Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Martinez-Carrion, M., & Jenkins, W. T. (1965) *J. Biol. Chem. 240*, 3538-3546.
Martinez-Carrion, M., Turano, C., Chiacone, E., Bossa, F., Giartosio, A., Riva, F., & Fasella, P. (1967) *J. Biol. Chem. 242*, 2397-2409.
Matsuzawa, T., Katsunuma, T., & Katunuma, N. (1968) *Biochem. Biophys. Res. Commun. 32*, 161-166.
Meister, A. (1954) *J. Biol. Chem. 206*, 587-596.
Moses, U., & Churchich, J. E. (1980) *Biochim. Biophys. Acta 613*, 392-400.
Nishizuka, Y., Takeschita, M., Kuno, S., & Hayaishi, O. (1959) *Biochim. Biophys. Acta 33*, 591-593.
Peraino, C., Bunville, L. G., & Tahmissian, T. N. (1969) *J. Biol. Chem. 244*, 2241-2249.
Roberts, E. (1954) *Arch. Biochem. Biophys. 48*, 395-401.
Roberts, E., & Bregoff, H. M. (1953) *J. Biol. Chem. 201*, 393-398.
Roberts, E., Ayengar, P., & Posner, I. (1953) *J. Biol. Chem. 203*, 195-204.
Rothstein, M. (1965) *Arch. Biochem. Biophys. 111*, 467-476.
Schousboe, A., Wu, J.-Y., & Roberts, E. (1973) *Biochemistry 12*, 2868-2873.
Soda, K., & Misono, H. (1968) *Biochemistry 7*, 4110-4119.
Tamino, I. (1964) *J. Biochem. (Tokyo) 56*, 499.
Taylor, R. T., & Jenkins, W. T. (1966) *J. Biol. Chem. 241*, 4396-4405.
White, H. L., & Sato, T. L. (1978) *J. Neurochem. 31*, 41-47.
Yonaha, K., & Toyama, S. (1980a) *Arch. Biochem. Biophys. 200*, 156-164.
Yonaha, K., & Toyama, S. (1980b) *Anal. Biochem. 101*, 504-506.
Yonaha, K., Misono, H., Yamamoto, T., & Soda, K. (1975) *J. Biol. Chem. 250*, 6983-6989.
Yonaha, K., Toyama, S., & Kagamiyama, H. (1983) *J. Biol. Chem. 258*, 2260-2265.
Yonaha, K., Suzuki, K., & Toyama, S. (1985a) *J. Biol. Chem. 260*, 3265-3268.
Yonaha, K., Suzuki, K., & Toyama, S. (1985b) *Eur. J. Biochem. 146*, 101-106.

Unique Rearrangement of Ergocalciferol Side Chain in Vitro: Production of a Biologically Highly Active Homologue of 1,25-Dihydroxyvitamin D_3[†]

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ABSTRACT: In vitro incubation of 24-epi-25-hydroxyvitamin D_2 with chicken kidney homogenate produced several compounds, one of which had an affinity equal to that of 1,25-dihydroxyvitamin D_2 for the chick intestinal receptor. The affinity of 24-epi-1,25-dihydroxyvitamin D_2 for the same receptor was found to be half that of 1,25-dihydroxyvitamin D_2. The unknown compound was produced only when homogenate was prepared from pooled kidneys taken from both vitamin D deficient and replete chickens. The compound has been tentatively identified as 1,25-dihydroxy-22-dehydro-26-homovitamin D_3 by ultraviolet absorption spectrophotometry and mass spectrometry. Chemical synthesis of 1,25-dihydroxy-22-dehydro-26-homovitamin D_3 provided additional evidence for the structure. Administration of this 26-homologue of 1,25-dihydroxyvitamin D_3 at the dose level of 650 pmol/rat stimulated bone calcium mobilization in the hypocalcemic rat equal to that of 1,25-dihydroxyvitamin D_3. Thus, this paper demonstrates unique methyl migration on the side chain of 24-epi-1,25-dihydroxyvitamin D_3 to form a more biologically potent analogue.

Vitamin D_2 undergoes the same functional metabolism as vitamin D_3, namely, 25-hydroxylation in liver and 1-hydroxylation in kidney, to exert its activity on the target tissues (Jones et al., 1976a,b). Biological potencies of vitamin D_2 and/or its metabolites are equal to that of vitamin D_3 in the rat (Jones et al., 1975; Suda et al., 1970) and in humans (Jones et al., 1954), suggesting that the ergocalciferol side chain, which is unsaturated at C_{22}–C_{23} and has a methyl group at C_{24}, is of equal acceptability for the expression of vitamin D activity in these species. In the bird, however, vitamin D_2 and its metabolites are about one-tenth as active as those derived from vitamin D_3 (Chen & Bosmann, 1964; Drescher et al., 1969; Hibberd & Norman, 1969; Jones et al., 1976a,b). Accumulating data suggest that the differential biological activity of vitamin D_2 in the bird is due to the presence of a 24-methyl rather than an unsaturated side chain (DeLuca et al., 1968). In order to study the functional importance of 24-methyl of vitamin D_2, 25-hydroxyvitamin D_2 (25-OH-D_2) and its C-24 epimers were synthesized in the authors' laboratory (Morzycki et al., 1984) and their biological activities and metabolism studied. It was found that 24-epi-25-OH-D_2

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(*R* configuration at C-24) exhibited significantly lower biological activity in the rat and in binding to chick intestinal cytosol receptor protein for 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] than did 25-OH-D_2 (24*S* configuration) (Y. Tanaka, C. Smith, and H. F. DeLuca, unpublished data). During the course of studies on the metabolism and function of these epimers, we have isolated 24-epi-1,25-$(OH)_2D_2$, 24-epi-24,25-$(OH)_2D_2$, and a new metabolite from incubations of chick kidney homogenate with epi-25-OH-D_2. This unidentified compound is as active as natural 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ and 2–3 times more active than 24-epi-1,25-$(OH)_2D_2$ in binding to chick intestinal cytosol receptor despite the fact that it was derived from a 24-epi substrate. By mass spectral analyses of this compound and subsequent chemical synthesis, the unknown compound was tentatively identified as (22*E*)-1α,25-dihydroxy-22-dehydro-26-homovitamin D_3. Thus, this paper presents evidence for a unique migration of a methyl group of an aliphatic chain on a steroid that improves its activity in binding to the steroid receptor.

Materials and Methods

Vitamin D Compounds. 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were generous gifts from the Hoffmann-La Roche Co. (Nutley, NJ). 1,25-$(OH)_2D_2$ and its 24-epimer were biologically produced by incubation of the corresponding isomers of 25-OH-D_2 with vitamin D deficient chick kidney homogenate. 25-OH-D_2 and its 24-epimers were synthesized by Morzycki et al. (1984). 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (sp act. 160 Ci/mmol) was prepared by a method described elsewhere (Napoli et al., 1980) and was supplied by Du Pont/New England Nuclear (Boston, MA).

Animals. White Leghorn 1-day-old chicks were purchased from Northern Hatcheries (Beaver Dam, WI) and fed a vitamin D deficient diet (Omdahl et al., 1971) for 4 weeks. Half the chicks were given 6.5 nmol of vitamin D_3 dissolved in 0.1 mL of propylene glycol subcutaneously each day for 4 days prior to use. Weanling male rats were obtained from the Holtzman Co. (Madison, WI) and fed a low-calcium, vitamin D deficient diet for 3 weeks (Suda & DeLuca, 1970).

In Vitro Incubation of Kidney Homogenate. Equal amounts of kidney taken from vitamin D deficient and from replete chickens were pooled, and a 20% (w/v) homogenate was prepared in ice-cold buffer containing 0.19 M sucrose, 15 mM tris(hydroxymethyl)aminomethane (Tris)–acetate buffer (pH 7.4 at room temperature), and 1.9 mM magnesium acetate. The incubation was carried out in 125-mL Erlenmeyer flasks that contained 1 g of kidney tissue, 0.19 M sucrose, 15 mM Tris–acetate, 1.9 mM magnesium acetate, and 25 mM sodium succinate in a 7.5-mL final volume. The reaction was initiated by addition of 70 μg of 24-epi-25-OH-D_2 dissolved in 50 mL of 95% ethanol to incubation flasks. The mixtures were incubated at 37 °C with shaking at 100 oscillations/min for 90 min.

Extraction and Chromatography. Incubation mixtures from three flasks similarly prepared were pooled and extracted with a methanol–dichloromethane mixture by a modified Bligh and Dyer (1959) procedure. Dichloromethane phases from a total of three extractions were combined, and the solvent was removed by rotary evaporation. The residue was dissolved in 1 mL of 10% 2-propanol in hexane and placed in a freezer (−20 °C) overnight. Supernatant was removed and used for chromatography. The sample was subjected to high-performance liquid chromatography (HPLC) on a Waters Model ALC/GPC 204 instrument equipped with a 254-nm absorbance detector (Waters Associate, Melford, MA). Chromatography was performed on a Zorbax-Sil column (4.6 mm × 25 cm, 7–8 μm, Du Pont Instruments, Wilmington, DE) under a pressure of 1000 psi at a flow rate of 2 mL/min with 10% 2-propanol in a hexane solvent mixture. A compound that eluted at 24 mL (Figure 1, cross-hatched peak) was collected and then purified on reverse-phase high-performance liquid chromatography (HPLC) with a Richrosorb RP-18 column (Merck, Darmstadt, West Germany) and 15% water in methanol as the eluting solvent. The desired product was collected and reapplied to the same straight-phase HPLC system described above. The purified product was then assayed for binding activity to chick intestinal receptor and subjected to physical characterization.

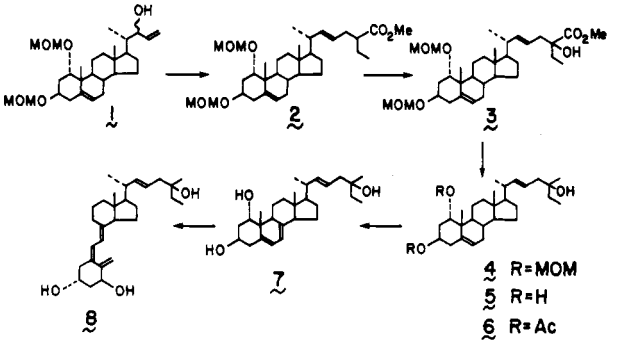

FIGURE 1: Synthesis scheme for 1α,25-dihydroxy-22-dehydro-26-homovitamin D_3.

General Methods. Melting points were determined with a hot-stage microscope and are uncorrected. ^{1}H NMR spectra were taken with a Hitachi R-24A (60 MHz) in $CDCl_3$ with Me_4Si as an internal standard unless otherwise noted. Ultraviolet absorption spectra were obtained in ethanol solution with either a Beckman Model 24 or a Shimazu UV-200 double-beam spectrophotometer. Mass spectra were obtained with either a Shimadzu QP-1000 mass spectrometer or an Associated Electric Industries 902 mass spectrometer. All spectra were run at 70 eV. Column chromatography was effected with silica gel (E. Merck, Kieselgel 60, 70–230 mesh). Preparative thin-layer chromatography was carried out on precoated plates of silica gel (E. Merck, Kieselgel 60 F_{254}, 0.25-mm thickness). The usual workup refers to dilution with water, extraction with an organic solvent indicated in parentheses, washing the extract to neutrality, drying over anhydrous magnesium sulfate, filtration, and removal of the solvent under reduced pressure.

Chemical Synthesis (Figure 1). (*A*) *(22E,25ξ)-1α,3β-Bis[(methoxymethyl)oxy]-26-homocholesta-5,22-dien-27-oic Acid Methyl Ester* (**2**). A solution of allylic alcohol **1** (Sai et al., 1986) (390 mg, 0.844 mmol), trimethyl ortho-*n*-butyrate (0.7 mL), and propionic acid (3 drops) in toluene (6 mL) was refluxed under argon for 2 h. Removal of the solvent under reduced pressure gave a crude product, which was applied to a column of silica gel (20 g). Elution with hexane–ethyl acetate (5:1) gave the ester **2** (446 mg, 97%) as an oil: ^{1}H NMR δ 0.68 (3 H, s, 18-H_3), 0.88 (3 H, t, J = 7 Hz, $-CH_2CH_3$), 0.98 (3 H, d, J = 6 Hz, 2-H_3), 1.03 (3 H, s, 19-H_3), 3.38 (3 H, s, $-OCH_3$), 3.43 (3 H, s, $-OCH_3$), 3.68 (3 H, s, $-CO_2CH_3$), 3.76 (1 H, m, 1β-H), 4.68 (2 H, s, 3β-OCH_2O–), 4.69 (2 H, ABq, J = Hz, ΔAB = 11 Hz, 1α-OCH_2O–), 5.27 (2 H, m, 22-H and 23-H), and 5.56 (1 H, m, 6-H).

(*B*) *(22E,25ξ)-1α,3β-Bis[(methoxymethyl)oxy]-25-hydroxy-26-homocholesta-5,22-dien-27-oic Acid Methyl Ester* (**3**). To a solution [prepared with diisopropylamine (0.13 mL, 0.929 mmol), 1.56 M *n*-butyllithium (0.59 mL), and tetrahydrofuran (THF) (2 mL)] ester **2** (437 mg, 0.800 mmol) in

THF (5 mL) was added, and the mixture was stirred under argon at −78 °C for 30 min. Oxygen was bubbled into this solution, and then triethyl phosphite (0.14 mL, 0.817 mmol) was added. The usual workup (ether for extraction) gave a crude product, which was applied to a column of silica gel (25 g). Elution with hexane–ethyl acetate (5:1) provided hydroxy ester **3** (303 mg, 67%) as an oil: ^{1}H NMR δ 0.68 (3 H, s, 18-H_3), 0.85 (3 H, t, J = 7 Hz, $-CH_2CH_3$), 0.98 (3 H, d, J = 6 Hz, 21-H_3), 1.02 (3 H, s, 19-H_3), 3.08 (1 H, br s, $W_{1/2}$ = 3 Hz, –OH), 3.38 (3 H, s, $-OCH_3$), 3.42 (3 H, s, $-OCH_3$), 3.76 (3 H, s, $-CO_2CH_3$), 4.68 (2 H, s, 3β-OCH_2O–), 4.68 (2 H, ABq, J = 7 Hz, ΔAB = 11 Hz, 1α-OCH_2O–), 5.32 (2 H, m, 22-H and 23-H), and 5.55 (1 H, m, 6-H).

(C) (22E,25ξ)-1α,3β-Bis[(methoxymethyl)oxy]-25-hydroxy-26-homocholesta-5,22-diene (**4**). To a solution of hydroxy ester **3** (294 mg, 0.539 mmol) in THF (5 mL) lithium aluminum hydride (20 mg, 0.526 mmol) was added, and this mixture was stirred at room temperature for 30 min. The usual workup (ether for extraction) gave a crude diol. This was treated with methanesulfonyl chloride (0.04 mL, 0.517 mmol) and pyridine (1.5 mL) at room temperature for 30 min. The usual workup (ether for extraction) gave a crude mesylate. To a solution of the crude mesylate in THF (5 mL) lithium aluminum hydride (20 mg, 0.526 mmol) was added, and the mixture was refluxed for 30 min. The usual workup (ether for extraction) gave a crude product, which was applied to a column of silica gel (20 g). Elution with hexane–ethyl acetate (5:1) provided alcohol **4** (190 mg, 70%) as an oil: ^{1}H NMR δ 0.71 (3 H, s, 18-H_3), 0.90 (3 H, t, J = 7 Hz, $-CH_2CH_3$), 1.03 (3 H, d, J = 6 Hz, 21-H_3), 1.03 (3 H, s, 19-H_3), 1.12 (3 H, s, 27-H_3), 3.36 (3 H, s, $-OCH_3$), 3.40 (3 H, s, $-OCH_3$), 3.74 (1 H, m, 1β-H), 4.66 (2 H, s, 3β-OCH_2O–), 4.67 (2 H, ABq, J = 7 Hz, ΔAB = 11 Hz, 1α-OCH_2O–), 5.35 (2 H, m, 22-H and 23-H), and 5.54 (1 H, m, 6-H).

(D) (22E,25ξ)-1α,3β,25-Trihydroxy-26-homocholesta-5,22-diene (**5**). A solution of dimethoxymethyl ester **4** (181 mg, 0.349 mmol) in THF (5 mL) was treated with 6 N HCl (1 mL) at 50 °C for 1.5 h. The usual workup (ethyl acetate for extraction) gave a crude product, which was applied to a column of silica gel (15 g). Elution with hexane–ethyl acetate (1:2) provided triol **5** (147 mg, 98%): mp 85–87 °C (hexane–dichloromethane); ^{1}H NMR δ 0.69 (3 H, s, 18-H_3), 0.89 (3 H, t, J = 7 Hz, $-CH_2CH_3$), 1.02 (3 H, s, 19-H_3), 1.13 (3 H, s, 27-H_3), 3.85 (1 H, m, 1β-H), 3.98 (1 H, m, 3α-H), 5.40 (2 H, m, 22-H and 23-H), and 5.60 (1 H, m, 6-H).

(E) (22E,25ξ)-1α,3β-Diacetoxy-25-hydroxy-26-homocholesta-5,22-diene (**6**). A solution of triol **5** (100 mg, 0.233 mmol) in pyridine (1 mL) was treated with acetic anhydride (1 mL) at room temperature for 15 h. The usual workup (ethyl acetate for extraction) gave a crude product, which was applied to a column of silica gel (10 g). Elution with hexane–ethyl acetate (5:1) provided diacetate **6** (101 mg, 85%) as an amorphous solid: ^{1}H NMR δ 0.68 (3 H, s, 18-H_3), 0.88 (3 H, t, J = 7 Hz, $-CH_2CH_3$), 0.98 (3 H, d, J = 6 Hz, 21-H_3), 1.08 (3 H, s, 19-H_3), 1.12 (3 H, s, 27-H_3), 2.03 (3 H, s, acetyl), 2.06 (3 H, s, acetyl), 4.98 (1 H, m, 3α-H), 5.06 (1 H, m, 1β-H), 5.37 (2 H, m, 22-H and 23-H), and 5.53 (1 H, m, 6-H).

(F) (22E,25ξ)-1α,3β,25-Trihydroxy-26-homocholesta-5,7,22-triene (**7**). A solution of 5,22-diene **6** (38 mg, 0.0739 mmol) and *N*-bromosuccinimide (19 mg, 0.107 mmol) in carbon tetrachloride (3 mL) was refluxed under argon for 20 min. After being cooled to 0 °C, the resulting precipitate was filtered off. The filtrate was concentrated below 40 °C to leave the residue. The THF (5 mL) solution of this residue was treated with a catalytic amount of tetra-*n*-butylammonium bromide at room temperature for 50 min. Then, the mixture was treated with a solution of tetra-*n*-butylammonium fluoride in THF (0.3 mL, 0.3 mmol) at room temperature for 30 min. The usual workup (ethyl acetate for extraction) gave a crude triene. This triene in THF (5 mL) was treated with 5% KOH–MeOH (4 mL) at room temperature for 14 h. The usual workup (ethyl acetate for extraction) gave a crude product, which was submitted to preparative thin-layer chromatography (benzene–ethyl acetate, 1:1, developed 6 times). The band of R_f value 0.45 was scrapped off and eluted with ethyl acetate. Removal of the solvent provided 5,7,22-triene **7** (8.7 mg, 40%): UV λ_{max}^{EtOH} 293, 282, 271 nm.

(G) (22E,25ξ)-1α,25-Dihydroxy-22-dehydro-26-homovitamin D_3 (**8**). A solution of triene **7** (4.4 mg, 0.0103 mmol) in benzene (90 mL) and ethanol (40 mL) was irradiated with a medium-pressure mercury lamp through a Vycor filter at 0 °C under argon for 2.5 min. The reaction mixture was refluxed under argon for 1 h. Removal of the solvent under reduced pressure gave a crude product, which was submitted to preparative thin-layer chromatography (benzene–ethyl acetate, 1:1, developed 6 times). The band of R_f value 0.49 was scraped off and eluted with ethyl acetate. Removal of the solvent provided vitamin D_3 analogue **8** (0.91 mg, 21%): UV λ_{max}^{EtOH} 265 nm, λ_{min}^{EtOH} 282 nm; mass spectrum, m/z 428 (M^+), 410, 392, 356, 338, 320, 287, 269, 251, 152, 134, 73; ^{1}H NMR (400 MHz) δ 0.56 (3 H, s, 18-H_3), 0.91 (3 H, t, J = 7.6 Hz, $-CH_2CH_3$), 1.04 (3 H, d, J = 6.8 Hz, 21-H_3), 1.13 (3 H, s, 27-H_3), 4.23 (1 H, m, $W_{1/2}$ = 18.4 Hz, 3α-H), 4.43 (1 H, m, $W_{1/2}$ = 16.9 Hz, 1β-H), 5.00 (1 H, br s, $W_{1/2}$ = 3.2 Hz, 19-H), 5.32 (1 H, br s, $W_{1/2}$ = 3.2 Hz, 19-H), 5.37 (2 H, m, 22-H and 23-H), 6.02 (1 H, d, J = 11.5 Hz, 7-H), and 6.38 (1 H, d, J = 11.5 Hz, 6-H).

Receptor Binding Assay. The competitive binding assay was performed as previously described by Shepard et al. (1979). Intestinal mucosal extract prepared from vitamin D deficient chicks was incubated with 1,25-$(OH)_2$-[26,27-^{3}H]D_3 and various concentrations of unlabeled compounds for 18 h at 6 °C. Specific binding was determined by subtraction of nonspecific binding [binding in the presence of excess of unlabeled 1,25-$(OH)_2D_3$] from total binding (Figures 4 and 6).

Measurement of Biological Activity in Rat. Six hundred fifty picomoles of the test compound was dissolved in 0.05 mL of 95% ethanol and administered intrajugularly 7 h prior to determination of serum calcium concentration. Rats in the control group were given 0.05 mL of ethanol in the same manner. Rats were killed by decapitation and blood was collected. Calcium concentration in serum obtained by centrifugation of blood was determined with a Perkin-Elmer atomic absorption spectrometer (Perkin-Elmer, CT) in the presence of 0.1% lanthanum chloride.

Results

Figure 2 illustrates an HPLC profile of the lipid extract from kidney homogenate incubated with 24-epi-25-OH-D_2 as detected by UV absorption monitor set at 254 nm. Unchanged substrate, 24-epi-25-OH-D_2, was eluted at 8 mL (not shown). Compounds eluting just prior to the elution positions of authentic 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were isolated in pure form and identified as 24-epi-24,25-$(OH)_2D_2$ and 24-epi-1,25-$(OH)_2D_2$, respectively, by UV spectrophotometry and mass spectrometry (Y. Tanaka and H. F. DeLuca, unpublished data). The cross-hatched peak eluting just prior to 24-epi-1,25-$(OH)_2D_2$ produced in vitro was obtained only when a mixture of vitamin D deficient and replete chick homogenates was used as a source of enzyme. When kidney homogenate

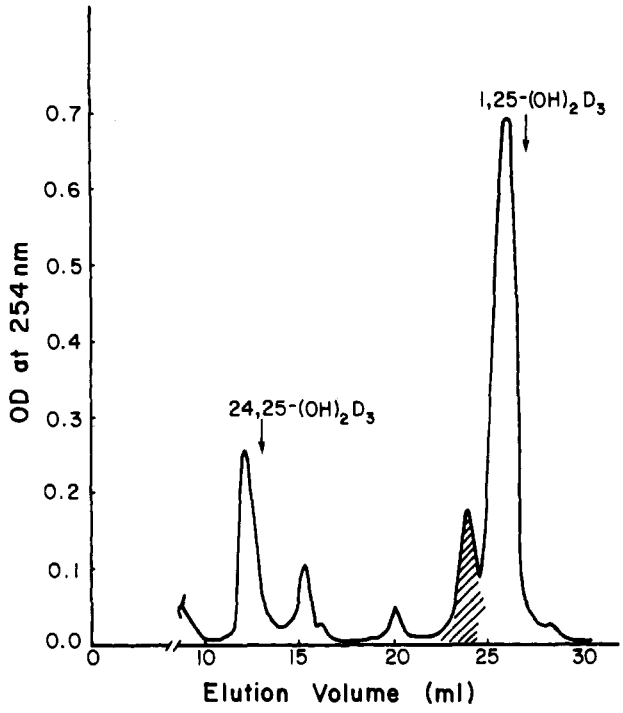

FIGURE 2: Ultraviolet absorption profile of an HPLC chromatogram of an extract of an incubation mixture containing 24-epi-25-OH-D_2 and kidney homogenate from both vitamin D deficient and replete chickens. A Zorbax-Sil column was eluted with 10% 2-propanol in hexane at a pressure of 1000 lb/in.^{2} and a flow rate of 2 mL/min. Arrows indicate elution positions of 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$. A cross-hatched peak indicates the unknown compound produced in vitro.

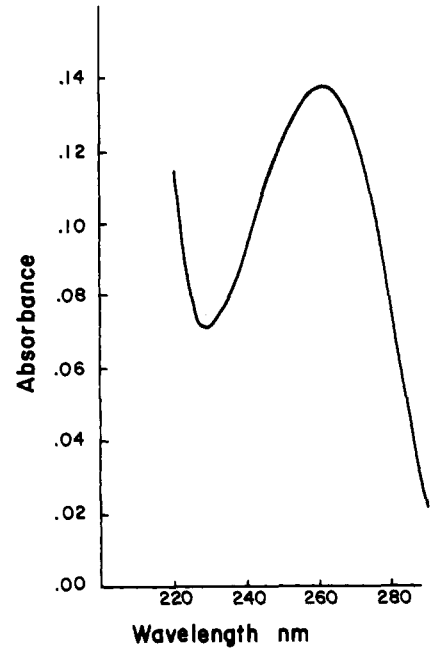

FIGURE 3: Ultraviolet absorption spectrum of purified unknown compound.

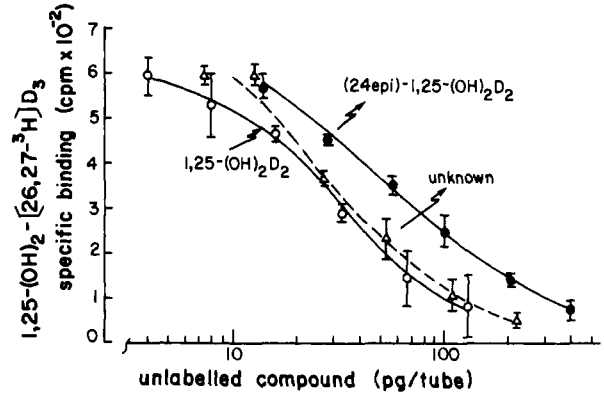

FIGURE 4: Binding affinity of unknown compound and 24-epimers of 1,25-$(OH)_2D_2$ for chick intestinal receptor. Displacement of 1,25-$(OH)_2$-[26,27-^{3}H]D_3 from the receptor protein by the test compound was carried out by the method described by Shepard et al. (1979). Various concentrations of compounds were dissolved in 50 μL of 95% ethanol and added to the cytosol receptor in the presence of 1,25-$(OH)_2$-[26,27-^{3}H]D_3. Each point represents the mean value of triplicate determinations with standard deviation from the mean. Specific binding of 1,25-$(OH)_2$-[^{3}H]D_3 was calculated by subtraction of nonspecific binding from total binding. Concentration of the unknown compound was estimated by the assumption that the compound has same extinction coefficient and molecular weight as those of 1,25-$(OH)_2D_2$ isomers.

from vitamin D deficient chickens was used, 24-epi-1,25-$(OH)_2D_2$ was a sole product, while 24-epi-24,25-$(OH)_2D_2$ was a major product when kidney homogenate from vitamin D replete chickens was used (profile not shown). The unknown compound was not produced when 24-epi-25-OH-D_2 was incubated with boiled homogenate or when fresh homogenate was incubated without addition of the substrate. Furthermore, it was not produced when 25-OH-D_2 was used as substrate with the mixtures of tissues. A base-line separation of the unknown compound from 24-epi-1,25-$(OH)_2D_2$ for purification was achieved by either a straight-phase HPLC with a 6% 2-propanol in hexane solvent system or a reverse-phase HPLC with 15% H_2O in methanol (profile not shown).

As shown in Figure 3, the UV absorption spectrum of the purified unknown product exhibited the typical vitamin D triene system with λ_{max} 265 nm, λ_{min} 228 nm, and $\lambda_{max}/\lambda_{min}$ = 1.8. Assuming that this compound has the same molar extinction coefficient and molecular weight as 1,25-$(OH)_2D_2$ (18 200 and 428, respectively), a total of 3.4 μg of the unknown compound was produced.

On the basis of the assumed amount, the potency of the compound to compete with 1,25-$(OH)_2D_3$ for binding to the chick intestinal receptor protein was examined. The unknown compound binds to the receptor as well as does 1,25-$(OH)_2D_2$, while 24-epi-1,25-$(OH)_2D_2$ is 2–3 times less active than 1,25-$(OH)_2D_2$ (Figure 4). In our hands, 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ binds equally well to the 1,25-$(OH)_2D_3$ receptor in chick intestine.

The mass spectrum (Figure 5B) of the unknown product contains a molecular ion at m/z 428 along with ions and m/z 410 and 392, which represent elimination of one and two molecules of H_2O. Loss of the entire steroid side chain (cleavage of C_{17}–C_{20} bond) of the compound results in the fragment of m/z 287, which by elimination of one and two molecules of H_2O gives rise to the peaks at m/z 269 and 251. The spectrum shows prominent peaks at m/z 152, which represents ring A + C_6 + C_7, and at m/z 134, which represents elimination of one molecule of H_2O. These two peaks are diagnostic for the 1α,3β-dihydroxy A ring of vitamin D (Paaren et al., 1978). It was thus confirmed that this compound has a 1α-hydroxylated A ring, *cis*-triene system, and intact C and D rings of vitamin D. These fragmentation patterns are identical with those found in the spectrum of 24-epi-1,25-$(OH)_2D_2$ as shown in Figure 5A. However, the spectrum of 24-epi-1,25-$(OH)_2D_2$ contains an intense ion at m/z 59 that results from cleavage of the C_{24}–C_{25} bond and is characteristic of 25-hydroxylated vitamin D compounds with no additional substitution on C_{26} or C_{27} (Blunt et al., 1968), while the spectrum of the unknown does not show the peak at m/z 59 but a very intense peak at m/z 73 as shown in

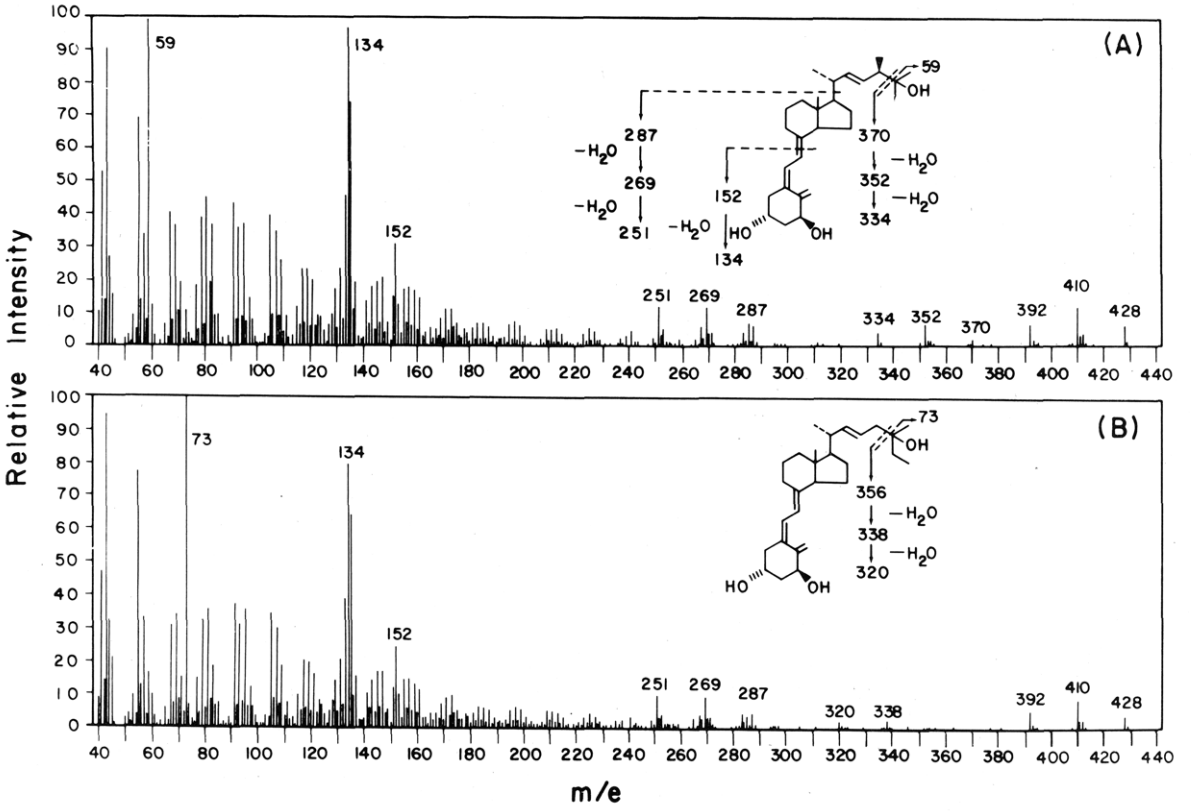

FIGURE 5: Mass spectrum (A) of in vitro produced 24-epi-1,25-$(OH)_2D_2$ and (B) of the unknown compound with suggested structure for the compound.

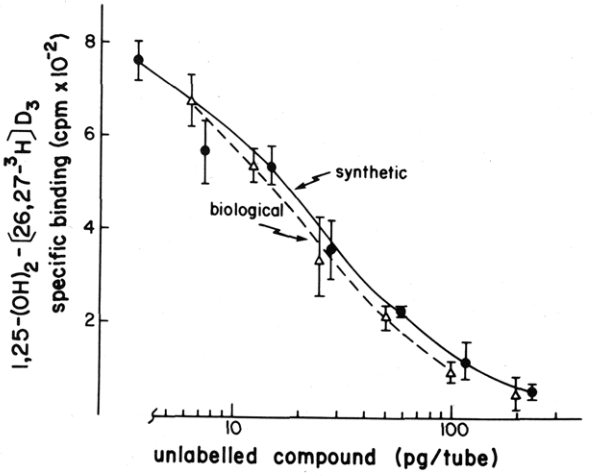

FIGURE 6: Binding affinity of biologically produced or chemically synthesized 26-homologue. Method is described in the Figure 4 legend.

Figure 5B. The absence of a prominent peak at m/z 59 and the presence of a peak at m/z 73 suggest that $-CH_2$ was added on C_{26} or C_{27}. On the basis of all available data, a possible structure of the unknown compound was proposed as 1α,25-dihydroxy-22-dehydro-26-homovitamin D_3. The proposed structure is shown in Figure 5B.

To gather further evidence for the assigned structure, chemical synthesis of the compound was performed by the route shown in Figure 1. It was found that the UV absorption spectrum and mass spectrum of the synthetic (22*E*)-1α,25-dihydroxy-22-dehydro-26-homovitamin D_3 are identical with those obtained from the biologically produced compound (**8**). Further evidence for identification of the biological compound was presented by chromatography and receptor binding of the synthetic compound. The synthetic analogue comigrated with the biologically generated compound on both straight- and reverse-phase HPLC systems described above (profiles not shown). As shown in Figure 6, the synthetic compound has identical binding affinity for the receptor as the biological product. Therefore, the structure of the compound enzymatically produced from 24-epi-25-OH-D_2 is likely (22*E*)-1α,25-dihydroxy-22-dehydro-26-homovitamin D_3.

Table I: Increase in Serum Calcium Concentration in Response to 1,25-Dihydroxyepi-Δ^{22}-26-homovitamin D_3 or 1,25-$(OH)_2D_3$ Administration[a]

compd	serum calcium concn (mg/100 mL)
ethanol	3.4 ± 0.3[b]
1,25-$(OH)_2$-epi-Δ^{22}-26-homo-D_3	4.6 ± 0.3[c]
1,25-$(OH)_2D_3$	4.5 ± 0.2[c]

[a] Rats that had been fed a low-calcium, vitamin D deficient diet for 3 weeks were divided into three groups of six rats each. They were given 650 pmol of either Δ^{22}-26-homo-1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$, respectively, dissolved in 0.05 mL of 95% ethanol, intrajugularly 7 h prior to sacrifice. Rats in the control group were given an ethanol vehicle in the same manner. Serum calcium concentration was determined as described in the text. [b] Standard deviation from the mean. [c] Significantly different from value for ethanol at $p < 0.001$.

Table I provides the results of in vivo biological assay of the 26-homo analogue in the rat. Chemically synthesized analogue was tested at the dose level of 650 pmol/100 g of body weight in comparison with 1,25-$(OH)_2D_3$ at an identical dose level. Either compound increased serum calcium concentration equally well at 7-h postadministration.

Discussion

The in vitro generated compound described in this paper is a side-reaction product in the preparation of 24-epi-1,25-$(OH)_2D_2$ and 24-epi-24,25-$(OH)_2D_2$. Since its elution position on the HPLC systems employed as similar to that of 24-epi-1,25-$(OH)_2D_2$, the unknown compound was purified during the isolation of 24-epi-1,25-$(OH)_2D_2$. However, the high

affinity of the unknown compound for the 1,25-$(OH)_2D_3$ receptor attracted our attention. It seemed unlikely that a metabolite derived from 24-epi-25-OH-D_2 should be more active than 24-epi-1,25-$(OH)_2D_2$. The requirement of kidney homogenate prepared from vitamin D deficient chicks for in vitro generation of the unknown compound suggests that the compound is 1-hydroxylated (Fraser & Kodicek, 1970), while the requirement for kidney homogenate prepared from vitamin D treated chicks suggests that the molecule is further hydroxylated on the side chain such as on C_{23}, C_{24}, or C_{26} DeLuca, 1981) or lactonized at C_{23} and C_{26} (DeLuca, 1981). However, in the case of 1,25-$(OH)_2D_3$, such side-chain modification markedly reduces receptor activity (Wichmann et al., 1979). Such modification would be expected to increase polarity on HPLC rather than decrease it as shown in Figure 2. Therefore, hydroxylated or lactonized 24-epi-1,25-$(OH)_2D_2$ seemed unlikely for the structure of the unknown compound. Another speculation for the structure might be epimerization of the 24-methyl to give natural 1,25-$(OH)_2D_2$. In fact, the unknown compound comigrated with 1,25-$(OH)_2D_2$ on straight-phase HPLC performed with 10% 2-propanol in the hexane solvent system. Together with the equal binding competency to the intestinal receptor, the assumption seemed reasonable. However, the mass spectrum of the unknown compound revealed that it was neither 1,25-$(OH)_2D_2$ nor an oxidation product but a product with a rearranged side chain.

A possible structure of the unknown compound was assigned to account for the mass spectrum. Enzymatic rearrangement of vitamin D side chain, however, has never been reported. Moreover, it is difficult to believe that a 24-methyl could migrate to form a 26-homologue that results in a compound biologically as active as natural 1,25-$(OH)_2D_3$. Thus, chemical synthesis of the compound was considered of fundamental importance for assignment of the structure. The synthetic compound was found to be identical in every respect including mass spectrum, chromatographic behavior, and receptor binding affinity with the enzymatically produced compound. The binding affinity of the synthetic compound to the receptor seemed slightly but not significantly less than that of the enzymatically produced compound. This may be because the synthetic compound is likely a C_{25} epimeric mixture. Determination of the 25-position stereochemical configuration must await stereospecific chemical synthesis of the compound.

In addition to the 26-homo analogue, another homologue of 1,25-$(OH)_2D_3$, (22*E*)-1α,25-dihydroxy-22-dehydro-24-homovitamin D_3, was synthesized (H. Sai, N. Ikekawa, Y. Tanaka, and H. F. DeLuca, unpublished data), considering that it might also account for the mass spectrum obtained from the biologically produced compound. However, that compound did not comigrate with the enzymatically produced compound on straight-phase HPLC, and the mass spectrum differed substantially. Although this excludes the 24-homologue as the biologically generated compound, the results to data strongly support but do not absolutely prove the structure of the biologically synthesized compound from epi-24-hydroxyvitamin D_2.

Enzymatic catalyzed migration of methyl group(s) is a rare phenomenon enzymatically. It has been known that the "NIH shift" involves the migration of groups from the position of hydroxyl attack on the aromatic system to an adjacent position on the aromatic ring (Guroff et al., 1967). For example, the methyl group of *p*-methylphenylalanine migrates to an adjacent position on the ring by phenylalanine hydroxylase to produce *m*-methyltyrosine (Daly & Guroff, 1968). It was also reported that an alkyl side chain of *p*-hydroxyphenyl pyruvate migrates to the adjacent position on the ring, but in this case with decarboxylation (Schepartz & Gurin, 1949). The present case is quite unique because a methyl group on an aliphatic chain migrates without involvement of hydroxylation of the position to which the group is attached. It is unknown at this moment whether this rearrangement occurs in vivo when 24-epi-25-OH-D_2 is administered. It is also unknown whether the side-chain rearrangement will occur with 25-OH-D_2 as the substrate.

The substrate 24-epi-25-OH-D_2 undergoes two modifications to yield the isolated product. First, 1α-hydroxylation must occur, and second, the side-chain modification must occur. Undoubtedly, kidneys from vitamin D deficient animals are required for 1α-hydroxylation, while kidney from vitamin D sufficient rats is needed for the side-chain modification. It is not clear which modification occurs first, a subject of current investigation. However, in a normal animal, both reactions should be occurring, suggesting that although the mixture of tissue was required to produce sufficient amounts to be detected, small amounts might be found under in vivo circumstances.

Although the 26-homo-1,25-dihydroxy compound is as active as natural 1,25-$(OH)_2D_2$ in receptor binding, this might not be reflected in vivo because of possible metabolic transformation or inefficient transport to the target tissues. Thus, we examined activity of the 26-homo analogue in elevating serum calcium of calcium and vitamin D deficient rats. Although the relative potency of the 26-homologue to that of 1,25-$(OH)_2D_3$ must await a thorough comparison, our bioassay showed it approximately equal to that of 1,25-$(OH)_2D_3$. Regardless of the physiological significance of the present findings, the data presented here demonstrate that lengthening the terminal side chain of 1,25-$(OH)_2D_3$ by one carbon does not diminish biological activity appreciably; second, they provide an unexpected methyl migration in a steroid system and on an aliphatic side chain.

Registry No. **1**, 99741-49-8; **2**, 103732-09-8; **3**, 103732-10-1; **4**, 103732-11-2; **5**, 103732-12-3; **6**, 103732-13-4; **7**, 103732-14-5; **8**, 103732-08-7; $PrC(OMe)_3$, 43083-12-1; Ca, 7440-70-2; $EtCO_2H$, 79-09-4; 24-epi-25-hydroxyvitamin D_2, 90191-28-9; 1,25-dihydroxyvitamin D_2, 55248-15-2; 24-epi-1,25-dihydroxyvitamin D_2, 95783-08-7.

References

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Blunt, J. W., DeLuca, H. F., & Schnoes, H. K. (1968) *Biochemistry 7*, 3317–3322.

Chen, P. S., Jr., & Bosmann, H. B. (1964) *J. Nutr. 83*, 133–139.

Daly, J. W., & Guroff, G. (1968) *Arch. Biochem. Biophys. 125*, 136–141.

DeLuca, H. F. (1981) *Annu. Rev. Physiol. 43*, 199–209.

DeLuca, H. F., Weller, M., Blunt, J. W., & Neville, P. F. (1968) *Arch. Biochem. Biophys. 124*, 122–128.

Drescher, D., DeLuca, H. F., & Imrie, M. H. (1969) *Arch. Biochem. Biophys. 130*, 657–661.

Fraser, D. R., & Kodicek, E. (1970) *Nature (London) 228*, 764–766.

Guroff, G., Daly, J. W., Jerina, D. M., Renson, J., Whtkop, B., & Udenfriend, S. (1967) *Science (Washington, D.C.) 157*, 1524–1526.

Hibberd, K. A., & Norman, A. W. (1969) *Biochem. Pharmacol. 18*, 2347–2355.

Jones, G., Schnoes, H. K., & DeLuca, H. F. (1975) *Biochemistry 14*, 1250–1256.

Jones, G., Baxter, L. A., DeLuca, H. F., & Schnoes, H. K. (1976a) *Biochemistry 15*, 713–716.
Jones, G., Schnoes, H. K., & DeLuca, H. F. (1976b) *J. Biol. Chem. 251*, 24–28.
Jones, J. H., Kramer, B., & Knof, A. (1954) in *The Vitamins* (Sebrell, W. H., Jr., & Harris, R. S., Eds.) Chapter 8, pp 253–266, Academic, New York.
Morzycki, J. W., Schnoes, H. K., & DeLuca, H. F. (1984) *J. Org. Chem. 49*, 2148–2151.
Napoli, J. L., Mellon, W. S., Fivizzani, M. A., Schnoes, H. K., & DeLuca, H. F. (1980) *Biochemistry 19*, 2515–2521.
Omdahl, J., Holick, M., Suda, T., Tanaka, Y., & DeLuca, H. F. (1971) *Biochemistry 10*, 2935–2940.
Paaren, H. E., Hamer, D. E., Schnoes, H. K., & DeLuca, H. F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2080–2081.
Sai, H., Takatsuto, S., Ikekawa, N., Tanaka, Y., & DeLuca, H. F. (1986) *Chem. Pharm. Bull.* (in press).
Schepartz, B., & Gurin, S. (1949) *J. Biol. Chem. 180*, 663–666.
Shepard, R. M., Horst, R. L., Hamstra, A. J., & DeLuca, H. F. (1979) *Biochem. J. 182*, 55–69.
Suda, T., DeLuca, H. F., & Tanaka, Y. (1970) *J. Nutr. 100*, 1049–1052.
Wichmann, J. K., DeLuca, H. F., Schnoes, H. K., Horst, R. L., Shepard, R. M., & Jorgensen, N. A. (1979) *Biochemistry 18*, 4775–4780.

Evidence for Posttranslational O-Glycosylation of Fetuin†

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ABSTRACT: Fetuin, a major glycoprotein in the serum of fetal calves that contains three N-linked and three O-linked carbohydrate side chains, was found to be synthesized in the liver with an 18 amino acid signal peptide, Met-X-X-X-X-Leu-Leu-X-Cys-Leu-Ala-X-Leu-X-X-Cys-X-X, and to undergo cotransational N-glycosylation. In order to examine O-glycosylation, fetuin peptidyl-tRNA was purified from liver and analyzed for O-linked carbohydrate by quantitating the released [^{3}H]GalNAcitol produced after β-elimination in the presence of NaB^3H_4. Within the limits of the assay, <1.3% of the O-linked chains had been initiated. Additionally, rough microsomes were used to program a cell-free protein synthesis system. A radiolabeled fetuin intermediate was isolated by immunoprecipitation and shown to contain N-linked carbohydrate by binding to concanavalin A and by susceptibility to cleavage by endoglycosidase H. However, this fetuin intermediate was not detectably bound (<1%) by GalNAc-specific lectins, which were shown to bind asialoagalactofetuin. These results suggest that O-glycosylation of fetuin is a posttranslational event.

Many serum proteins are glycosylated, and their carbohydrate side chains are frequently attached through a glycosylamine linkage to specific asparagine residues. It is well established that the formation of these N-linked side chains begins in the rough endoplasmic reticulum (RER) with the cotranslational transfer of a core oligosaccharide unit from a lipid carrier to protein asparagine residues (Presper & Heath, 1983; Hanover & Lennarz, 1981; Staneloni & Leloir, 1982). Carbohydrate attached through a glycosidic linkage to serine or threonine residues occurs in serum proteins less frequently. These O-linked carbohydrate side chains are synthesized by the sequential addition of individual monosaccharide units from nucleotide sugars starting with the attachment of an α-linked GalNAc residue. Although Strous has presented evidence that O-glycosylation is initiated cotranslationally (Strous, 1979), most of the data currently available indicate that O-glycosylation is posttranslational, occurring in the Golgi apparatus [Hanover and Lennarz (1981) and Berger et al. (1982) for reviews].

Fetuin, an abundant serum protein found in fetal calves, has three N-linked and three O-linked carbohydrate side chains (Spiro & Bhoyroo, 1974). Fetuin was found to be a major protein synthesized by the liver and, as for many other secretory proteins, was found to be synthesized with a signal peptide on membrane-bound polysomes and to undergo cotranslational N-glycosylation. Therefore, fetal bovine liver peptidyl-tRNA was analyzed for the presence of O-linked GalNAc by two independent methods of analysis in order to compare the temporal and spatial relationship between polypeptide synthesis and both N- and O-glycosylation.

Experimental Procedures

Materials. Fetuin, purified by the method of Spiro, was purchased from GIBCO Laboratories (Grand Island, NY). Protein A coupled to Sepharose and bovine submaxillary mucin were from Sigma Chemical Co. (St. Louis, MO). Concanavalin A–agarose and endo-β-*N*-acetylglucosaminidase H were purchased from Miles Laboratories, Inc. (Elkhart, IN). The

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