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Selenium as a Component of Glutathione Peroxidase Isolated from Ovine Erythrocytes†

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ABSTRACT: By a nine-step procedure, glutathione peroxidase (glutathione:H_2O_2 oxidoreductase, EC 1.11.1.9) was purified 4000-fold from ovine erythrocytes to homogeneity as determined by polyacrylamide gel electrophoresis and gel filtration chromatography. During purification the selenium concentration per unit of protein increased 3000-fold indicating that this enzyme accounted for about 75% of the total ovine erythrocyte selenium. The purified enzyme contained 0.34% selenium and the approximate molecular weight as determined by gel filtration and polyacrylamide gel electrophoresis was 88,000, giving a stoichiometry of 3.8 g-atoms of selenium/mol of glutathione peroxidase. This is considered to represent 4 g-atoms of selenium/mol of enzyme, presumably one selenium per enzyme subunit since sodium dodecyl sulfate gel electrophoresis suggested the presence of 4 subunits/mol of enzyme. Glutathione peroxidase is the first positively identified selenoenzyme of animal tissues.

Previous studies in our laboratory (Rotruck *et al.*, 1971) demonstrated that dietary selenite exerted a marked protective effect against oxidant damage to the membrane and hemoglobin of rat erythrocytes incubated *in vitro* in the presence of ascorbate or H_2O_2. This effect depended upon the presence of glucose in the incubation medium and was clearly distinguishable from the protective effects of vitamin E. The Se–glucose relationship was shown to involve the need for glucose metabolism in the continued regeneration of reduced glutathione (GSH) in the erythrocyte and a role for Se in the utilization of this GSH in protecting the cell against oxidant damage (Rotruck *et al.*, 1971, 1972). Because GSH protects the cell, at least in large part, by serving as the hydrogen donor in the glutathione peroxidase catalyzed reduction of H_2O_2 and various hydroperoxides (Mills and Randall, 1958; Clive and Obrien, 1968), we attempted to determine if Se is a necessary component of this enzyme. It was found that most of the ^{75}Se of rat erythrocytes labeled *in vivo* by injecting rats 2–4 weeks earlier with $Na^{75}SeO_3$ followed glutathione peroxidase activity through two highly effective purification steps, but we did not purify the rat enzyme to homogeneity (Rotruck *et al.*, 1973). In order to obtain a larger amount of glutathione peroxidase than was easily provided by rat blood, we selected ovine blood as a source material in our further attempts at

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purification of the enzyme. This paper describes these studies which resulted in a homogeneous preparation of ovine erythrocyte glutathione peroxidase containing approximately 4 g-atoms of Se/mol of enzyme. These studies were reported in a preliminary communication (Hoekstra *et al.*, 1973) and have recently been confirmed for crystalline bovine erythrocyte glutathione peroxidase by Flohe *et al.* (1973).

Experimental Section

Materials. Glutathione (reduced), 5,5′-dithiobis(2-nitrobenzoic acid) and 2,3-diaminonaphthalene were purchased from Aldrich Chemical Co. DEAE-Sephadex and Sephadex G-150 were purchased from Pharmacia Fine Chemicals. Hydroxylapatite was freshly made in our laboratory by the method of Tiselius *et al.* (1956). Disodium carbamate used in the protein determinations was purchased from Sigma Chemical Co.

Assay of Enzymic Activity. Glutathione peroxidase activity was assayed at 37° by a modification of Mills' (1959) procedure II. The incubation medium contained 0.04 M sodium phosphate (pH 7.0), 0.08 mM EDTA, 1.0 mM sodium azide, 0.4 mM GSH, and 0.25 mM H_2O_2. An enzyme unit was defined as a decrease in concentration of GSH of 0.001 log unit/min, after the nonenzymic reaction rate was subtracted. Protein was determined by the microbiuret method of Westley and Lambeth (1960) and specific activity of the enzyme was expressed as number of enzyme units (EU) per milligram of protein. Protein estimation by this method has advantages of simplicity and reliability regardless of the particular protein analyzed, but it is necessary to minimize the amount of salts present in the enzyme solution. All samples except the original hemolysate were dialyzed against distilled water for 48 hr before protein determination.

Quantitative Analysis of Selenium. Selenium was determined by a fluorometric microanalysis procedure modified from the method of Hoffman and Westerby (1968). Samples were predigested with nitric acid overnight at room temperature before applying the wet digestion procedure with sulfuric acid and perchloric acid as described. The digest mixed with 2,3-diaminonaphthalene and 0.02 M EDTA was adjusted to pH 2.0 by adding 40% NH_4OH solution and was incubated at 60° for 45 min. After the extraction of the Se–2,3-diaminonaphthalene complex with pure cyclohexane, the fluorescence was measured with a Turner Model 110 photofluorometer fitted with a no. 7-60 (365 mμ) primary filter and a no. 58 (525 mμ) secondary filter.

Purification of Glutathione Peroxidase. Ovine blood (2.5 l.) was collected from the jugular vessels of a freshly killed 1-year-old sheep. The blood was prevented from clotting by adding 5 g of crystalline sodium citrate/l. of blood. The citrated blood was centrifuged for 15 min at 3000*g*, the plasma was discarded, and the packed cells were washed twice with isotonic saline sodium phosphate buffer (0.05 M PO_4, pH 7.4) and centrifuged at 3000*g* for 20 min. The collected red blood cells were hemolyzed by adding cold distilled water to make the same volume as the initial whole blood.

STEP 1. SOLVENT DENATURATION. The hemoglobin in the hemolysate was denatured by a method modified from that described by Keilin and Mann (1940). For each 100 ml of hemolysate (4°), 82 ml of 95% ethanol (−30°) was added while stirring followed by slow addition over a 2-min period of 35 ml of CCl_4 (25°). As soon as the bulk of the precipitate was formed, 3 l. of distilled water/l. of hemolysate was added and the bulk of the precipitate was removed by filtering through cheesecloth. The remaining precipitate and excess CCl_4 were removed by centrifugation at 2000*g* for 15 min and decanting the supernatant.

STEP 2. DEAE-SEPHADEX (A-50) CHROMATOGRAPHY. The supernatant from step 1 was applied to columns (7 × 50 cm) packed with DEAE-Sephadex (A-50) gel equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) and was eluted stepwise with two concentrations of pH 7.0 phosphate buffer (0.02 M, then 0.10 M). The enzyme activity was found in the 0.10 M phosphate buffer eluate.

STEP 3. AMMONIUM SULFATE PRECIPITATION. The eluate from step 3 was adjusted to give a protein concentration of about 1.8 mg/ml by using 0.10 M sodium phosphate buffer (pH 7.0), and crystalline ammonium sulfate was added to the solution to a concentration of 65% saturation which precipitated the enzyme. After standing overnight at 4°, the precipitate and supernatant were transferred into funnels containing Whatman No. 50 filter paper. The funnel outlets were connected to Tygon tubing closed with screw clamps. After an additional 10 hr at 4°, the screw clamps were slowly opened to keep the precipitate undisturbed during filtration. The precipitated protein which collected on filter paper was dissolved in 0.02 M phosphate buffer (pH 7.0).

STEP 4. SEPHADEX G-150 CHROMATOGRAPHY. The protein solution from step 3 was concentrated to a small volume by ultrafiltration (Amicon Model 202, PM 10) and applied onto columns (2.8 × 90 cm) of Sephadex G-150 which were then eluted with 0.02 M sodium phosphate buffer (pH 7.0) at a rate of 0.4 ml/min. The eluate was monitored for glutathione peroxidase activity and the active fractions representing a single peak were pooled.

STEP 5. HYDROXYLAPATITE CHROMATOGRAPHY. Freshly prepared hydroxylapatite gel was packed into a column (5 × 20 cm). The active fraction from step 4 was applied and eluted stepwise with phosphate buffer (0.05 M, 0.15 M, pH 6.8) at 4°. The enzymatically active fraction eluted in 0.15 M phosphate buffer was concentrated by ultrafiltration for the next step.

STEP 6. SEPHADEX G-150 CHROMATOGRAPHY. This step was repeated as described in step 4 on the active fractions from the hydroxylapatite column.

STEPS 7 AND 8. DEAE-SEPHADEX (A-50) CHROMATOGRAPHY. The active fraction from step 6 was applied to a 4 × 20 cm column of DEAE-Sephadex (A-50) previously equilibrated with 0.01 M phosphate buffer (pH 7.0) and was eluted with 2 l. of phosphate buffer (pH 7.0) in a linear gradient from 0.02 to 0.20 M. The combined fractions containing enzyme activity were diluted with deionized water to give an approximate phosphate concentration of 0.02 M and rechromatographed through a column prepared in the same way.

STEP 9. SEPHADEX G-150 CHROMATOGRAPHY. Further purification of the enzyme was attempted by this column as described in step 4 but elution was with 0.05 M phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min. The fractions containing glutathione peroxidase were pooled and mixed with the same amount of 48% ethanol for longer storage.

All steps of purification except the solvent denaturation step were carried out in the cold room at 4°. The enzymatically active fractions obtained at each step were analyzed for Se, protein, and glutathione peroxidase activity. Column eluates were monitored for protein by absorbance at 280 nm, glutathione peroxidase activity and Se content.

Results and Discussion

Purification of Ovine Erythrocyte Glutathione Peroxidase. Data obtained from the analysis of the pooled enzymatically

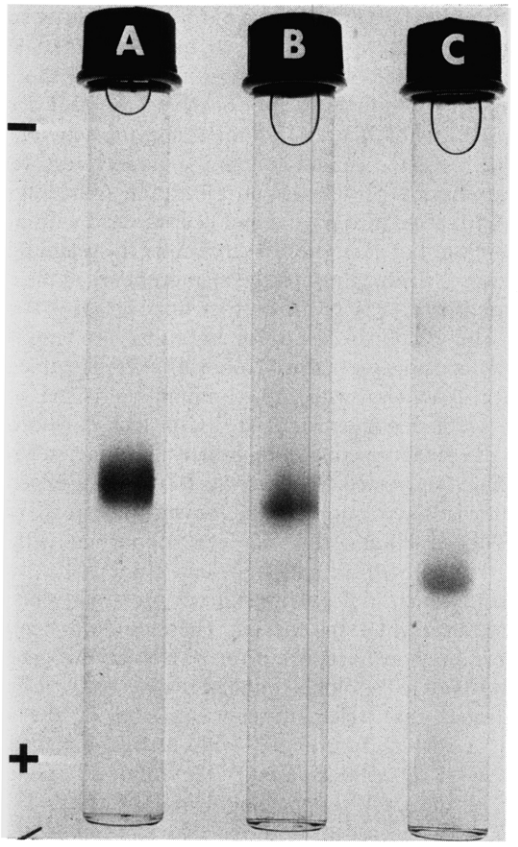

FIGURE 1: Polyacrylamide disc gel (7%) electrophoresis pattern of glutathione peroxidase purified from ovine erythrocytes. Electrophoresis was at a low temperature (5°) with Tris buffer (pH 8.3) and the gel was stained with Coomassie Brilliant Blue followed by destaining with 25% methanol and 10% acetic acid solution: gel A, 100 μg of protein; gel B, 50 μg of protein; gel C, 50 μg of protein pretreated with 8 M urea, 1% sodium dodecyl sulfate, and 0.1% dithiothreitol (DTT); gel C contained 8 M urea and 0.1% sodium dodecyl sulfate.

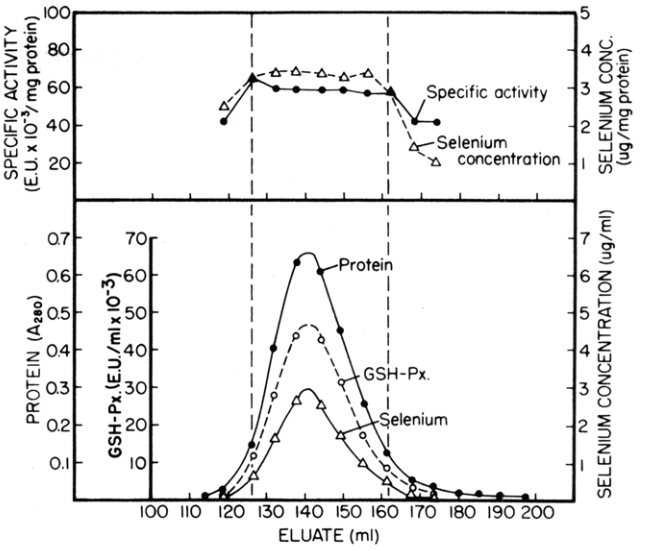

FIGURE 2: Sephadex G-150 chromatography of glutathione peroxidase (GSH–Px) from ovine erythrocytes (final purification step). Enzymatically active fractions obtained from DEAE-Sephadex column (step 8) were chromatographed with 0.05 M sodium phosphate buffer (pH 7.0) at a rate of 0.5 ml/min. Specific enzymatic activity and selenium concentration in the fractions are shown at the top. Protein was estimated from absorbance at 280 nm.

active fractions following each purification step are summarized in Table I. The enzyme was purified 4000-fold with an overall yield of 23.8% of the enzyme activity of the initial hemolysate. Two of the steps (1 and 4) resulted in an activation, or removal of inhibition, of the enzyme so that the actual yield is probably somewhat less than that indicated. Particularly effective purification steps were the solvent denaturation and ammonium sulfate precipitation steps, but it was necessary in these steps to carefully follow the conditions described to prevent extensive loss of enzymatic activity. The final preparation of glutathione peroxidase appeared to be homogeneous both by disc gel electrophoresis whereby a single, but somewhat diffuse, band was observed (Figure 1) and by Sephadex G-150 chromatography (final purification step) in which a close correspondence between protein, selenium, and enzyme activity was seen in the elution profile (Figure 2). In addition, no substantial increase of specific activity occurred in the last purification step and the absence of any appreciable absorption peak at 400–500 nm indicated lack of substantial contamination from heme proteins.

Selenium Content of Glutathione Peroxidase. Flohe *et al.* (1971) reported earlier that glutathione peroxidase prepared

TABLE I: Purification of Ovine Erythrocyte Glutathione Peroxidase (GSH-Px). Data are for the Pooled Enzymatically Active Fractions Following Each Purification Step.[a]

Purifcn Step	Total Protein (mg)	Total GSH–Px Act. EU, 37° (× 10⁶)	Enzyme Yield (%)	Sp Act. (EU/mg of Protein)	-fold Purified	Total Se (μg)	μg of Se/ g of Protein
Hemolysate	400,000	6.00	100.0	15	1	440	1.1
1. Solvent denaturation (EtOH + CCl_4)	15,200	7.00	116.7	460	31	380	25
2. DEAE-Sephadex (A-50) column	5,500	3.60	60.0	654	44	358	65
3. Ammonium sulfate (65%)	960	3.40	56.7	3,540	236	300	312
4. Sephadex G-150 column	550	2.50	41.7	4,540	303	209	380
5. Hydroxylapatite column	410	3.25	54.1	7,930	528	189	460
6. Sephadex G-150 column	108	2.10	35.0	19,600	1300	114	1060
7. DEAE-Sephadex (A-50) column	42.0	2.00	33.3	47,600	3180	95.6	2280
8. DEAE-Sephadex (A-50) column	28.5	1.70	28.3	59,600	3980	92.5	3250
9. Sephadex G-150 column	23.8	1.43	23.8	60,100	4000	81.0	3400

[a] See text under Experimental Section for description of methods and definition of enzyme unit.

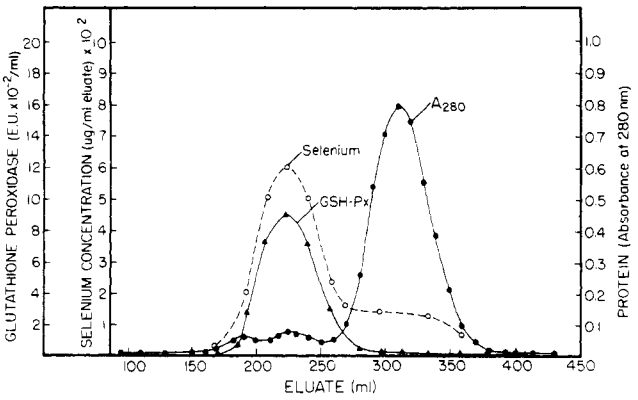

FIGURE 3: Elution pattern of glutathione peroxidase activity (GSH-Px), selenium, and protein from the Sephadex G-150 column during the sixth step in the purification.

from bovine erythrocytes contains no non-protein prosthetic group, but this conclusion was based only on spectrophotometric observations. In the present study, the selenium concentration per unit of protein increased dramatically during enzyme purification and a 3000-fold increase in Se concentration was achieved in comparing the initial hemolysate with the purified enzyme. This finding implies that a large proportion, probably about 75%, of the ovine erythrocyte Se is present in glutathione peroxidase. Such a conclusion is also supported by our finding of much lower amounts of Se in other protein peaks observed during chromatography as is exemplified by the 6th step of the enzyme purification shown in Figure 3. The final preparation of glutathione peroxidase contained 0.34% selenium. The molecular weight of native ovine erythrocyte glutathione peroxidase was 89,000 as evaluated by its elution volume from Sephadex G-150 in comparison to those for γ-globulin, bovine serum albumin, ovalbumin, and cytochrome *c* as reference standards with molecular weights of 160,000, 69,000, 42,000, and 13,000, respectively (log molecular weight was linearly related to the ratio of elution volume to void volume). Sodium dodecyl sulfate gel electrophoresis of the purified enzyme after treatment with 8 M urea, 1% sodium dodecyl sulfate, and 0.1% dithiothreitol in comparison to reference standards of catalase, ovalbumin, chymotrypsinogen A, and cytochrome *c* with subunit molecular weights of 60,000, 42,000, 25,000, and 13,000, respectively, gave a molecular weight of 22,000 for the enzyme subunit (migration distance on electrophoresis was linearly related to log subunit molecular weight). The subunit molecular weight when multiplied by 4 gives what we consider to be the best estimate of the molecular weight of the native enzyme of 88,000.

On this basis, our purified enzyme contained 3.8 g-atoms of Se/mol of enzyme. Allowing for a small degree of impurity and inaccuracies in methodology, we consider this to represent a stoichiometry of 4 g-atoms of Se/mol of enzyme. This stoichiometry has recently been confirmed for crystalline bovine glutathione peroxidase by neutron activation analysis for Se (Flohe *et al.*, 1973) and probably represents one Se in each enzyme subunit, although this remains to be proven.

Some Additional Properties of the Enzyme. The purified enzyme was rather stable if stored in cold 24% ethanol at neutral pH. The storage of glutathione peroxidase at −14° in 24% ethanol and 0.05 M phosphate buffer (pH 7.0) for 30 days allowed recovery of 82% of original enzyme activity. Selenium bound in the enzyme was not dialyzable at neutral pH against water (4°) for 48 hr and was not removed from protein after heat denaturation at 65° for 2 hr or after standing at room temperature (25°) for 48 hr. More than 90% of original enzyme activity was recovered following the dialysis or storage at room temperature; however, the heat denaturation caused loss of 70% of the initial enzyme activity. A low molecular weight selenium-containing moiety was removed from the enzyme protein by ultrafiltration (Amicon Model 202, PM 10) after the enzyme had been treated with a strong acid or a base. For example, about 50% of the selenium in the enzyme was filterable through the membrane when the enzyme was treated with 40% NH_4OH (to a final pH of 9.4) or with 1 N HCl (to a final pH of 1.6) for 24 hr at room temperature. The selenium moiety obtained following these treatments was tested for reactivity with 2,3-diaminonaphthalene with or without a reducing agent (0.01 M $FeCl_2$); lack of fluorescence showed the selenium-containing moiety to be neither selenite nor selenate. The results of two isoelectric focusing experiments showed the isoelectric point of the enzyme to be in the range of 6–6.5. Polyacrylamide disc gel electrophoresis with 0.1% sodium dodecyl sulfate and 8 M urea (Figure 1) showed a single band which indicates that in all probability no hetero subunits are present in the enzyme. These results are consistent with those previously reported for bovine erythrocyte glutathione peroxidase by Flohe *et al.* (1971). Recently, it has been demonstrated that selenium concentration in the culture medium is directly correlated with formic dehydrogenase activity in *Escherichia coli* and ^{75}Se followed the enzyme through partial purification (Shum and Murphy, 1972). In addition, it has been demonstrated that a protein component of the glycine reductase system of *Clostridium stricklandii* is a selenoprotein (Turner and Stadtman, 1973). These findings imply that selenium is an integral part of these microbial enzymes as well as animal glutathione peroxidase; however, in all three cases the nature of the selenium moiety in these proteins remains obscure and further studies are required. The identification of glutathione peroxidase as a selenoenzyme explains, at least in part, why selenium is necessary for the normal growth and health of animals. The detailed function of selenium in the catalytic process is unknown at the present time, but most likely it carries out a redox or electron-transfering role.

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Affirmation of Critical Proton Magnetic Resonance Data on the Solution Conformation of the Valinomycin–Potassium Ion Complex†

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ABSTRACT: Two hundred and twenty MHz proton magnetic resonance spectra are presented for the valinomycin–potassium ion complex in methanol-d_4 which demonstrate the valyl α-CH–β-CH coupling constants to be 11.0 ± 0.3 Hz. Data reported at 100 MHz are consistent with the 220-MHz data, but the $^3J_{\alpha CH-\beta CH}$ values of 3.5–4.0 Hz, interpreted from the 100-MHz data, are incorrect. Accordingly, proposed conformations which are based on the valyl α-CH–β-CH coupling constants of 3.5–4.0 Hz require revision. Data are presented which show the 11-Hz coupling constant to be maintained over the temperature range from −35 to 74°. This demonstrates the conformation of the complex to be very stable, but more importantly, the results require a conformation in which the α-CH–β-CH dihedral angle of each valyl residue is constrained in a predominantly trans orientation. The proposal of such a conformation is already in the literature.

The solution conformation of the valinomycin–potassium ion complex, (L-Lac-L-Val-D-OHVal-D-Val)$_3$–K^+, has generated a great deal of interest over the last several years. The result is a general consensus on the secondary structure, yet important details required for a complete solution description remain in question.

Starting with a secondary structure of a series of six β turns in which all of the peptide NH protons are hydrogen bonded, the α-CH–β-CH dihedral angle of the valyl residues have been presented as a criterion for delineating between solution conformations (Mayers and Urry, 1972). The vicinal coupling constant, $^3J_{\alpha CH-\beta CH}$, was reported by Ivanov *et al.* (1969) and by Patel and Tonelli (1973) to be 3.5–4.0 Hz whereas a value of 11 Hz was reported by Ohnishi and Urry (1970) and Urry and Ohnishi (1970). This is the difference between predominantly gauche and predominantly trans orientations of the valyl α-CH–β-CH bonds. As indicated by Patel and Tonelli (1973) the observation of a small coupling constant would reverse the choice of conformation made by Mayers and Urry (1972). The point of basic concern is, of course, the correct magnitude of the coupling constant.

Fundamental to the correct interpretation of the experimental data in CD_3OD is the question of whether the valyl α-CH doublets in the proton magnetic resonance spectra are separate or overlapping. The results of Ivanov *et al.* (1969) and of Patel and Tonelli (1973) were at 100 MHz and they took the doublets to be nonoverlapping, whereas those of Ohnishi and Urry (1970) and Urry and Ohnishi (1970) were obtained at 220 MHz and they took the doublets to be overlapping. However, none of the workers published the spectra; each reported only the interpreted value of J.

Experimental Section

Spectra were recorded on a Varian Associates HR-220 spectrometer. Chemical shifts were measured relative to an internal standard of tetramethylsilane. The spectra were carefully calibrated by introducing side bands which were generated by modulation of the tetramethylsilane reference. The probe temperature was measured by determining chemical shift differences between resonances of ethylene glycol or of methanol.

The valinomycin–potassium ion complex was formed by dissolving valinomycin (Calbiochem, San Diego, Calif., Lot No. 010172 and 860031) and a 10% molar excess of KBr in a sample tube using a CD_3OD–H_2O (9:1 v/v) solvent mixture. The sample was then dried under high vacuum and twice dissolved in CD_3OD and redried under high vacuum. This assures complete exchange of the peptide NH proton. The complex was redissolved in CD_3OD and the sample tube was sealed. The same procedure was followed when using KSCN.

Results and Discussion

Magnetic resonance spectra for the α-proton region of the valinomycin–potassium ion complex formed with KBr and KSCN salts are given in Figures 1 and 2. Bracketing the spectra on the left and on the right are modulation side bands precisely positioning the indicated frequencies. In both figures the α-CH proton of the L-lactic acid residue is near 1100 Hz; the α-CH of the D-hydroxyisovaleric acid residue is near 1030 Hz, and near 850 Hz are the α-CH resonances of the L- and D-valine residues. At 74° the solvent peak is near 950 Hz whereas at 3 and 6° it overlaps with the α-CH of the L-lactic acid residue. That the chemical shifts are so similar over a 70° range of temperature (compare Figures 1 and 2) is indicative of a stable complex. This is to be contrasted with the dramatic changes in chemical shift observed in the absence

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