sugars may have also contained dolichol-11.

The question concerning the origin of dolichol-11 in pig liver arises in view of the fact that the proportion of three internal trans isoprene residues in this molecule is rather typical for plant undecaprenol (Feeney and Hemming, 1967). As revealed by reversed phase thin-layer chromatography, the polyprenols of pig liver form two different families (group A and group B, Figure 1). The more abundant are the longer chain dolichols ranging from C_{80} to C_{110} and possessing two internal trans isoprene residues (cf. also Feeney and Hemming, 1967). The components of group B, in which dolichol-11 is present, occur in lower amounts. One can assume that dolichol-11 may arise from plant polyprenols present in the diet, though direct evidence is lacking on the possibility of its adsorption from the intestine and specific enzymic reduction of its OH-terminal isoprene residue.

Using the procedure of selective hydrogenation of undecaprenol, we recently performed the synthesis of dihydrosolanesol with saturated OH-terminal isoprene residue from *all-trans*-(C_{45})solanesol. This shows that the method presented here for selective hydrogenation is convenient with respect to both cis/trans- and all-trans polyprenols and also is irrespective of the configuration of the OH-terminal isoprene unit.

Acknowledgments

We thank Professor Gustav Dallner of the University of Stockholm for a sample of Lipidex-5000 and Mrs. Josephine Hertel for her skilled technical assistance.

References

Barr, R. M., and Hemming, F. W. (1972), *Biochem. J. 126,* 1193-1202.

Burgos, J., Hemming, F. W., Pennock, J. F., and Morton, R. A. (1963), *Biochem. J. 88,* 470-482

Chojnacki, T., Jankowski, W., Mańkowski, T., and Sasak, W. (1975), *Anal. Biochem. 69,* 114-119.

Dunphy, P. J., Kerr, J. D., Whittle, K. J., and Pennock, J. F. (1966), *Chem. Ind. (London)* 1549-1550.

Dunphy, P. J., Kerr, J. D., Pennock, J. F., Whittle, K. J., and Feeney, J. (1967), *Biochim. Biophys. Acta 136,* 136-147.

Feeney, J., and Hemming, F. W. (1967), *Anal. Biochem. 20,* 1-15.

Gajda, A., Jankowski, W., Mańkowski, T., and Chojnacki, T. (1974), Abstracts, 7th International Symposium on Carbohydrate Chemistry, Bratislava, Czechoslovakia, p 233.

Gough, D. P., and Hemming, F. W. (1970), *Biochem. J. 118,* 163-166.

Gough, D. P., Kirby, A. L., Richards, J. B., and Hemming, F. W. (1970), *Biochem. J. 118,* 167-170.

Hemming, F. W. (1974), *MTP Int. Rev. Sci. Biochem., Ser. One 4,* 39-98.

Sasak, W., and Chojnacki, T. (1973), *Acta Biochim. Pol. 20,* 343-350.

Stone, K. J., Butterworth, P. W. H., and Hemming, F. W. (1967a), *Biochem. J. 102,* 443-455.

Stone, K. J., Wellburn, A. R., Hemming, F. W., and Pennock, J. F. (1967b), *Biochem. J. 102,* 325-330.

Walton, M. J., and Pennock, J. F. (1972), *Biochem. J. 127,* 471-479.

Isolation of an Inhibitor of 25-Hydroxyvitamin D_3-1-Hydroxylase from Rat Serum†

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ABSTRACT: An inhibitor of chick kidney mitochondrial 25-hydroxyvitamin D_3-1-hydroxylase has been isolated from rat serum by ammonium sulfate precipitation, gel filtration, ion-exchange chromatography, and preparative polyacrylamide disc gel electrophoresis. The purified protein was shown to contain iron and has a mol wt of 52 000. The protein is indistinguishable on gel electrophoresis from a similar inhibitor found in rat kidney tissue. The physiological significance of the inhibitor is not known; however, it seems possible that it is responsible for the failure to demonstrate in vitro 25-hydroxyvitamin D_3-1-hydroxylation with rat and other mammalian tissues.

1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$),[1] the most active metabolite of vitamin D_3 known in both induction of intestinal calcium transport and bone mobilization (Holick et al., 1971a; Haussler et al., 1971; Omdahl et al., 1971), is synthesized from 25-hydroxyvitamin D_3 (25-OH-D_3) in vivo in the kidney of vitamin D-deficient chicks and rats (Holick et al., 1971a, b; Fraser and Kodicek, 1970; Gray et al., 1971).

Hydroxylation of 25-OH-D_3 in the 1 position has been demonstrated in vitro using kidney tubules, homogenates, and isolated mitochondria from vitamin D-deficient chicks (Shain, 1972; Fraser and Kodicek, 1970; Gray et al., 1972; Norman et al., 1971). However, although experiments with nephrectomized animals have shown that the kidney is the site of 1,25-$(OH)_2D_3$ formation in the rat in vivo (Gray et al., 1971), it has not yet been possible to demonstrate this reaction with rat kidney tubules, homogenates, or mitochondria in vitro.

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[1] Abbreviations used are: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; EDTA, ethylenediaminetetraacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]-benzene; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

The presence of a factor in rat kidney homogenates which inhibits in vitro 1-hydroxylation of 25-OH-D_3 by chick kidney preparations has been reported previously (Botham et al., 1974). A similar inhibitor was found in some other rat tissues and to a much less extent in chick tissue, the most potent sources being rat serum and intestinal homogenates.

This paper describes the isolation of the inhibitory factor and some of its properties.

Materials and Methods

Animals. One-day-old white Leghorn cockerels (Northern Hatcheries, Beaver Dam, Wis.) were maintained on a vitamin D-deficient purified diet (Omdahl et al., 1971) for 4–6 weeks before use.

Male albino rats (Holtzman Co., Madison, Wis.) were housed in hanging wire cages and fed a vitamin D-deficient diet containing 0.47% calcium and 0.3% phosphorus (Suda et al., 1970). Rats were allowed food and water ad libitum for 5–8 weeks before being used in experiments.

Animals were killed by decapitation and blood was collected and centrifuged at 300*g* for 10 min to obtain serum. Kidneys were removed immediately after death and placed in ice-cold buffered sucrose (0.32 M sucrose, 0.05 M Tris-acetate, pH 7.4).

Radioactive 25-OH-D_3. 25-OH-[26,27-^{3}H]D_3 (1.2 and 9.2 Ci/mmol) was synthesized in this laboratory by the method of Suda et al. (1970). The radioactive material was purified periodically by chromatography on a Sephadex LH-20 column (1 × 60 cm) eluted with 50:50 chloroform–Skellysolve B (Holick and DeLuca, 1971).

Incubation Conditions. Chick kidney mitochondria were prepared as described by Knutson and DeLuca (1974). The final pellet was resuspended in buffered sucrose solution to give a concentration of approximately 16 mg of protein/ml. This preparation (0.5 ml) was incubated with 1 ml of cofactor solution (5 mM succinate, 1.87 mM $MgCl_2$: Omdahl et al., 1972), 1 ml of rat protein preparation to be tested for inhibition in 0.05 M Tris-acetate buffer (pH 7.4) (except where noted), and 10 ng of 25-OH-[26,27-^{3}H]D_3 (1.2 Ci/mmol) in 10 μl of 95% ethanol. The total volume in each flask was 2.5 ml. In control flasks, 1 ml of buffer replaced the rat protein preparation. Incubations were carried out for 15 min at 37 °C in the presence of 100% oxygen with continuous agitation of 80 oscillations/min with 1-in. stroke length. Reactions were stopped with 15 ml of 2:1 methanol–chloroform.

Extraction. The one-phase system obtained with 2:1 methanol–chloroform addition was allowed to stand at room temperature for 1 h after which time 5 ml of chloroform and 4 ml of water were added to produce two phases. After separation, the chloroform phase was taken to dryness on a rotary evaporator in the presence of a small amount of ethanol, added to remove residual water. The residue was dissolved in chloroform and a small aliquot taken for radioactive determination. Ninety to one-hundred percent recovery of the tritium added to the incubation flask was obtained.

Thin-Layer Chromatography. Further aliquots of the chloroform solution were applied to thin-layer chromatograms (silica gel G 100 μm thick 2 × 20 cm, Eastman Kodak, Rochester, N.Y.), and developed in 95% chloroform–glacial acetic acid (Gray et al., 1971). The chromatogram was then dried, cut into 1-cm strips (16), and eluted with chloroform. After 1 h, the strips were removed and the chloroform was evaporated under a stream of air.

The residue was dissolved in 4 ml of toluene counting solution (Bhattacharyya and DeLuca, 1973). Samples were assayed for radioactivity in a Nuclear-Chicago Model 6868 Isocap liquid scintillation counter. Efficiency of counting was 48–50%. Recovery of radioactivity from chromatograms was 75–90% and the presence of lipids in the extracts did not alter the migration of either 25-OH-D_3 or 1,25-$(OH)_2D_3$ on the thin-layer chromatographic strips.

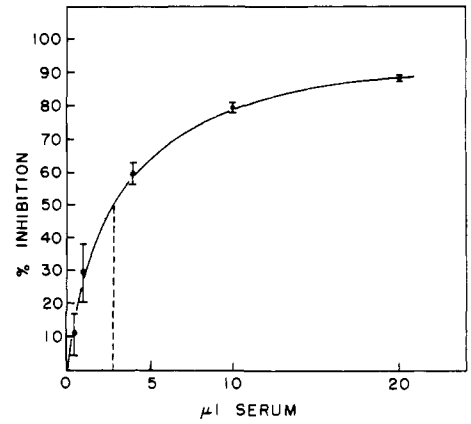

FIGURE 1: Standard curve for assay of inhibitor. Flasks were incubated as described in the text. Bars represent standard deviation of five determinations.

The relative position of the metabolites was determined in separate experiments by chromatography of the pure compounds. 1,25-$(OH)_2D_3$ and 25-OH-D_3 have R_f values of 0.3 and 0.75, respectively. Values for radioactivity recovered as 1,25-$(OH)_2D_3$ and 25-OH-D_3 from thin-layer chromatograms agreed (within 5%) with those obtained by the well-established system of column chromatography on Sephadex LH-20 (Holick and DeLuca, 1971).

Using the assay system described above, a standard curve of percent inhibition vs. milliliters of serum protein added to the incubation was plotted (Figure 1). One unit of inhibition was defined as that amount of protein required to produce 50% inhibition of the chick kidney 25-OH-D_3-1-hydroxylase. The curve was found to be reproducible and was used to quantitate the inhibitor in the isolation procedure.

Sephadex LH-20 Chromatography. For some experiments (Figure 7), the lipid extract from each incubation mixture was chromatographed on a 20-g Sephadex LH-20 column (2 × 30 cm) equilibrated with 65:35 chloroform–Skellysolve B. The column was eluted with the same solvent and 40 10-ml samples were collected into counting vials. The solvent was dried in a stream of air and the residue redissolved in 5 ml of toluene counting solution (Bhattacharyya and DeLuca, 1973). Radioactivity was counted in a Beckman LS-100 C liquid scintillation spectrometer. The relative elution positions of 25-OH-D_3 and 1,25-$(OH)_2D_3$ in this chromatographic system have been previously determined (Holick and DeLuca, 1971).

Ammonium Sulfate Precipitation. Solid ammonium sulfate was added to rat serum with continuous stirring to the appropriate percent saturation (Di Jeso, 1968). The precipitates were collected by centrifugation at 20 000*g* for 15 min. Pellets were dissolved in 0.05 M Tris-acetate buffer (pH 7.4) and supernatants were further treated with ammonium sulfate to the required saturation point.

Column Chromatography. Gel filtration was carried out on 2.6 × 60 cm columns of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) and equilibrated with 0.05 M Tris-acetate buffer (pH 7.4). Proteins were eluted with the same buffer and 100 3-ml fractions were collected.

Ion-exchange chromatography was performed on 2.4 × 45 cm columns of DEAE-Sephadex A-50 (Pharmacia Fine

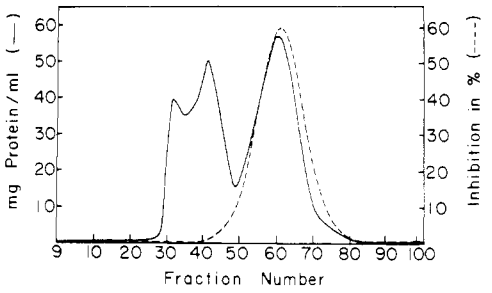

FIGURE 2: Gel filtration on Sephadex G-200 of protein fraction from rat serum precipitating between 30 and 60% saturation with ammonium sulfate. Fractions containing 2.8 ml were collected.

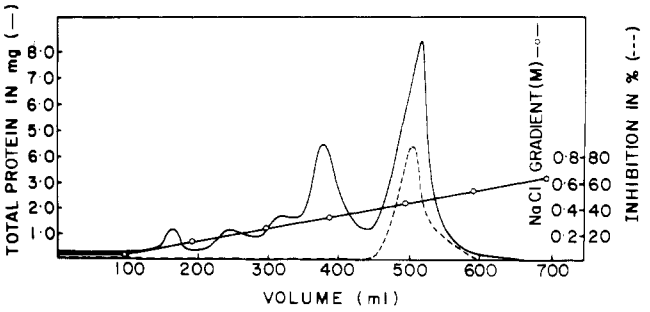

FIGURE 3: Ion-exchange chromatography on DEAE-Sephadex A-50 of inhibitor band eluted from the Sephadex G-200 column (Figure 2). Four-milliliter fractions were collected.

Chemicals) equilibrated with 0.05 M Tris-acetate buffer (pH 7.4). A continuous salt gradient of 0–1 M sodium chloride was used.

Dilute protein solutions were concentrated by ultrafiltration under 40 psi of nitrogen in an Amicon (Lexington, Mass.) ultrafiltration cell with a UM2 membrane.

Electrophoresis. Analytical disc gel electrophoresis was carried out on 5% polyacrylamide gels as described by Davis (1962). Migration of the inhibitor was not affected by the presence of 25-OH-D_3. Protein was recovered from these gels by slicing into pieces of appropriate length, crushing in a tissue press, and eluting with 5 ml of 0.05 Tris-acetate buffer (pH 7.4) for 2 h. The crushed polyacrylamide was then filtered off, and the filtrate was adjusted to pH 7.4 with 1 N hydrochloric acid. This solution could then be tested for inhibition in the assay described above.

Gels were stained for protein with 0.025% Coomassie Brilliant Blue R (St. Groth et al., 1963). Sudan Black B was used to test for the presence of lipoprotein (Prat et al., 1969). Iron-containing proteins were detected by their color reaction with benzidine hydrochloride (Ornstein, 1963) and staining for proteins containing carbohydrate was performed using Schiff reagent-periodic acid (Zacharius et al., 1969) and Toluidine Blue O (Maurer, 1971).

Sodium dodecyl sulfate treated gels were used to determine molecular weights by the method of Weber et al. (1972).

Preparative electrophoresis was run at 4 °C using the Canalco Prep-disc apparatus (Canalco, Rockville, Md.). The gel system was as described for analytical electrophoresis. The Tris-acetate (0.05 M) at pH 7.4 was the eluting buffer at a flow rate of approximately 0.5 ml/min.

Sucrose Density Gradients. Linear 10–30% sucrose gradients (3.55 ml) were prepared by layering 0.71 ml each of 30, 25, 20, 15, and 10% sucrose in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, buffer into 4-ml polyallomer tubes. At least 6 h at 4 °C was allowed for diffusion of the layers.

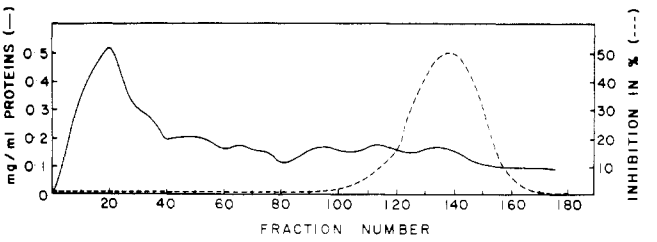

FIGURE 4: Preparative disc gel electrophoresis profile obtained by applying the inhibitor band eluted from ion-exchange chromatography (Figure 3) to a polyacrylamide gel prepared as described in the text. Flow rate of the elution buffer was approximately 0.5 ml/min. Four-milliliter fractions were collected.

Binding Experiment. Purified rat inhibitor protein was diluted to an appropriate concentration with 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 buffer. Aliquots (0.5 ml) of diluted protein were added to glass tubes containing 25-OH-[^{3}H]D_3 (9.2 Ci/mmol) in 0.015 ml of 95% ethanol. The contents of the tubes were mixed thoroughly and placed in a shaking water bath maintained at 37 °C. After 15 min, the tubes were placed on ice and 0.2 ml of each incubation mixture was layered onto the top of a 10–30% linear sucrose gradient. The gradients were contained in an SW60 rotor and centrifuged in a Beckman L5-50 ultracentrifuge at 257 000*g* for 18 h at 4 °C. The gradients were fractionated from the bottom by displacement with mineral oil dispensed from a 5-ml Hamilton syringe (Jensen et al., 1968). Fractions (0.1 ml) were collected in counting vials and radioactivity was determined in 5 ml of scintillation fluid containing 1:3 Triton X-100:toluene, PPO, Me_2POPOP, and sufficient water to effect clarification. Radioactivity remaining in the oil layer and the gradient tube was also determined. Samples were counted in a Beckman LS-100C liquid scintillation spectrometer at 36% efficiency.

Other Methods. Protein was determined as described by Lowry et al. (1951). The side-chain cleavage enzyme of cholesterol was prepared from bovine adrenal cortex and incubations were carried out as described by Simpson and Boyd (1967).

Results

Isolation of Inhibitor from Rat Serum. Preliminary experiments in which proteins were separated by analytical polyacrylamide gel electrophoresis then eluted and tested in the chick kidney 25-OH-D_3-1-hydroxylase assay showed that in both rat serum and kidney preparations inhibition was associated with a protein having α-globulin-like mobility. In the isolation procedure, rat serum was used as it was found to be the most convenient source of the inhibitor. This inhibitor is heat labile (Botham et al., 1974) and is destroyed by trypsin, demonstrating that it is at least in part a protein.

Preliminary experiments revealed that 80% of the inhibitor was associated with proteins precipitating between 30 and 60% salt saturation. This fractionation procedure was found to give the maximum recovery of inhibitor with the maximum removal of unwanted protein. The 30–60% ammonium sulfate precipitate was dissolved in 5 ml of Tris-acetate buffer (pH 7.4) and chromatographed on a Sephadex G-200 column previously equilibrated with this buffer. The protein profile from this column showed three major bands (Figure 2), while the inhibitor was eluted in a single peak. The fractions associated with the inhibitor were combined and applied to DEAE-Sephadex A-50. The ion-exchange column had been previously equilibrated with 0.05 M Tris-acetate buffer (pH 7.4). A salt gradient of 0–1 M NaCl was applied after the first 20 fractions

TABLE I: Purification of 25-Hydroxyvitamin D_3-1-Hydroxylase Inhibitor from Rat Serum.

Treatment	Total Protein (mg)	Total Units of Inhibitor	Specific Activity (units/mg of protein)
None (serum)	842	2259	2.7
Ammonium sulphate	418	1588	3.8
Gel filtration (G-200)	221	1356	6.1
Ion-exchange chromatography	104	1224	11.8
Preparative electrophoresis	0.63	1074	1705

had been collected. Figure 3 shows the elution profile of protein and inhibitor obtained. Those fractions containing the inhibitor were combined, dialyzed overnight against 0.05 M Tris-acetate buffer (pH 7.4), and concentrated by ultrafiltration to a volume of 2–3 ml.

Analytical polyacrylamide disc gel electrophoresis of aliquots of the concentrate resolved two major and several minor protein bands. It seemed likely that these could be separated by preparative disc gel electrophoresis.

Figure 4 shows the elution profile for protein and inhibitor obtained after subjecting the concentrated solution from the ion-exchange column to preparative polyacrylamide disc gel electrophoresis. The inhibitor was associated with a minor protein band well separated from the major protein peaks.

The fractions showing inhibition were pooled and concentrated by ultrafiltration. Analytical polyacrylamide disc gel electrophoresis of aliquots of the concentrate showed a single band after staining for protein (Figure 5). Table I summarizes the purification of inhibitor protein from rat serum.

Chemical and Physical Properties of the Inhibitor. In order to detect any nonprotein constituents of the inhibitor, polyacrylamide gels were stained with reagents giving color reactions with lipids, carbohydrates, and metals. The inhibitor did not give a positive test for lipid or carbohydrate, but did give a positive test for iron by the benzidine hydrochloride test.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate separates polypeptide chains according to their molecular weight (Shapiro et al., 1967), there being an inverse linear relationship between relative migration distance and log molecular weight. Electrophoresis of the inhibitor and a series of standard proteins on sodium dodecyl sulfate-treated polyacrylamide gels indicated that the inhibitor is a monomer with a mol wt. of 52 000. This was in agreement with molecular weight estimates from gel filtration and ultracentrifugation on sucrose density gradients. The 52 000 mol wt species was the only band detected on sodium dodecyl sulfate gels.

A protein concentrate prepared from rat kidney microsomes by suspension in hypotonic buffer (Botham et al., 1974) was shown to contain a protein band which comigrated with the isolated inhibitor on polyacrylamide disc gel electrophoresis. This protein was eluted from the gel as described under Materials and Methods, and shown to cause inhibition of the chick kidney 25-OH-D_3-1-hydroxylase. Other proteins eluted from the same gel did not inhibit the chick enzyme system.

It has been established that cytochrome P-450 is involved in the activity of 25-OH-D_3-1-hydroxylase (Ghazarian and DeLuca, 1974; Ghazarian et al., 1974) and that the system requires a flavoprotein and an iron–sulfur protein (Ghazarian et al., 1974). Low levels of the purified inhibitor (1 unit) completely blocked the production of 1,25-$(OH)_2D_3$ from 25-OH-D_3 in the reconstituted enzyme system composed of beef adrenal ferredoxin reductase, chick renal ferredoxin, chick renal mitochondrial cytochrome P-450, and NADPH (Ghazarian et al., 1974; Pedersen et al., 1976). However, the inhibitor did not prevent the reduction of cytochrome *c* in the presence of NADPH, beef adrenal ferredoxin reductase, and chick renal mitochondrial ferredoxin (measured spectrophotometrically as the increase in absorbance at 550 nm). This demonstrates that the inhibitor either specifically inhibits the 1-hydroxylase cytochrome P-450 or binds the 25-OH-D_3 substrate.

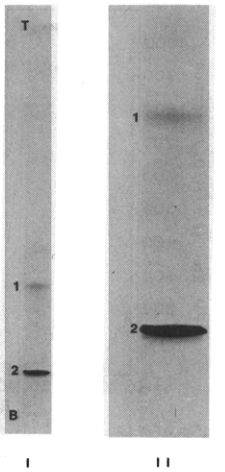

FIGURE 5: Analytical polyacrylamide disc gel electrophoresis of protein after preparative gel electrophoresis. Protein was concentrated by ultrafiltration applied to polyacrylamide gels, and stained as described in the text. (1) Protein band; (2) tracking dye. Fifty-micrograms of protein was applied. T, top, B, bottom. II is an enlargement of the lower half of gel I.

The enzyme system present in bovine adrenal cortex mitochondria which cleaves the side chain of cholesterol to form pregnenolone has also been shown to involve cytochrome P-450 (Simpson and Boyd, 1967). Levels of inhibitor which reduced the production of 1,25-$(OH)_2D_3$ from 25-OH-D_3 by the chick kidney 1-hydroxylase to 20–30% that of the uninhibited enzyme had no effect on the formation of pregnenolone from cholesterol in the in vitro system of Simpson and Boyd (1967).

Binding of 25-OH-D_3 by the 25-OH-D_3-1-Hydroxylase Inhibitor. To examine the question of whether the binding of substrate might be the mechanism of inhibition, the inhibitor was incubated at 37 °C with 25-OH-[^{3}H]D_3 and then subjected to centrifugation on a 10–30% linear sucrose gradient (Figure 6). There is no doubt that the inhibitor binds the 25-OH-[^{3}H]D_3 and the complex has a sedimentation coefficient of 4 S. Extraction and chromatography of the ^{3}H bound to the inhibitor after incubation revealed that the 25-OH-[^{3}H]D_3 remained unchanged during this procedure. A series of such binding studies permitted a determination of the binding capacity of the inhibitor (7.5 μg/mg of protein). Increasing the substrate concentration above the binding capacity of the inhibitor increases the reaction rate (Figure 7). The velocity of the 1-hydroxylase at 34 ng of substrate is 0.25 pmol min^{-1} mg^{-1} of protein in the absence of inhibitor. The velocity of the 1-hydroxylase at 50 ng of substrate in the presence of inhibitor was 0.19 pmol min^{-1} mg^{-1} of protein. Since the inhibitor at capacity binds 16 ng of substrate, the effective substrate concentration should be 34 ng in this case. The velocities are,

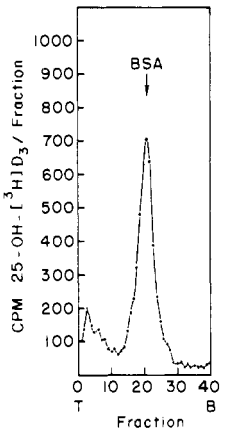

FIGURE 6: Binding of 25-OH-[^{3}H]D_3 to purified rat serum inhibitor protein. Rat serum inhibitor protein was diluted with 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, buffer to a final concentration of 0.006 mg/ml. Then 0.5 ml was incubated with 1.25 ng of 25-OH-[^{3}H]D_3 (9.2 Ci/mmol) contained in 0.015 ml of 95% ethanol for 15 min at 37 °C. Binding of 25-OH-[^{3}H]D_3 to rat serum inhibitor was analyzed on 10–30% sucrose density gradients as described in the text. The arrow indicates the position of sedimentation of bovine serum albumin. The early peak of radioactivity represents unbound 25-OH-[^{3}H]D_3.

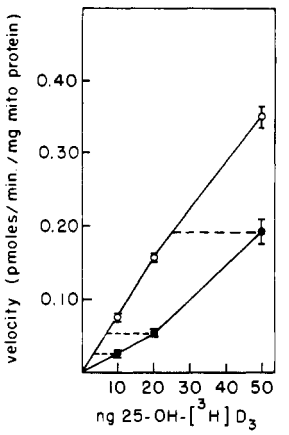

FIGURE 7: The effect of increasing substrate concentration on 25-OH-D_3-1-hydroxylase by chick kidney mitochondria in the presence and absence of purified rat serum inhibitor protein. Chick kidney mitochondria (8.2 mg) was incubated with either 10, 20, or 50 ng of 25-OH-[^{3}H]D_3 (1.2 Ci/mmol) in the presence (●) or absence (○) of 2 μg of rat serum inhibitor for 15 min at 37 °C. 1,25-$(OH)_2$-[^{3}H]D_3 was determined by Sephadex LH-20 chromatography of the lipid extract of each incubation. The rate of 1-hydroxylation is expressed as pmol of 1,25-$(OH)_2D_3$ produced min^{-1} mg^{-1} of mitochondrial protein. The amount of inhibitor added to each of the indicated vessels was sufficient to bind a maximum of 16 ng of 25-OH-[^{3}H]D_3 as determined by sedimentation analysis.

therefore, similar when effective substrate concentration is considered.

Discussion

The factor present in rat kidney and other tissues which inhibits the conversion of 25-OH-D_3 to 1,25-$(OH)_2D_3$ by chick kidney preparations (Botham et al., 1974) has been isolated from rat serum by ammonium sulfate precipitation, gel filtration, ion-exchange chromatography, and preparative disc gel electrophoresis. The protein has been shown to be a monomer containing iron with a mol wt of 52 000. The data indicate that the serum inhibitor is indistinguishable on gel electrophoresis from that found in rat kidney preparations.

It is possible that the inhibitor exerts its effect by interfering with the respiratory chain and/or oxidative phosphorylation, thus reducing the availability of reducing equivalents to the 25-OH-D_3-1-hydroxylase. The finding that the inhibitor was effective when chick kidney mitochondria were swollen with 10 mM calcium (Botham et al., 1974), conditions where extramitochondrial NADPH can support 1-hydroxylation in the absence of Krebs cycle intermediates and in the presence of respiratory chain inhibitors and uncouplers of oxidative phosphorylation (Ghazarian and DeLuca, 1974), suggested that this was not the case. It has now been shown that the reduction of cytochrome *c* by the chick kidney mitochondrial NADPH–cytochrome *c* reductase is not affected by the purified inhibitor. Thus, the effect of the inhibitor upon the 1-hydroxylase may involve a direct interaction with the 1-hydroxylase cytochrome P-450 or with the substrate.

The presence of cytochrome P-450 in chick kidney mitochondria has been demonstrated by carbon monoxide difference spectra, and its involvement in 25-OH-D_3-1-hydroxylation has been shown by reconstitution studies with the solubilized cytochrome, flavoprotein, iron–sulfur protein, and NADPH (Ghazarian et al., 1974). The possible inhibition of other cytochrome P-450 systems was investigated by studying the effect of the inhibitor on another well-established mitochondrial P-450 system, the side-chain cleavage enzyme which forms pregnenolone from cholesterol (Simpson and Boyd, 1967). No inhibition was seen in this system.

It is likely that the inhibitor acts by binding the substrate 25-OH-D_3 more strongly than does the 1-hydroxylase enzyme. There is no doubt that the inhibitor binds 25-OH-D_3 (Figure 6). Its electrophoretic behavior, molecular weight, and sedimentation constant suggest that it may be identical with the 25-OH-D_3 transport protein in the plasma (Rikkers and DeLuca, 1967; Edelstein et al., 1974; Edelstein, 1974; Haddad and Birge, 1971, 1975). In support of the idea that the inhibitor acts by binding the substrate, the results in Figure 7 demonstrate that the reduced 1-hydroxylase velocity in the presence of inhibitor can be explained almost entirely by substrate binding.

All attempts made in our laboratory to demonstrate substantial 25-OH-D_3-1-hydroxylase activity in homogenates and cell fractions from rat kidney have been unsuccessful. Midgett et al. (1973) have reported the production of 1,25-$(OH)_2D_3$ from 25-OH-D_3 using human, dog, and rat kidney tissue in vitro; however, the reported levels could not account for the amounts produced by rat kidney in vivo. The physiological significance of the inhibitor in the rat is not clear; however, it seems possible that it is responsible for the failure to find in vitro 25-OH-D_3-1-hydroxylation with rat and other mammalian tissues. It is hoped that the purification and characterization of this protein will enable experiments to be designed in which the inhibitory effect can be overcome, thus allowing the study of the mammalian 25-OH-D_3-1-hydroxylase in vitro under controlled conditions.

Acknowledgment

We are grateful to Dr. Jan Pedersen for spectrophotometric measurements of cytochrome *c* reduction, Dr. R. R. Burgess for advice on sodium dodecyl sulfate polyacrylamide gel electrophoresis, and Dr. N. Orme-Johnson for advice on the cholesterol side-chain-cleavage enzyme preparation.

References

Bhattacharyya, M. H., and DeLuca, H. F. (1973), *J. Biol. Chem. 248,* 2969.

Botham, K. M., Tanaka, Y., and DeLuca, H. F. (1974), *Bio-*

chemistry 13, 4966.
Davis, B. J. (1962), Preprint "Disc Electrophoresis", Distillation Products Division, Eastman Kodak Co., Rochester, N.Y.
Di Jeso, F. (1968), *J. Biol. Chem. 243,* 2022.
Edelstein, S. (1974), *Biochem. Soc. Spec. Publ. 3,* 43.
Edelstein, S., Lawson, D. E. M., and Kodicek, E. (1973), *Biochem. J. 135,* 417.
Fraser, D. R., and Kodicek, E. (1970), *Nature (London) 228,* 765.
Ghazarian, J. G., and DeLuca, H. F. (1974), *Arch. Biochem. Biophys. 160,* 63.
Ghazarian, J. G., Jefcoate, C. R., Knutson, J. C., Orme-Johnson, W. H., and DeLuca, H. F. (1974), *J. Biol. Chem. 249,* 3026.
Gray, R. W., Boyle, I. T., and DeLuca, H. F. (1971), *Science 172,* 1232.
Gray, R. W., Omdahl, J. L., Ghazarian, J. G., and DeLuca, H. F. (1972), *J. Biol. Chem. 247,* 7528.
Haddad, J. G., and Birge, S. J. (1971), *Biochem. Biophys. Res. Commun. 45,* 829.
Haussler, M. R., Boyce, D. W., Littledike, E. T., and Rasmussen, H. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 177.
Holick, M. F., and DeLuca, H. F. (1971), *J. Lipid Res. 12,* 460.
Holick, M. F., Schnoes, H. K., and DeLuca, H. F. (1971a), *Proc. Natl. Acad. Sci. U.S.A. 68,* 803.
Holick, M. F., Schnoes, H. K., DeLuca, H. F., Suda, T., and Cousins, R. J. (1971b), *Biochemistry 10,* 2799.
Jensen, E. V., Suzuki, T., Kawaskima, T., Stumpf, W. E., Jungblut, P. W., and DeSombre, E. R. (1968), *Proc. Natl. Acad. Sci. U.S.A. 59,* 632.
Knutson, J. C., and DeLuca, H. F. (1974), *Biochemistry 13,* 1543.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Maurer, H. R. (1971), in Disc Electrophoresis, New York, N.Y., Walter de Gruyter, p 72.
Midgett, R. J., Spielvogel, A. M., Coburn, J. W., and Norman, A. W. (1973), *J. Clin. Endocrinol. Metab. 36,* 1153.
Norman, A. W., Midgett, R. J., Myrtle, J. F., and Nowicki, H. G. (1971), *Biochem. Biophys. Res. Commun. 42,* 1082.
Omdahl, J. L., Holick, M. F., Suda, T., Tanaka, Y., and DeLuca, H. F. (1971), *Biochemistry 10,* 2935.
Ornstein, L. (1963), Enzyme Analysis, C.8, Canalco.
Pedersen, J. I., Ghazarian, J. G., Orme-Johnson, N. R., and DeLuca, H. F. (1976), *J. Biol. Chem.* (in press).
Prat, J. P., Lamy, J. N., and Weill, J. D. (1969), *Bull. Soc. Chim. Biol. 51,* 1367.
Rikkers, H., and DeLuca, H. F. (1967), *Am. J. Physiol. 213,* 380.
Shain, S. A. (1972), *J. Biol. Chem. 247,* 4393.
Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28,* 815.
Simpson, E. R., and Boyd, G. S. (1967), *Eur. J. Biochem. 2,* 275.
St. Groth, S. F., Webster, R. G., and Datyner, A. (1963), *Biochim. Biophys. Acta 71,* 377.
Suda, T., DeLuca, H. F., and Hallick, R. B. (1971), *Anal. Biochem. 43,* 139.
Suda, T., DeLuca, H. F., and Tanaka, Y. (1970), *J. Nutr. 100,* 1049.
Weber, K., Pringle, J. R., and Osborn, M. (1972), *Methods Enzymol. 26,* 3.
Zacharius, R. M., Zell, T. E., Morrison, J. H., and Woddlock, J. J. (1969), *Anal. Biochem. 30,* 148.