inhibition appeared to be of the competitive type (see preceding paper, Figure 2).

The model predicts that phosphatidylethanolamine plays two roles. (1) The phospholipid interacts with lipopolysaccharide to form a lipopolysaccharide–phospholipid complex whereby binding sites on the polysaccharide are made accessible to the enzyme. Both didecanoyl- and *A. agilis* phosphatidylethanolamine are presumed to be effective in this role since both compounds formed binary complexes with lipopolysaccharide, and both binary complexes were also capable of binding the enzyme (Figures 8 and 11). The failure of dipalmitoyl- and hydrogenated *A. agilis* phosphatidylethanolamine to be active in the enzyme system is ascribed to their inability to form binary complexes with lipopolysaccharide (Figure 8). (2) Phosphatidylethanolamine provides a second binding site for the enzyme. Didecanoylphosphatidylethanolamine is presumed to be ineffective in this role as indicated by its failure to bind to the transferase enzyme (Figure 9b) in the absence of lipopolysaccharide.

Binding to both the polysaccharide and phospholipid are considered necessary for the full catalytic activity of the enzyme. If the model is correct, the inhibition of the transfer reaction by didecanoylphosphatidylethanolamine can be ascribed to removal of lipopolysaccharide from the reaction by formation of lipopolysaccharide–phosphatidylethanolamine complexes which are inactive because of the inability of the phospholipid portion of the complex to interact effectively with the enzyme. Both the decreased yield of the reaction (Figure 6) and the competitive nature of the kinetics (Figure 7) are consistent with this view.

The evidence in support of the scheme is indirect and the model is presented here as a guide to further experimentation.

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The Isolation and Identification of 25-Hydroxyergocalciferol*

T. Suda,† H. F. DeLuca,‡ H. K. Schnoes, and J. W. Blunt§

ABSTRACT: A polar metabolite of vitamin D_2 (314 μg) has been isolated in pure form from the blood of four pigs given 500,000 IU of ergocalciferol/day for 26 days. It has been identified as 25-hydroxyergocalciferol by means of ultraviolet spectra, gas–liquid partition chromatography, nuclear magnetic resonance spectra, mass spectra, and mass spectra of its trimethylsilyl ether derivative. It is about 1.5 times more active than vitamin D_3 or D_2 in curing rickets in rats.

Lund and DeLuca (1966) described for the first time a polar metabolite of vitamin D_3 which appeared to be at least as biologically active as the parent vitamin in curing rickets in rats. This metabolite was described in man (DeLuca *et al.*, 1967; Avioli *et al.*, 1967), chicks (Drescher *et al.*, 1969), and hogs (Blunt *et al.*, 1968a), proved to possess all the biological activities of the parent vitamin, and acted more rapidly (Lund and DeLuca, 1966; Morii *et al.*, 1967). This metabolite was isolated in pure form and identified as 25-hydroxycholecalciferol (Blunt *et al.*, 1968a,b). Evidence has been obtained that it represents the metabolically active form of the vitamin (Blunt *et al.*, 1968c; Trummel *et al.*, 1969).

Recently it was shown that the peak IV metabolite is also formed from vitamin D_2 in both rats and chicks (Drescher *et al.*, 1969). This metabolite has now been demonstrated in hogs, isolated in pure form, and identified as 25-hydroxyergocalciferol. It is the purpose of this communication to report these findings.

Methods and Results

General Procedures. All radioactive determinations were carried out by means of a Packard Tri-Carb Model 3003 liquid scintillation counter equipped with an automatic external standardization system. Samples to be counted were evaporated to dryness with a stream of air, dissolved in toluene-counting solution (2 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)benzene] per l. of toluene), and counted.

Ultraviolet spectra were recorded with a Beckman DB-G spectrophotometer. Samples in this case were dissolved in diethyl ether.

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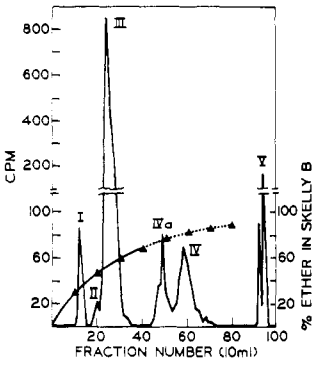

FIGURE 1: Silicic acid column profile of ^3H in extracts during the isolation of 25-hydroxyergocalciferol. (▲—▲ · · · ▲) Gradient; (—) radioactivity; 10-ml fractions were collected and tubes 53–67 were used in further purification.

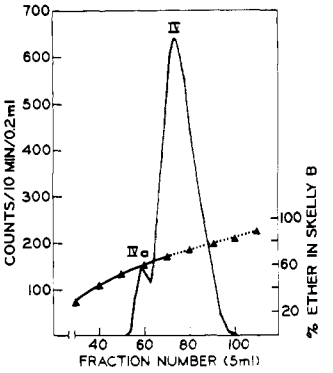

FIGURE 2: Chromatography of peak IV obtained from Figure 1 on a silicic acid multibore column. (▲—▲ · · · ▲) Gradient of diethyl ether in Skelly B; (—) radioactivity; 5-ml fractions were collected and tubes 63–98 were used in further purification.

Nuclear magnetic resonance spectra were recorded in $CDCl_3$ solution using a Varian Associates Model HA-100 spectrometer coupled to a time-averaging computer, the latter being essential because of the small amount of metabolite isolated. Tetramethylsilane was used as the internal standard. Data are recorded as δ in parts per million, relative to tetramethylsilane ($\delta = 0$).

Gas–liquid partition chromatography was carried out in an F & M Model 402 using a 4 ft × 0.25 in. glass column packed with 3% W-98 on 80–100 mesh Diatoport S.

Conventional mass spectra were obtained with C. E. C.-110B and A. E. I. MS-9 mass spectrometers, by direct introduction into the ion source, operated at a temperature of *ca.* 150°, electron potential of 70 eV, and ionizing current of 100 μA. High-resolution mass spectra were recorded on an A. E. I. MS-9 mass spectrometer coupled on-line to a Sigma-7 (Scientific Data Systems) computer (Burlingame, 1968; Burlingame *et al.*, 1968); source temperature 150°, electron energy 70 eV, ionizing current 400 μA.

Isolation of Metabolites. Four pigs of mixed breed weighing 165–200 lb were fed stock rations to which was added water-dispersible vitamin D_2 at a level of 70,000 IU/lb of feed. This supplied 500,000 IU of vitamin D_2 daily per pig. After 26 days the pigs were slaughtered and their blood was collected. It was immediately mixed with one-tenth volume of 0.1 M sodium oxalate to prevent clotting. Plasma was separated from the cells by means of a DeLaval blood separator (DeLaval Co.). The 4.1 l. of plasma thus obtained was made 70% saturated with $(NH_4)_2SO_4$ and allowed to stand at 4° for 7 days. The precipitate was collected by centrifugation at 25,000 rpm for 25 min in a Sharples AS-16-P centrifuge. The protein precipitate was extracted with 6.6 l. of methanol–chloroform (2:1) with a heavy-duty stirrer and allowed to stand for 24 hr. An additional 2.2 l. of chloroform was added and stirred. The denatured protein was removed by filtration through glass wool and reextracted with another 4.4 l. of methanol–chloroform. The phases were allowed to separate. The aqueous phase was drawn off and reextracted with 2 l. of chloroform. The combined chloroform layers were washed with 10 l. of tap water and allowed to stand for 24 hr. The $CHCl_3$ phases were concentrated with a rotary flash evaporator to 50 ml. This oily black residue was washed with saturated sodium chloride and dried over anhydrous $MgSO_4$. It was then taken to dryness with the flash evaporator and dissolved in 100 ml of Skelly B (a petroleum fraction, bp 65 67°).

Radiochemically pure [^3H]vitamin D_2 was prepared by exposing 1 g of vitamin D_2 to 3.0 Ci of tritiated acetic acid (Nuclear-Chicago Corp.) for 2 weeks after which time it was chromatographed three times on a multibore silicic acid column and one time on a reversed-phase column both described previously (Neville and DeLuca, 1966) to constant specific activity. Radiochemical purity was established as described by Neville and DeLuca (1966). The [^3H]vitamin D_2 had a specific activity of 1200 dpm/IU or 8.6 mCi/mmole. Ten rats (weighing 400 g) fed a low vitamin D stock ration (Steenbock, 1923) were given 10,000 IU of [^3H]vitamin D_2 intraperitoneally in 0.1 ml of ethanol; 24 hr later blood was drawn by cardiac puncture giving 40 ml of plasma after centrifugation. This was extracted with methanol–chloroform (2:1) as described earlier (Blunt *et al.*, 1968a). The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness in a flash evaporator. The residue was dissolved in Skelly B and combined with the extract from the hogs.

The combined extract was divided into five equal aliquots and each aliquot was treated as follows. It was first chromatographed on a 60 × 1 cm silicic acid column described earlier (Blunt *et al.*, 1968a). The radioactivity profile from such a column is shown in Figure 1. Note that peaks I and II, esters of vitamin D and an unidentified metabolite, appeared. Peak III represents unchanged vitamin D_2. A relatively large peak IVa appeared as well as peak IV. Peak V is actually a mixture of metabolites all of which are biologically inactive by the "line test" rickets cure test in rats (U. S. Pharmacopoeia, 1955). Peak IVa possesses low if any antirachitic activity in rats. Peak IV was approximately 1.5 times more active than peak III (unchanged vitamin D_2). The peak IV fractions from the five silicic acid columns were collected and evaporated to dryness in a flash evaporator. It was next rechromatographed on a multibore silicic acid column as described by Neville and DeLuca (1966) except that the mixing chamber contained 250 ml of Skelly B and the holding chamber contained 400 ml of 85% diethyl ether in Skelly B. As soon as the holding chamber became empty, it was filled with 300 ml of 100% diethyl ether. The elution profile is shown in Figure 2. Tubes 63–98 were combined and chromatographed on a Celite par-

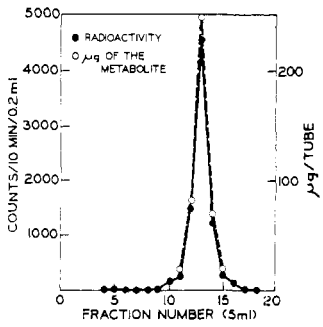

FIGURE 3: Partition chromatography of the peak IV metabolite from porcine plasma. Celite was used as the support. The stationary phase consisted of 80% methanol–20% water equilibrated with Skelly B while the mobile phase was the Skelly B which was equilibrated with the 80% methanol–20% water; 5-ml fractions were collected.

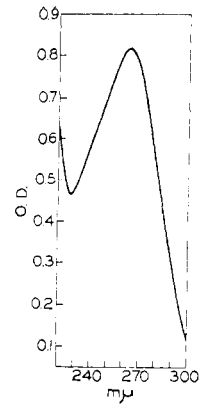

FIGURE 4: Ultraviolet spectrum of 25-hydroxyergocalciferol.

tition column. For this, 200 ml of Skelly B was equilibrated at 10° with an equal volume of 80% methanol–20% water; 15 ml of the methanolic phase was mixed with 20 g of Celite and dry packed into a 60 × 1 cm column in 2-cm portions. The upper phase was used as the mobile phase. The residue obtained from tubes 63–98 from the silicic acid column (after removal of solvent by a flash evaporator) was applied in a small volume of mobile phase (1–3 ml) to the partition column. The column was developed with mobile phase with 5-ml fractions collected. Tubes 11–17 contained the peak IV metabolite. These were recombined, evaporated to dryness as before, and rechromatographed on another identical partition column. The profile of radioactivity from that column is shown in Figure 3. Optical density at 265 mμ was also measured in each fraction and the calculated micrograms of peak IV metabolite are plotted assuming a molar extinction coefficient of 19,400. Clearly the absorbancy at 265 mμ exactly coincides with the radioactivity plot. The ultraviolet spectrum of the material is shown in Figure 4. A total of 0.314 mg of metabolite was isolated. The purity of the peak IV metabolite thus obtained from the Celite column was examined by gas–liquid partition chromatography, as shown in Figure 5. Note that a double peak of pyro and isopyro derivatives of the metabolite is evident having a retention time of 14.0 and 15.8 min. This together with the ultraviolet λ_{max} at 265 mμ demonstrates that the triene structure of the parent vitamin D_2 is retained by the metabolite. In any case both the ultraviolet and gas–liquid partition chromatographic data demonstrate a high degree of purity for the isolated metabolite. It may be of interest that the retention times for the pyro- and isopyroergocalciferol are 7.0 and 7.9 min under the same conditions.

For the purpose of identification it was necessary to prepare the trimethylsilyl ether derivative of the metabolite. Isolated metabolite (30 μg) was taken to dryness under a stream of dry N_2. A few drops of a reagent (14 ml of dry pyridine, 4 ml of hexamethyldisilazane, and 2 ml of trimethylsilyl chloride) was added and flushed with N_2, and the mixture was allowed to stand in the dark at room temperature for 3 hr.; 3 ml of Skelly B was added followed by 2 ml of 10% H_2SO_4. The mixture was vigorously mixed and then allowed to separate. The Skelly B phase was dried over a small amount of anhydrous Na_2SO_4. It was then chromatographed on silicic acid column as described by Lund *et al.* (1967). In tubes 19–27 the disilyl ether derivative was located (15 μg) and was found to be essentially pure by gas–liquid partition chromatography. It was then used for determination of mass spectra.

Identification of 25-Hydroxyergocalciferol. As already pointed out, the ultraviolet spectra and the gas–liquid partition chromatographic data clearly suggest that the triene structure remains unchanged in the metabolite structure. The mass spectrum of the metabolite (Figure 6) shows intense peaks at *m/e* 136 ($C_9H_{12}O$) and 118 (C_9H_{10}) deriving from ring A of the molecule including C-6 and -7 (see Figure 7) and are present also in the spectrum of vitamin D_2 (Figure 6).

The mass spectrum of the metabolite (Figure 6) indicated a molecular ion of *m/e* 412, the increase of 16 mass units over the molecular weight of vitamin D_2 (Figure 6), suggesting the incorporation of an additional oxygen atom. Confirmation was provided by the high-resolution mass spectrum, since the exact mass of the molecular ion, 412.3341, required the composition of $C_{28}H_{44}O_2$ (calcd 412.3341). The location of the additional oxygen substituent in the side chain is apparent from comparison of the spectra of vitamin D_2 (Figure 6) and its metabolite (Figure 6). Both show peaks at *m/e* 271 ($C_{19}H_{27}O$ 271.2065, by high-resolution mass spectrometry) resulting from loss of the entire side chain by cleavage of the C_{17}–C_{20} bond. In addition the high-resolution spectrum of the metabolite contains minor peaks which are probably due to the side chain itself, *m/e* 141.1255 ($C_9H_{17}O$) and 123.1181 (C_9H_{15}).

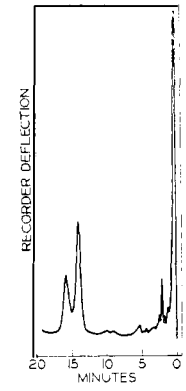

FIGURE 5: Gas–liquid partition chromatogram of 25-hydroxyergocalciferol.

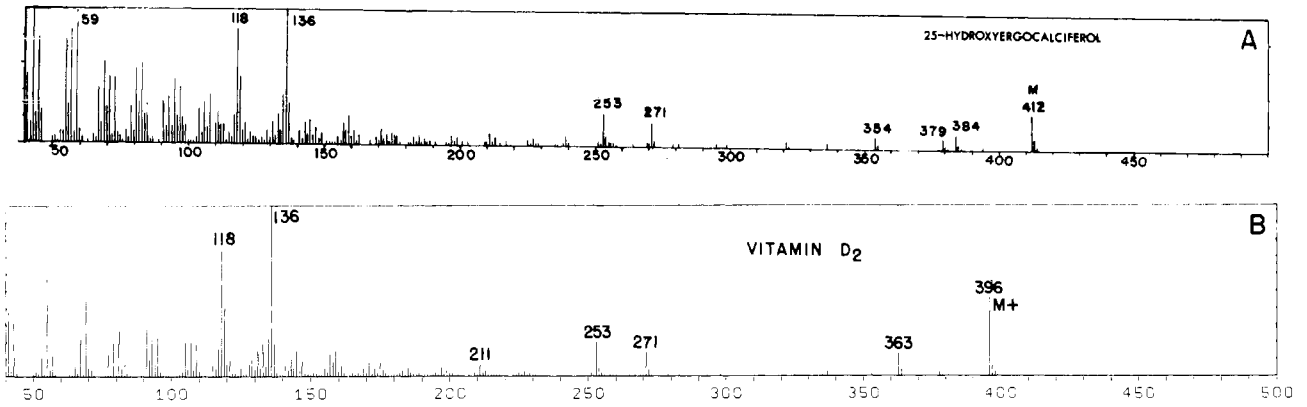

FIGURE 6: Mass spectra of 25-hydroxyergocalciferol and ergocalciferol.

A fragment of mass 59 (C_3H_7O, as determined by high-resolution mass spectrometry) and the elimination of 58 mass units from the molecular ion to give a peak at *m/e* 354 (354.2900 $C_{25}H_{38}O$), both present in the mass spectrum of the metabolite but absent in that of vitamin D_2, provided evidence for a hydroxyl function at C-25. The former peak would arise by simple cleavage of the C-24–C-25 bond to give the ion $(CH_3)_2C{=}O^+H$, the latter by hydrogen rearrangement with elimination of the elements of acetone, a process not uncommon for homoallylic alcohols.

Proof of the position of the hydroxyl grouping was obtained from the mass spectrum of the silyl ether derivative of the metabolite which exhibited a molecular ion at *m/e* 556, as required for a ditrimethylsilyl ether, and the expected intense peak at *m/e* 131 (base peak), shown to correspond to $C_6H_{15}SiO$, $(CH_3)_2C{=}O^+Si(CH_3)_3$, by high-resolution mass spectrometry. This peak, which also occurs in the spectra of the trimethylsilyl ether derivatives of 25-hydroxycholesterol and 25-hydroxycholecalciferol (J. W. Blunt, H. F. DeLuca, and H. K. Schnoes, unpublished data), but not in the spectra of the silyl ethers of vitamin D_2 or D_3, thus requires C-25 as the position of the additional hydroxyl function.

The nuclear magnetic resonance spectrum of the metabolite (Figure 8) fully corroborated these deductions and provided independent structural evidence: an intense singlet at δ = 1.24 ppm can be assigned to the two methyl groupings at C-25, while two doublets at δ = 0.79 (J = 6.5 cps) and 0.81 ppm (J = 7.0 cps) must be due to the remaining methyl substituents of the side chain (C-21 and -28). The spectra of 25-hydroxycholesterol and 25-hydroxycholecalciferol exhibit a singlet for the C-26, 27 methyl groups at essentially the same position (δ = 1.20 ppm). It should be noted that two doublets at δ 0.87 (J = 7.0 cps) and 0.98 (J = 6.0 cps) originally present in the ergocalciferol spectrum (Figure 9) are absent in that of the metabolite. This elimination of two doublets which must correspond to two methyl groups can be explained only by hydroxyl substitution at C-25. The remaining identifiable feature is the singlet due to the C-18 angular methyl group. These data, therefore, establish the structure of the metabolite as 25-hydroxyergocalciferol (Figure 10).

The isolated metabolite was finally assayed for antirachitic activity in rats (U. S. Pharmacopoeia, 1955) and by other techniques. Routinely it assayed at 60–64 IU of antirachitic activity per micrograms of metabolite. Thus by this test its activity is essentially that of 25-hydroxycholecalciferol and 1.4–1.5 times more active than ergocalciferol or cholecalciferol.

Discussion

There is now mounting evidence that vitamin D_3 (cholecalciferol) must be converted into its metabolically active form

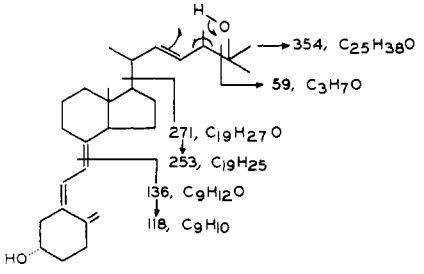

FIGURE 7: Schematic representation of bond ruptures leading to prominent ions in the mass spectrum of 25-hydroxyergocalciferol.

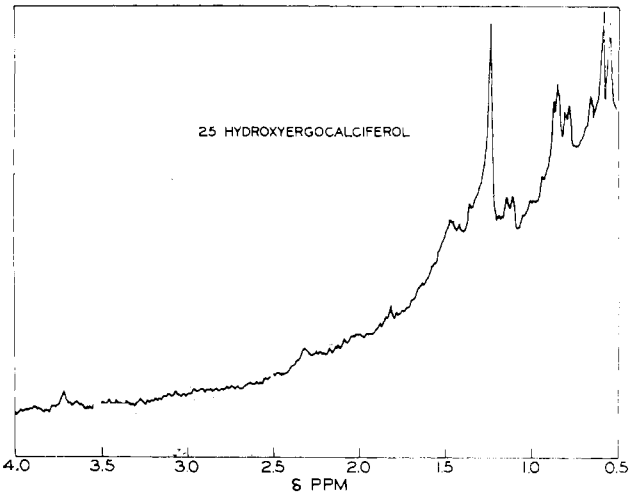

FIGURE 8: Nuclear magnetic resonance spectrum of 25-hydroxyergocalciferol. The 0.54-ppm peak is a spinning side band of the trimethylsilane while the 0.58 ppm is due to the C-18 protons.

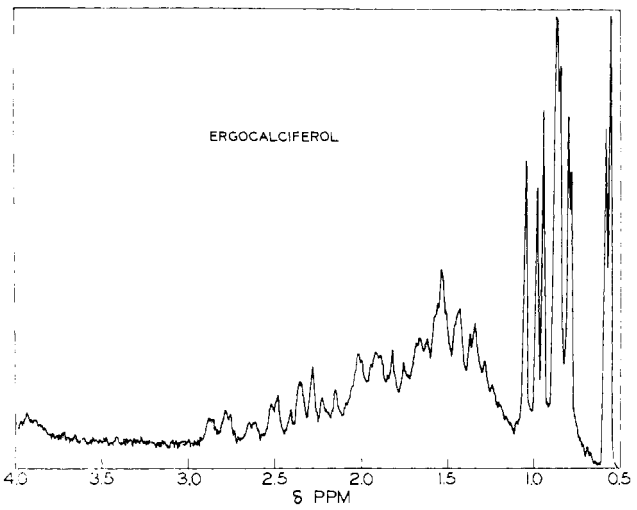

FIGURE 9: Nuclear magnetic resonance spectrum of ergocalciferol. The 0.54-ppm peak is a spinning side band of trimethylsilane while the 0.58 ppm is due to the C-18 protons.

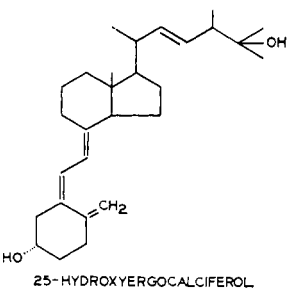

FIGURE 10: Structure of 25-hydroxyergocalciferol.

before it can produce its characteristic physiologic responses (Blunt *et al.*, 1968c; DeLuca, 1969; Trummel *et al.*, 1969). In this laboratory it has been shown that radioactive vitamin D_2 (Drescher *et al.*, 1969), vitamin D_3 (Lund and DeLuca, 1966; Blunt *et al.*, 1968a,b; DeLuca, 1967), and vitamin D_4 (H. F. DeLuca, 1969, unpublished data) are converted into polar, highly biologically active metabolites. Recently it was possible to isolate in milligram quantities the metabolite derived from vitamin D_3 and identify its structure as 25-hydroxycholecalciferol (Blunt *et al.*, 1968a,b). The structure was unequivocally confirmed when 25-hydroxycholecalciferol was synthesized chemically and shown to be identical in all respects with the isolated material (Blunt and DeLuca, 1969). This metabolite has been shown to stimulate intestinal Ca^{2+} transport and bone mobilization more rapidly than does vitamin D_3 when given to D-deficient animals (Blunt *et al.*, 1968c). It also induces bone mobilization in tissue culture (Trummel *et al.*, 1969) and calcium transport in perfused intestine (E. B. Olson and H. F. DeLuca, 1969, unpublished data) while vitamin D_3 itself has little or no effect. Evidence is therefore strong that 25-hydroxycholecalciferol is the metabolically active form of vitamin D_3. We have now been able to show the production of the analogous 25-hydroxyergocalciferol from vitamin D_2 in pigs. This metabolite is also more biologically active than either vitamin D_3 or D_2 in curing rickets in rats. It is therefore likely that the metabolically active form of vitamin D_2 is 25-hydroxyergocalciferol and that all D vitamins are converted into the 25-hydroxy derivative before they can act.

The identification of the 25-hydroxyergocalciferol bears some comment. It was first of all necessary to feed higher levels of vitamin D_2 to pigs before adequate peak IV metabolite could accumulate in the plasma than was the case with vitamin D_3. Next, another metabolite (peak IVa) was difficult to separate from the peak IV and could best be achieved by a multibore silicic acid column. When the metabolite was finally purified, the nmr data were more difficult to interpret because it was difficult to assign positions of the C-21, C-28, C-26, and C-27 doublets of ergocalciferol. Because two doublets were absent from the metabolite nuclear magnetic resonance spectrum (as compared to ergocalciferol) (δ = 0.87 ppm, J = 7.0 cps; δ = 0.98 ppm, J = 6.0 cps) and an intense singlet appeared at δ = 1.24 ppm, it was apparent that only an hydroxyl at the 25 position could satisfy this shift. This suggests that δ = 0.79 ppm (J = 6.0 cps) and δ = 0.81 ppm (J = 7.0 cps) represent the 28 and 21 methyl groups of ergocalciferol.

Acknowledgments

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Brain Hexokinase: Immunohistochemical Localization at the Light Microscopic Level*

Patricia A. Craven† and R. E. Basford

ABSTRACT: The indirect fluorescent antibody technique was used to localize hexokinase at the light microscopic level. Antibodies were produced in rabbits to soluble bovine brain hexokinase. Evidence of purity was obtained from agar gel diffusion and the precipitin test. The antiserum completely inhibited soluble hexokinase but only partially (60–70%) inhibited the activity associated with intact mitochondria or with the outer membrane fraction of mitochondria. When these antibodies were used to demonstrate the presence of hexokinase in thin sections of brain cortex, bright fluorescent granules were observed in the cytoplasm of nerve cells and in the intervening tissue. These corresponded in size and distribution to mitochondria demonstrated either by the succinate-dependent reduction of tetranitro blue tetrazolium or a classical supravital staining method. An unsuccessful attempt was made to confirm these observations in the electron microscope using the peroxidase-conjugated antibody technique.

Although it is generally believed that mitochondria contain the enzymes necessary to oxidize pyruvate completely to carbon dioxide and water, the location of the glycolytic enzymes has been the subject of many conflicting reports. In 1956, Gallagher *et al.* reported that brain mitochondria, unlike those isolated from other sources, possessed the ability to oxidize glucose completely to carbon dioxide and water. On the other hand, the preparations of Brody and Bain (1952) and Aldridge (1957), did not possess this ability. The subsequent preparation of brain mitochondria which are relatively free of contaminating microsomes, nerve ending particles, and glycolytic capacity has led to the conclusion that glycolysis is probably not a function of brain mitochondria (Løvtrop and Svennerholm, 1963; Beattie *et al.*, 1964; Ruscak *et al.*, 1967). Nevertheless, the work of Crane and Sols (1953) and Johnson (1960) has revealed that 75–90% of the hexokinase activity of brain homogenates is sedimented with the mitochondrial fraction, whereas the other glycolytic enzymes are present in the soluble fraction. Basford *et al.* (1964) have shown that the addition of mitochondria to the supernatant fraction of brain homogenates results in a severalfold stimulation of glycolytic activity which can be duplicated by the addition of yeast hexokinase alone. Furthermore, direct assay revealed that both hexokinase and phosphofructokinase were concentrated with the mitochondria. The association of both of the ATP-requiring enzymes of glycolysis with the mitochondria might be a way of allowing more efficient integration of ATP production and glucose utilization. This type of mechanism would be particularly important in brain where the major source of energy is supplied by blood glucose.

The uncertainty of the results obtained from studies involving the differential centrifugation of brain homogenates has prompted this investigation into the *in situ* location of hexokinase at the light microscopic level in thin sections of brain cortex. In the following paper (Craven *et al.*, 1969), evidence for the association of hexokinase with the outer mitochondrial membrane will be presented.

Materials

The following materials were obtained commercially: glucose 6-phosphate dehydrogenase (type 5), ATP, TPN^+, and EDTA from Sigma Chemical Co., St. Louis, Mo.; Sephadex gels from Pharmacia, Uppsala, Sweden; DEAE-cellulose (Cellex D) from Bio-Rad Laboratories, Richmond, Calif.; tetranitro-BT[1] from Nutritional Biochemicals Corp., Cleveland, Ohio; bovine serum albumin from Armour Pharmaceutical Co., Kankakee, Ill.; Freund's complete adjuvant and fluorescein isothiocyanate from Baltimore Biological Laboratories, Baltimore, Md.; Ouchterlony double-diffusion plates, pattern B from Hyland Laboratories, Los Angeles, Calif.; and Tris from General Biochemicals, Chagrin Falls, Ohio. The sheep antirabbit 7S γ-globulin was the generous gift of Dr.

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[1] Abbreviation used is: tetranitro-BT, tetranitro blue tetrazolium.