparations have also been analyzed for their content of nine other metals by atomic absorption spectrophotometry after removal of extraneous contaminants by gel filtration on Sephadex G-25 in the presence of 0.05 M Tris·Cl (pH 7.4) containing 1 mM EDTA. The data obtained are summarized in Table III and indicate that none of these latter metal ions are present at detectable levels in stage 3 preparations of avimanganin. The limit of detection for these various metal ions did not exceed 0.2 g-atom/mole of protein in any case (Table III). Avimanganin appears therefore to be a manganese metalloprotein. Since the manganese content is not significantly affected by incubation of this protein with a variety of chelating agents including EDTA, EGTA,[1] CyDTA, 8-hydroxyquinoline-5-sulfonate, and 1,10-phenanthroline at concentrations in the range 1.0–10.0 mM, this metal ion appears to be tightly bound to avimanganin. The bound manganese is however released by denaturation of the protein in

[1] Abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate; CyDTA, 1,2-cyclohexanediaminetetraacetate; SDS, sodium dodecyl sulfate; PRR, the longitudinal nuclear magnetic relaxation rate ($1/T_1$) of water protons.

TABLE II: Manganese Content of Avimanganin.

Method[a]	Manganese Content		No. of Prepns Examined
	μg/mg[b]	g-atoms/mole[c]	
Atomic absorption	0.6–0.75	0.99–1.22	5
Neutron activation	0.74	1.17	1
PRR after treatment with $HClO_4$	0.62	0.97	1
Weighted average		1.09 ± 0.1 (std dev)	

[a] Magnanese concentrations were estimated by atomic absorption spectrophotometry as described in Experimental Section; by neutron activation analysis essentially as described previously (Wishnick *et al.*, 1970); and by PRR analysis by reference to the relaxivity of standard Mn^{2+} solutions in the same medium (Scrutton *et al.*, 1966). In all cases the manganese content of the solutions subjected to analysis was at least an order of magnitude greater than the limit of detection for this metal ion in the procedure used. I am grateful to Mr. John Kelly of Industrial Reactor Laboratories Inc. for the neutron activation analysis. [b] Protein was determined by the method of Warburg and Christian (1941) and a correction was applied to give true protein concentration (mg of spectrophotometric protein × 1.36), which was derived by comparison of protein concentrations obtained by the spectrophotometric and biuret methods. [c] On the basis of a molecular weight of 89,000.

TABLE III: Survey Analysis of Bound Metal Content of Avimanganin.[d]

Metal Ion	Metal Concn[a] Required for ΔA = 0.01 μg/ml	Observed Metal Content (μg/ml)		Net Metal Content[c] (g-atoms/mole)
		Buffer	Buffer + Protein[b]	
Mg	0.020	0.11	0.10 (0.8)	<0.1
Ca	0.24	0.12	0.10 (2.0)	<0.2
Cr	0.32	0.08	0.07 (1.2)	<0.2
Mn	0.15	0.03	0.78 (1.2)	1.05
Fe	0.27	0.07	0.08 (1.6)	<0.2
Co	0.29	0.03	0.03 (1.6)	<0.2
Ni	0.30	0.04	0.04 (1.6)	<0.2
Cu	0.23	0.02	0.03 (1.2)	<0.15
Zn	0.052	0.01	0.01 (1.0)	<0.1
Cd	0.075	0.01	0.01 (1.0)	<0.1

[a] At the appropriate wavelength for each metal ion. Calculated from the increment in absorbance which is observed when an aliquot of a standard metal solution is added to the buffer. The limit of detection did not exceed ΔA = 0.002 for any metal ion examined. [b] The figures in parentheses indicate the protein concentration (in milligrams per milliliter) employed in each analysis. [c] On the basis of a molecular weight of 89,000. [d] These analyses were conducted using the "spike-height" procedure described by Duckworth and Coleman (1970). A total sample volume of 0.15 ml was employed.

the presence of 0.1% sodium dodecyl sulfate or 12% perchloric acid.

IV. Absorption Spectrum. Concentrated solutions (~20 mg/ml) of stage 3 preparations of avimanganin have a brown to pink color. The absorption spectrum of this protein in the visible region is shown in Figure 4. The main absorption bands are located at 480 and 600 mμ. This latter band appears as a well-defined shoulder on the main peak. The extinction coefficients (M^{-1} cm^{-1}) of these absorption bands are calculated as 508 (480 mμ) and 250 (600 mμ). The slight shoulder observed at approximately 410 mμ may be due to contamination with a trace of the hemoprotein from which avimanganin is separated during stage 3 of the purification procedure. A minimal extinction coefficient of 1.12×10^5 M^{-1} cm^{-1} may be calculated for this hemoprotein from the absorbance at 410 mμ and the iron content of the fractions which emerge at the void volume of the DEAE-Sephadex column (Figure 1). Utilizing this minimal extinction coefficient it may be estimated that less than 0.5% contamination of stage 3 preparations of avimanganin with the hemoprotein could give rise to the slight shoulder observed at approximately 410 mμ in Figure 4. This level of contamination would not have been detected by any of the procedures used to evaluate the purity of these preparations. The characteristics of the visible absorption spectrum of avimanganin are not affected by addition of either mild reducing ($NaBH_4$, $Na_2S_2O_4$) or mild oxidizing (H_2O_2) agents. Although detailed analysis is deferred to the Discussion, the data described in Figure 4 strongly suggest that high-spin Mn(III) is present in avimanganin (Dingle, 1966).

In the ultraviolet region avimanganin exhibits a typical protein absorption spectrum (Wetlaufer, 1962). The absorption band arising from the aromatic residues of the protein exhibits little fine structure and a maximum of 280 mμ. The ratio of absorbance at 280 mμ to absorbance at 260 mμ is 1.61.

V. Magnetic Resonance Studies. The environment of the bound manganese in avimanganin has been studied further by examination of the effect of this protein on the longitudinal

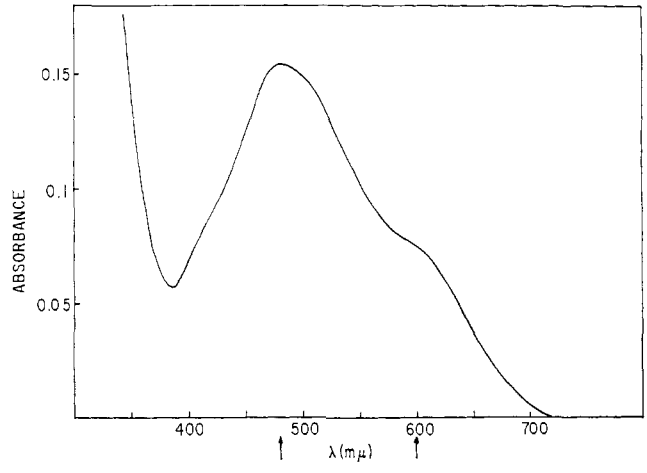

FIGURE 4: Absorption spectrum of avimanganin in the visible region. Avimanganin (25.2 mg/ml; 0.31 mM Mn) in 0.01 M Tris-Cl (pH 7.1) containing 0.03 M NaCl was examined using a Cary 14 double-beam spectrophotometer equipped with the 0–0.2-A slide-wire. The calibration of the instrument was checked as described in Methods.

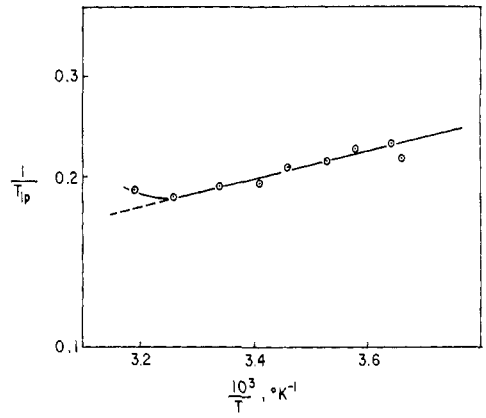

FIGURE 5: The paramagnetic contribution to the longitudinal relaxation rate ($1/T_{1p}$) of water protons measured as a function of temperature in the presence of avimanganin. The $1/T_1$ of water protons was measured at 24.3 MHz using a pulsed nmr spectrometer (Mildvan and Cohn, 1963) for solutions containing buffer (0.01 M Tris-Cl (pH 7.1) + 0.13 M NaCl) and buffer + avimanganin (9.3 mg/ml; specific activity 11.2 mμmoles/mg). Temperature control was to ±0.5°.

($1/T_1$) and transverse ($1/T_2$) relaxation rates of water protons. Addition of avimanganin causes a small increase in $1/T_1$ of water protons. A further increase in $1/T_1$ is observed on denaturation of this protein with $HClO_4$ (Table IV) and epr studies demonstrate that the manganese is present in the $HClO_4$ extract as Mn(II). If, as suggested by the absorption spectrum, manganese is present as Mn(III) in native avimanganin, denaturation appears to be accompanied by reduction of Mn(III) to Mn(II), which may account for part (or all) of the increase in the paramagnetic contribution to the relaxation rate ($1/T_{1p}$) (Table IV). A similar reduction of Mn(III) to Mn(II) is observed on denaturation of superoxide dismutase (Keele *et al.*, 1970) and is expected since Mn(III) is a powerful oxidizing agent which is only stable in the presence of a strong ligand field (Cotton and Wilkinson, 1966). Since a value cannot be obtained for the effect of an equivalent concentration of Mn(III) (aqueous) on $1/T_1$, it is not possible to calculate an enhancement factor (ϵ_b) (Mildvan and Cohn, 1970) for manganese in avimanganin. However, the increase in $1/T_1$ observed on addition of $HClO_4$ (Table IV) indicates that the relaxivity of manganese in avimanganin is reduced approximately fivefold as compared with that of $Mn(H_2O)_6^{2+}$.

TABLE IV: Effect of Avimanganin on the $1/T_1$ for Water Protons under Various Conditions.[a]

System	$1/T_1$ (sec^{-1})
Buffer (0.01 M Tris-Cl (pH 7.1) + 0.03 M NaCl)	0.41
Avimanganin (9.3 mg/ml)	0.63
Avimanganin + 12% $HClO_4$	1.39
Avimanganin + 83 mM $Na_2S_2O_4$	0.61
Avimanganin + 77 mM $NaBH_4$	0.63
Avimanganin + 8 mM NaEDTA	0.62
Avimanganin + 19 mM potassium oxalate	0.59
Avimanganin + 19 mM KCN	0.64

[a] $1/T_1$ was measured using a pulsed nmr spectrometer as described in Methods.

TABLE V: Comparison of the Effect of Manganese Deficiency on the Manganese Content of Pyruvate Carboxylase and of Avimanganin.

Diet Manganese Content (mg/kg):	0.3	4.8	58.0
Tissue Preparation (purification stage)	Manganese Concn of Fractions (mg-atoms/mg of Protein)		
Mitochondrial extraction (1)	0.035[a]	0.11[a]	0.42[a]
A. Pyruvate carboxylase			
0–33% $(NH_4)_2SO_4$ precipitate (2A)	0.02	0.27	1.11
DEAE-Sephadex chromatography (pooled fractions) (3A)	0.07	1.4	6.1
B. Avimanganin			
72–95% $(NH_4)_2SO_4$ precipitate (2)	0.6	2.95	3.47
DEAE-Sephadex chromatography (pooled fractions) (3)	3.1	16.5	16.9

[a] After gel filtration on a 20 × 1 cm column of Sephadex G-25 equilibrated with 0.01 M Tris-Cl (pH 7.2) containing 0.05 M NaCl and 5 mM EDTA to remove free and loosely bound manganese. Similar treatment has no effect on the manganese content of other fractions. [b] Protein (spectrophotometric method) and manganese contents were estimated as described in Experimental Section. Avimanganin was purified as described in Methods. Pyruvate carboxylase was purified from stage IIA by an unpublished procedure involving chromatography on DEAE-Sephadex A-50.

Neither reducing agents ($NaBH_4$, $Na_2S_2O_4$) nor ligands which exhibit a high affinity for Mn(II) and Mn(III) (Sillen and Martell, 1964) cause significant alterations in the effect of avimanganin on $1/T_1$ of water protons (Table IV).

When the effect of temperature on this system is examined, a linear relationship of positive slope is observed for the logarithmic variation of $1/T_{1p}$ as a function of the reciprocal of the absolute temperature over the greater part of the temperature range (0–44°) (Figure 5). From the slope of this linear relationship the activation energy for the process which dominates $1/T_{1p}$ is obtained as -1.2 ± 0.2 kcal/mole. Additionally at the three temperatures examined (0, 20, and 35°) $1/T_{2p}$ for the avimanganin solution is approximately equal to $1/T_{1p}$. The equality of $1/T_{1p}$ and $1/T_{2p}$ and the weak paramagnetic effect of the bound manganese on $1/T_{1p}$ (Figure 5) are consistent with the suggestion that an outer sphere mechanism dominates the relaxation process (Mildvan and Cohn, 1970). However, this suggestion does not provide a unique interpretation since if the electron spin relaxation time (τ_s) is very short but still modulates the relaxation process, approximate equality of $1/T_{2p}$ and $1/T_{1p}$ may be observed when an inner sphere mechanism, *i.e.*, relaxation of coordinated water protons, is dominant. Although this latter possibility is considered less likely in the light of other data, it should be noted that (i) a very short τ_s is characteristic of Mn(III) (Schwartz and Carlin, 1970) and (ii) the low activation energy and posi-

TABLE VI: Characteristics of Visible Absorption Spectra of Model Complexes of Mn(II) and Mn(III).

Type of Complex	Example	Location of Absorption Bands	Extinction Coef (M^{-1} cm^{-1})	Ref
Mn(II)-octahedral	$Mn(H_2O)_6^{2+}$	350–600 mμ (multiple)	0.01–0.05	Cotton and Wilkinson (1966)
Mn(II)-tetrahedral	$(MnBr_4)^{2-}$	350–500 mμ (multiple)	1.0–4.0	Cotton and Wilkinson (1966)
Mn(III)-low spin	$[Mn(CN)_6]^{3-}$	None in visible		Cotton and Wilkinson (1966)
Mn(III)-high spin				
(i) Trisbidentate	(Trisoxalato)manganese(III)	480–500 mμ (multiple)	200–300	Dingle (1966)
	(Tris(diethyldithiocarbamato))manganese(III)	480–500 mμ (multiple)	200–300	Dingle (1966)
		600–620 mμ (shoulder)	100–150	
(ii) Bisbidentate (diaquo)	(Bisoxalato)manganese(III)-diaquo)	440–460 mμ (multiple)	100–130	Dingle (1966)

tive slope observed for the variation of $1/T_{1p}$ with temperature (Figure 5) would be expected for modulation of an inner sphere relaxation mechanism by τ_s (Mildvan and Cohn, 1970).

VI. Effect of Manganese Deficiency. Avimanganin has also been purified from the livers of chickens raised on diets containing various levels of manganese in order to investigate the effect of deprivation of this metal ion on the manganese content of avimanganin. As shown in Table IV, avimanganin retains its manganese tenaciously. The manganese content of this protein is decreased only fivefold when the dietary manganese is lowered from an optimal level (56 mg/kg) to the lowest level attained in these studies (0.3 mg/kg). In contrast this decrease in dietary manganese causes a 100-fold decrease in the manganese content of pyruvate carboxylase. At an intermediate level of dietary manganese (4.8 mg/kg), the manganese content of avimanganin is not significantly depleted whereas the manganese content of pyruvate carboxylase is decreased 77%. Significant deficiency symptoms (weight loss, perosis) are observed at both the low (0.3 mg/kg) and intermediate (4.8 mg/kg) levels of dietary manganese.

Cellulose acetate strip and polyacrylamide gel electrophoresis of the stage 3 preparations of avimanganin obtained from chickens grown on the lowest level of dietary manganese showed the presence of a single protein band. Hence these preparations do not appear significantly less pure than the stage 3 preparations obtained from chickens raised on diets containing optimal concentrations of manganese. However, when calculated with reference to total mitochondrial protein, the yield of avimanganin is markedly decreased in severe manganese deficiency, suggesting that either the rate of synthesis of this protein is depressed or that the properties of the protein are altered under these conditions.

Analysis for other metal ions has shown the absence of significant concentrations (<0.2 g-atom/mole) of zinc, copper, magnesium, iron, or nickel from stage 3 preparations obtained from manganese-deficient chickens. However, the molecular weight of the metal-deficient protein is obtained as 87,500 ± 5000 by gel filtration (Andrews, 1964), in agreement with the value of 89,000 obtained for native avimanganin (section II).

The absorbance at 480 mμ of concentrated solutions (15–25 mg/ml) of avimanganin purified from manganese-deficient chickens approximates 0.03–0.04 with no indication of an absorption band in this region of the spectrum whereas in the ultraviolet region the spectrum appears similar to the native protein. Hence, the A_{480}:A_{280} ratio for avimanganin prepared from manganese-deficient chickens is less than 2×10^{-3} *A* as compared with the A_{480}:A_{280} ratios of 8–9 $\times 10^{-3}$ *A* which are characteristic of preparations obtained from normal chickens. This decrease in the A_{480}:A_{280} ratio which accompanies depletion of the manganese content of avimanganin provides support for the proposal that the presence of manganese is responsible for the absorption characteristics of avimanganin in the region 400–700 mμ (Figure 4).

Discussion

As noted above (section VI) the relationship between the A_{480}:A_{280} ratios observed for avimanganin purified from normal and manganese-deficient chickens provides definitive evidence linking the absorption bands at 480 mμ (ε 508 M^{-1} cm^{-1}) and 600 mμ (ε 250 M^{-1} cm^{-1}) to the presence of bound manganese in this protein. However the visible absorption spectrum observed for avimanganin differs in several respects from that observed for other manganese metalloproteins, *e.g.*, pyruvate carboxylase from avian liver (Scrutton *et al.*, 1966), Mn(III)-transferrin (Ulmer and Vallee, 1963), superoxide dismutase from *Escherichia coli* (Keele *et al.*, 1970). The visible absorption spectrum of Mn(III)-transferrin which consists of a single band at 429 mμ (ε 4000 M^{-1} cm^{-1}) (Ulmer and Vallee, 1963) has been attributed to charge transfer interaction with the tyrosine residues which may provide ligands to the metal ion in this protein (Warner and Weber, 1953). A more complex visible absorption spectrum is observed for superoxide dismutase from *E. coli* with bands at 370, 423, 560, and 600 mμ (Keele *et al.*, 1970). This spectrum differs markedly from that observed for Mn(III)-transferrin in the extinction coefficient (ε 200 M^{-1} cm^{-1}) observed for the major band at 423 mμ[2] and also from the spectrum observed for any model Mn(III) complex described thus far. It should be noted that proteins containing manganese as Mn(II), *e.g.*, pyruvate carboxylase purified from avian liver (Scrutton *et al.*, 1966),[3] exhibit no significant absorbance in the visible region of the spectrum, in accord with observations on Mn(II)-model complexes (Table VI).

In the case of avimanganin some preliminary conclusions

[2] Although Keele *et al.* (1970) state that superoxide dismutase from *E. coli* B exhibits an absorption maximum at 473 mμ it is apparent from examination of Figure 5 in this reference that this statement is incorrect and that the maximum is actually at 423 mμ.

[3] G. H. Reed and M. C. Scrutton, unpublished observations (1969).

regarding the valence and spin states of the bound manganese and the nature of the ligand field provided by the protein can be formulated by comparison of the observed visible absorption spectrum (Figure 4) with the spectral characteristics of various Mn(II) and Mn(III) complexes as summarized in Table VI. Since the model complexes of Mn(II) (in either tetrahedral, *e.g.*, $(MnBr_4)^{2-}$, or octahedral, *e.g.*, $Mn(H_2O)_6^{2+}$, coordination) and of low-spin Mn(III), *e.g.*, $Mn(CN)_6^{3-}$, show no strong absorption bands in the visible region of the spectrum (Table VI) it is improbable that manganese is present in any of these forms in avimanganin. In the case of model complexes of high-spin Mn(III) the absorption spectrum of avimanganin resembles most closely those described for trisbidentate model complexes, and differs significantly from those for the bisbidentate complexes, *e.g.*, diaquobisoxalatomanganese(III) (Table VI). An especially close resemblance is observed in the case of trisbidentate model complexes in which nitrogen atoms provide some of the ligands to the metal ion, *e.g.*, tris(diethyldithiocarbamato)manganese(III) (Table VI). Although these comparisons appear to indicate that avimanganin contains hexacoordinate high-spin Mn(III) with nitrogen atoms providing some of the ligands, certain observations may be inconsistent with this postulate. First, the absorption coefficient of the major band in the visible absorption spectrum of this protein ($\epsilon_{480\ m\mu}$ 508 M^{-1} cm^{-1}) is increased approximately twofold as compared with that observed for the model complexes (Table VI). Such an increase would be expected if the ligand field is somewhat asymmetric in the case of the protein-bound manganese. Second, and more seriously, avimanganin does not appear to exhibit the bands of low extinction coefficient (ϵ 50–150 M^{-1} cm^{-1}) in the near-infrared region of the spectrum which characterize the trisbidentate complexes of high-spin Mn(III) (Dingle, 1966). Since at the limit of solubility of avimanganin in dilute salt solutions ($\approx$30 mg/ml) the manganese concentration is approximately 1.1 mM, it appears possible that, in the case of the protein, the apparent failure to observe these predicted absorption bands may be due to limitations of solubility.

The proposed model is consistent with the data obtained on the effect of avimanganin on the relaxation rates of water protons under various conditions (Table IV and Figure 5)[4] and with the failure to observe an epr spectrum when concentrated solutions (0.6 mM Mn) are examined. However, these magnetic resonance data do not exclude other possibilities (*cf.* section V). This analysis of the properties of avimanganin which are related to the presence of the bound manganese has also failed to provide any indication of the biological role of this protein in chicken liver. If the bound Mn(III) of avimanganin participates in catalysis, as appears to be the case for the Mn(III) present in superoxide dismutase purified from *E. coli B* (Keele *et al.*, 1970), it should be possible to demonstrate interaction of the bound metal ion with both solvent and ligands since such accessibility is a primary requirement for catalytic involvement (Mildvan, 1970). However, the data presented here (Figures 4 and 5; Tables IV and VI) appear most consistent with the absence of such a direct interaction and preliminary assays for several enzymes which are known either to contain bound manganese (superoxide dismutase, pyruvate carboxylate) or to require addition of this metal ion for activity (oxalacetate decarboxylase, arginase) have failed to demonstrate significant levels of any of these activities in stage 3 preparations. A role for avimanganin in electron transport, which would not require direct interaction of the metal ion with solvent or ligands (Mildvan *et al.*, 1967), is not excluded by the data presented, but the failure of oxidizing or reducing agents to affect the properties of the bound Mn(III) lends no support to this postulate.

[4] The statement that "Mn(III) is not paramagnetic" (Keele *et al.*, 1970) is incorrect even for the low-spin state of this metal ion with the possible exception of complexes in which the metal ion exists in an extremely strong ligand field. Few, if any, examples of diamagnetic low-spin Mn(III) have been described.

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Properties of a Collagen Molecule Containing Three Identical Components Extracted from Bovine Articular Cartilage*

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ABSTRACT: Incubation of bovine articular cartilage with papain at 4° followed by several washes with 0.15 M NaCl removes most of the hexosamines, uronic acid, sialic acid, and non-collagen-bound hexoses. Subsequent extraction with 0.45 M NaCl (pH 7.0) causes 18–20% of the collagen to appear in solution. This collagen is still associated with significant amounts of glycosaminoglycans and can be considerably purified by successive reprecipitations. It behaves like native collagen as judged by its viscosity, optical rotation, and melting profile and when denatured gives rise to one single component, with electrophoretic and chromatographic properties similar to the α1 chain from calf skin collagen. It differs from this type of collagen primarily by its high content of hydroxylysine (28 residues/1000) and glycosidically bound carbohydrate (8–9 glucose and 13 galactose residues per α chain). The fact that no other type of collagen chain was observed indicates that articular cartilage is composed entirely of a triple stranded molecule of the (α1-Type II)$_3$ conformation, similar to that first observed by Miller and Matukas ((1969), *Proc. Nat. Acad. Sci. U. S. 64*, 1264) in the sternal cartilage of lathyritic chicks. Evidence is also presented which suggests that collagen in articular cartilage may have lesser amounts of covalent cross-links than bone or skin, and may be stabilized to a great extent by interactions with the proteoglycan components of the ground substance.

Articular cartilage is a unique tissue in both structure and function. It is almost completely avascular, with a low cell density, composed primarily by extracellular substances such as collagen, proteoglycans, and glycoproteins.

So far, its principal components, collagen and proteoglycans, have been difficult to study in their native form due to problems involved in their isolation. The conventional procedures used to extract collagen using hypertonic salt solutions or dilute organic acids do not yield any material, and more drastic conditions yield degraded or denatured products. Because of this problem most studies have involved other types of cartilage, even though articular cartilage is of primary significance because of its involvement in the pathogenesis of joint disease. Sadjera and Hascall (1969) obtained several preparations of proteoglycans from bovine nasal cartilage by disruptive and dissociative techniques and Rosenberg *et al.* (1970) compared the yield and composition of the fractions isolated by similar methods. However, none of these procedures enhanced a subsequent extractability of collagen from articular cartilage. Miller *et al.* (1969) were only able to extract about 1% of the total collagen present in normal human articular cartilage using 5 M guanidine hydrochloride. Subsequently, Miller and Matukas (1969) and Miller (1971) described a new type of α1 chain in the sternal cartilage of lathyritic chicks. These investigators suggested that cartilage contained two types of collagen: one similar to that present in skin and bone, and one unique to this tissue containing only α1 chains. The two species of collagen present in lathyritic chick xiphoid cartilage were recently separated by Trelstad *et al.* (1970) after introducing intramolecular cross-links with formaldehyde followed by chromatography on CM-cellulose.

The present experiments describe the isolation and the properties of a unique type of collagen from articular cartilage containing three identical α1 chains.

Materials and Methods

Extraction Procedure. Bovine articular cartilage from long bones was obtained from a slaughterhouse. Cartilage was removed, freed from adhering tissues, rinsed with saline, and frozen. Sliced samples (5 g) were digested without shaking with 30 ml of 0.1% papain (Calbiochem) in 0.02 M phosphate buffer containing 2×10^{-2} M cysteine and 3×10^{-3} M EDTA at 4° for 48 hr. The suspension was centrifuged at 30,000*g* for 30 min. The sediment was resuspended in 0.15 M NaCl (pH 7.0) and the enzyme inactivated with 1×10^{-3} M iodoacetic acid, centrifuged, and washed twice with a similar solution. The insoluble material was dispersed in 0.45 M NaCl (pH 7.0)

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