affinities for 3′-nucleotide substrates and 2′-deoxy-3′-nucleotide substrate analogs; for ribonuclease $T_1$ Guo-2′-P > dGuo-3′-P, whereas for ribonuclease A dUrd-3′-P > Urd-3′-P (Walz, 1971). The significance that binding (or not binding) of the 2′-hydroxyl group has regarding the catalytic mechanisms of the two ribonucleases remains to be determined. It could be speculated that ribonuclease $T_1$ utilizes binding at the 2′-hydroxyl position of guanosine moieties in RNA as a significant contribution to the binding energy of the enzyme–substrate complexes in the absence of gross coulombic interactions which probably contribute to ribonuclease A–RNA binding (Richards and Wyckoff, 1971). Nevertheless, preliminary difference spectral experiments in our laboratory have indicated that ribonuclease $T_1$ can bind to single-stranded calf thymus DNA.

Future kinetic and equilibrium binding studies will consider the interactions of ribonuclease $T_1$ with diphosphate substrates and substrate analogs including P-5′-Guo-2′:3′-P, P-5′-Guo-3′-P, and P-5′-dGuo-3′-P. These planned studies were prompted by steady-state kinetic studies of the ribonuclease A catalyzed hydrolysis of P-5′-Urd-2′:3′-P which indicated a specific interaction of the substrate 5′-phosphomonoester group with the enzyme (J. Li and F. G. Walz, submitted for publication).

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# 24,25-Dihydroxyvitamin $D_3$. Synthesis and Biological Activity†

Hing-Yat Lam, Heinrich K. Schnoes, Hector F. DeLuca,* and Tai C. Chen‡

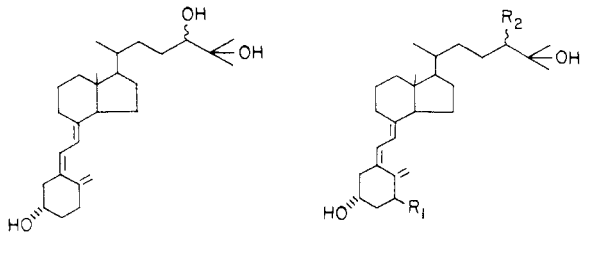
ABSTRACT: A kidney metabolite of vitamin $D_3$, 24,25-dihydroxyvitamin $D_3$, has been synthesized by an unambiguous route. Like the natural metabolite, the synthetic product elicits a pronounced and long-lasting intestinal calcium transport response, but has no effect on the bone calcium mobilization process.

The isolation and characterization of 24,25-dihydroxyvitamin $D_3$ (24,25-$(OH)_2D_3$)[1] (**1**) (Figure 1) was reported by us recently (Holick *et al.*, 1972). Although the function of this naturally occurring vitamin D metabolite is not completely understood at present, several findings suggest that the compound may play a significant role in vitamin D metabolism and its control: (a) 24,25-$(OH)_2D_3$, like the very potent 1$\alpha$,25-dihydroxyvitamin $D_3$ (1$\alpha$,25-$(OH)_2D_3$ (**2**) (Figure 1), is made in kidney tissue from 25-hydroxyvitamin $D_3$ (25-OH-$D_3$) (Holick *et al.*, 1972); (b) it is specifically active in promoting calcium transport in intestine and shows little or no effect on bone mineral mobilization (Boyle *et al.*, 1973); (c) its biological synthesis in kidney occurs at normal or high levels of serum calcium whereas low calcium levels induce production of 1$\alpha$,25-$(OH)_2D_3$ (Boyle *et al.*, 1971), an effect which can be shown to be mediated through parathyroid hormone (Garabedian *et al.*, 1972); and (d) it is further metabolized in the kidney to a more polar product (Boyle *et al.*, 1973) recently characterized (Holick *et al.*, 1973) as 1,24,25-trihydroxyvitamin $D_3$ (1,24,25-$(OH)_3D_3$) (**3**), the metabolite believed to be responsible for the intestinal calcium transport activity of **1**, since 24,25-$(OH)_2D_3$ itself can be shown to be inactive in anephric animals. These facts, which suggested promising further investigations into possible biological roles of metabolite **1**, and the need to confirm its structure, led us to undertake a synthesis of this compound.

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received July 16, 1973.* Supported by grants from the U. S. Public Health Service, Nos. AM-14881 and AM-15512, and the Wisconsin Alumni Research Foundation.

‡ Present address: Veterans Administration Hospital, LaJolla, Calif. 92161.

[1] Abbreviations used are: 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin $D_3$; 1$\alpha$,25-$(OH)_2D_3$, 1$\alpha$,25-dihydroxyvitamin $D_3$; 25-OH-$D_3$, 25-hydroxyvitamin $D_3$; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin $D_3$.



1

2: $R_1$ = OH, $R_2$ = H

3: $R_1$ = $R_2$ = OH

FIGURE 1: Vitamin D metabolites.

## Experimental Section

Ultraviolet absorption spectra were recorded on a Cary-15 instrument; infrared spectra (in chloroform) on a Beckman Model IR-5 spectrometer; mass spectra (at 70 eV) were obtained on an A.E.I. Model MS-902 mass spectrometer using a direct probe for introduction of samples (source temperature 130–150° above ambient); nuclear magnetic resonance (nmr) spectra were recorded on a Varian T-60 spectrometer or a Varian XL-100 spectrometer with deuteriochloroform as solvent and resonance signals are recorded with reference to $Me_4Si$ as internal standard. For thin-layer chromatography (tlc), air-dried silica gel G plates, developed with cyclohexane–ethyl acetate mixtures were used. Gas–liquid chromatography (glc) was carried out with an F&M Model 402 chromatograph equipped with a 0.25 in. × 4 ft glass column packed with 3% SE-30 on gas ChromZ, 100–120 mesh, operated isothermally at 255°, at a flow rate of 80 ml/min. Commercial Skellysolve B was distilled and the fraction boiling at 67–69° was used. Benzene (AR grade) was redistilled and dried over molecular sieves. Other solvents were reagent grade and used as such. Radioactive counting was carried out with a Packard Tri-Carb Model 3375 liquid scintillation counter equipped with an automatic external standard system. Optical rotations were measured in chloroform solution with a Perkin-Elmer Model 141 polarimeter. Melting points were measured on a hot-stage apparatus and are uncorrected. Elemental analyses were performed by Micro-Tech Laboratories, Skokie, Ill.

### *Synthesis of 24,25-$(OH)_2D_3$*

*3β,24ξ-Diacetoxy-27-norcholest-5-en-25-one* (**6**). The starting material, 3β-acetoxy-27-nor-5-cholesten-25-one (**4**) (7 g, mp 138–139°, $[\alpha]_D^{20}$ −45.6° (*c* 2.3, $CHCl_3$), obtained by acetylation of the corresponding 3β-hydroxy compound) was refluxed for 6 hr in 100 ml of $Ac_2O$ containing 570 mg of toluene–sulfonic acid hydrate. Water was then added to the reaction mixture, and the precipitated solid was filtered, taken up in ether, and combined with ether extracts of the aqueous solution. The ether solution, after washing with 1% ice-cold NaOH and water, was dried over $Na_2SO_4$ and evaporated *in vacuo*. Glc analysis of the residue showed the presence of three components; namely, starting material (**4**) and the two isomeric (cis and trans) $\Delta^{24}$-enol acetates. The residue was chromatographed on 400 g of activated silicic acid (4-cm diameter column) and eluted batchwise with ether–Skellysolve B mixtures (300 ml of 10% ether, 1200 ml of 20%, 200 ml of 30%, 300 ml of 40%, and finally pure ether) collecting 11-ml fractions. The major $\Delta^{24}$-enol acetate isomer eluted first (tubes 105–109) and was crystallized from methanol: mp 102.5–103.5°; nmr, δ 5.4 (1 H, m, C-6), 4.90 (1 H, m, C-24), 4.56 (1 H, m, C-3), 2.15 (3 H, s, C-25 OAc), 2.03 (3 H, s, C-3 OAc), 1.88 (C-26); mass spectrum, *m/e* (relative intensity) 410 (100, M − 60), 368 (26, M − 60 − 42), 255 (15). *Anal.* Calcd for $C_{30}H_{46}O_4$: C, 76.52; H, 9.91. Found: C, 76.55; H, 9.85. Mixtures of major and minor $\Delta^{24}$-enol acetates eluted next. These mixtures gave the same spectroscopic data as the major $\Delta^{24}$-enol acetate, except for the presence of two C-25 acetate signals in the nmr (at δ 2.15 C-25 acetate protons of the major isomer and δ 2.10 C-25 acetate of the minor isomer). These mixtures did not contain any $\Delta^{25(26)}$-enol acetate, however. All fractions containing $\Delta^{24}$-enol acetate were combined to give 3.54 g (46% yield) of a mixture of *cis*- and *trans*-$\Delta^{24}$-enol acetate (**5**). In later fractions, 3 g of starting material (**4**) was recovered which was recycled.

To the mixture of isomeric $\Delta^{24}$-enol acetates (4.73 g, major : minor = 2:1, based on glc) dissolved in 70 ml of $CH_2Cl_2$, 3.83 g of $Br_2$ in 70 ml of $CH_2Cl_2$ was added dropwise with stirring over a 5-min period. After evaporation of the solvent, the residue was taken up in 80 ml of benzene and treated with 24 g of NaI in 80 ml of 100% ethanol. After 45 hr at room temperature, ice–water was added, the solution extracted with ether, washed first with 1% NaOH, then with water, dried over $Na_2SO_4$, and evaporated. To the residue taken up in 400 ml of acetone was added 18 ml of acetic acid followed by 30 g of $KHCO_3$. After 18-hr reflux, water was added and the product was extracted into ether. The ether extracts were combined, washed (5% ice-cold $Na_2S_2O_3$, then water and finally saturated NaCl solution), and after drying ($Na_2SO_4$) and evaporation of solvent, the residue was chromatographed on 300 g of activated silicic acid. Batchwise elution with 100 ml of 10%, 100 ml of 20%, 600 ml of 30%, and 1000 ml of 40% ether in Skellysolve B gave in tubes 110–160 (11-ml fractions) 3.71 g (76%) of acetoxy ketone **6**, which was homogeneous on tlc and glc and crystallized from Skellysolve B–ether: mp 128–129.5°, $[\alpha]_D^{20}$ −42.4° (*c* 2.2, $CHCl_3$); nmr, δ 5.4 (1 H, m, C-6), 4.95 (1 H, m, C-24), 4.60 (1 H, m, C-3), 2.16 (6 H, s, C-24 OAc, C-26), 2.03 (3 H, s, C-3 OAc); ir, 1725 and 1230 $cm^{-1}$; mass spectrum, *m/e* (relative intensity) 426 (100, M − 60), 411 (9, M − 60 − 15), 255 (12); *Anal.* Calcd for $C_{30}H_{46}O_5$: C, 74.02; H, 9.52. Found: C, 74.32; H, 9.50.

*24,25-Dihydroxycholesterol* (**7**) *and Its Triacetate* **8**. A solution of 1.28 g of **6** in 30 ml of dry benzene was added to a solution of Grignard reagent (prepared by adding 2.2 ml of methyl iodide in 30 ml of ether to 0.66 g of Mg turnings with stirring until dissolution of Mg was complete) and the mixture was refluxed for 2 hr. After standing at room temperature for an additional 18 hr and addition of 5% aqueous $H_2SO_4$ and ice, the mixture was extracted with ether. Combined ether extracts were washed (5% $NaHCO_3$, $H_2O$, saturated NaCl) and dried over $Na_2SO_4$. Evaporation of solvent gave triol **7**, essentially pure on tlc; mass spectrum, *m/e* 418 ($M^+$).

Crude triol **7** was acetylated by refluxing for 4.5 hr in 15 ml of $Ac_2O$ and 10 ml of pyridine. After the usual work-up, crude triacetate was chromatographed on 100 g of silicic acid (2-cm column diameter), eluted with 10% ether in Skellysolve B (200 ml) and 20% ether in Skellysolve B (1200 ml). Crystallization from methanol gave 1.09 g (77% from **6**) of pure triacetate **8**: mp 119–120°, $[\alpha]_D^{20}$ −37.2° (*c* 1.7); nmr, δ 5.40 (1 H, m, C-6), 5.13 (1 H, m, C-24), 4.60 (1 H, m, C-3), 2.08 (3 H, s, OAc), 2.03 (3 H, s, C-3-OAc), 1.97 (3 H, s, OAc), 1.48 and 1.44 (6 H, s, C-26,27); mass spectrum, *m/e* (relative intensity) 484 (100, M − 60), 224 (56, M − 2 × 60), 382 (20), 381 (20), 365 (24), 264 (20), 255 (25), 253 (31); *Anal.* Calcd for $C_{33}H_{52}O_6$: C, 72.75; H, 9.62. Found: C, 73.04; H, 9.79.

*3β,24ξ,25-Trihydroxycholesta-5,7-diene* (**10**). Triacetate **8**

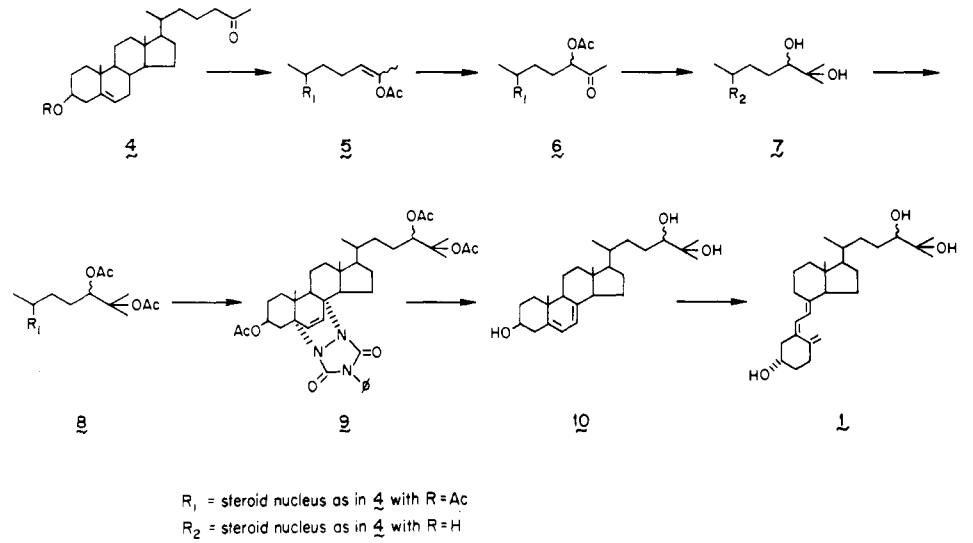



FIGURE 2: Reaction sequence followed in the synthesis of 24,25-$(OH)_2D_3$.

(218 mg) in 2 ml of Skellysolve B and 2 ml of benzene was brominated by treatment with 69 mg of dibromantin (*N,N*-dibromodimethylhydantoin) for 8 min at 70°. The reaction mixture was then cooled on an ice bath for 2 min, the precipitate filtered, and washed with 5 ml of ice-cold Skellysolve B, and filtrate and washings were combined to give after evaporation of all solvents a residue of the crude 7-bromo derivative (mass spectrum, *m/e* 542 (M − HBr)). A solution of this material in 0.2 ml of dry xylene was added dropwise to a solution of 1.4 ml of xylene and 0.2 ml of trimethyl phosphite, preheated to 132°. After 90 min at 132°, solvent was evaporated *in vacuo* and to the residue (consisting of the desired 5,7-diene, as well as 4,6-diene and starting material **8**), after dissolution in 4 ml of dioxane, a solution of 30 mg of 4-phenyl-1,2,4-triazoline-3,5-dione in 5 ml of dioxane was added dropwise with stirring until a pink color persisted for 1 min. Solvent was then evaporated *in vacuo*, and the residue was applied to a silicic acid column (35 g) and eluted with ether–Skellysolve B mixtures (100 ml of 20% ether in Skellysolve B; 100 ml of 35%, 300 ml of 50%, and 700 ml of 60%) to give 80 mg (28%) of pure triazoline adduct **9**: nmr, $\delta$ 7.45 (5 H, m, aromatic H), 6.36 (2 H, d, C-6 and C-7), 5.50 (1 H, m, C-3), 5.13 (1 H, m, C-24), 2.07 (3 H, s, OAc), 2.02 (3 H, s, OAc), 1.97 (3 H, s, OAc). To a solution of 80 mg of triazoline adduct **9** in 10 ml of benzene, 1 ml of 70% sodium bis(2-methoxyethoxy)aluminum hydride (Eastman Chemical Co.) in benzene was added. After reflux for 12 hr, water was added, precipitated salts were filtered, and the filtrate solution was evaporated *in vacuo*. The residue was applied to a silicic acid column (30 g), and eluted with Skellysolve B–ether (3:7) and pure ether. Trihydroxy-5,7-diene (**10**) was recovered in the pure ether fractions (24 mg, 52%); it was homogeneous on silica gel tlc ($R_F$ 0.34, ethyl acetate–cyclohexane, 1:1) and gave: nmr, $\delta$ 5.48 (2 H, dd, C-6,7), 1.15 and 1.19 (6 H, s, C-26,27); ultraviolet spectrum (EtOH) $\lambda_{max}$ 293.5, 282, 271 nm; mass spectrum, *m/e* (relative intensity) 416 (49, $M^+$), 398 (15, M − $H_2O$), 383 (39, M − $H_2O$ − Me), 351 (18), 271 (22).

*24ξ,25-Dihydroxycholecalciferol* (**1**). A solution of 4.2 mg of 5,7-diene **10** in 200 ml of ether was irradiated for 60 sec using the apparatus and conditions of Blunt and DeLuca (1969). After evaporation of solvent, the residue was applied to a multibore column of activated silicic acid (14 g). Gradient elution (going from a 3:7 ether–Skellysolve B mixture to pure ether and then to 5% MeOH in ether) gave the pre-vitamin D compound ($\lambda_{max}$ 260 nm). This material after warming to 70° for 2 hr under $N_2$ in 95% EtOH in the dark gave 0.9 mg of 24,25-$(OH)_2D_3$ (**1**): ultraviolet spectrum (EtOH), $\lambda_{max}$ 265 nm ($\epsilon$ 17,000) $\lambda_{min}$ 228 nm; mass spectrum *m/e* (relative intensity) 416 (63, $M^+$), 398 (9, M − $H_2O$), 383 (25), 271 (20), 253 (17), 136 (100), 118 (72); silica gel tlc $R_F$ 0.48 (ethyl acetate–cyclohexane, 1:1); nmr, $\delta$ 6.22, 6.00 (2 H, doublets, $J$ = 11 Hz, C-6 and -7), 5.03 and 4.80 (2 H, broad singlets, C-19), 1.15, 1.19 (6 H, s, C-26,27).

### *Biological Assays*

*Animals.* Weanling male rats (Holtzman Co., Madison, Wis.) were housed in overhanging wire cages. They were fed *ad libitum* a vitamin D deficient low calcium diet for 3–4 weeks prior to their use in the assays (Suda *et al.*, 1970).

*Intestinal Calcium Transport Assay.* Groups of four to six rats which were either bilaterally nephrectomized or intact, received 0.25 $\mu$g of the 24,25-$(OH)_2D_3$ intrajugularly in 0.05 ml of 95% ethanol. At the desired time after dosing, the animals were decapitated and the blood and duodena were collected. The duodena were prepared according to the procedure of Martin and DeLuca (1969) for measuring intestinal calcium transport activity by the everted gut sac technique. Aliquots from serosal and mucosal media were spotted on filter paper disks, dried, and placed in 20-ml counting vials containing 10 ml of scintillation counting solution.

*Bone Calcium Mobilization.* The blood from the rats was centrifuged and 0.1 ml of serum was mixed with 1.9 ml of a 0.1% $LaCl_3$ solution. Serum calcium concentration was determined with an atomic absorption spectrometer.

## Results

*Synthesis.* The synthesis of 24,25-$(OH)_2D_3$ (**1**) is outlined in Figure 2. We utilized ketone **4** as starting material[2] from which a mixture of *cis*- and *trans*-$\Delta^{24}$-enol acetates (**5**) was readily obtained under forcing acetylation conditions. From this mixture the desired 24-acetoxy 25-ketone **6** (mp 128–129.5°) was prepared *via* the corresponding bromo and iodo intermediates (Moffet and Weisblat, 1952). A Grignard reaction on **6** then led to 24,25-dihydroxycholesterol (**7**) which was

[2] We thank Professor Charles Sih for generously providing us with the corresponding 3$\beta$-hydroxy 25-ketone.

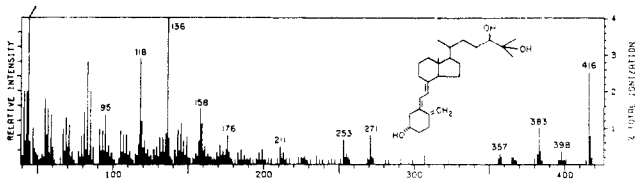


FIGURE 3: Mass spectrum of synthetic 24,25-$(OH)_2D_3$ (**I**).

converted to its crystalline triacetate **8**. Preparation of the required 5,7-diene (**10**) from triacetate **8** followed the usual scheme of bromination with *N,N*-dibromodimethylhydantoin (dibromantin) and dehydrobromination with trimethyl phosphite. Pure diene **10** was obtained by treating the crude reaction mixture after the dehydrobromination step with 4-phenyl-1,2,4-triazoline-3,5-dione (Zinner and Deucker, 1961) and chromatographic separations of the triazoline adduct **9** of the 5,7-diene (Barton *et al.*, 1971). Diene **10** was then generated from the adduct by hydride reduction (Barton *et al.*, 1971). Irradiation of **10** for 60 sec in ether (Blunt and DeLuca, 1969) and chromatography of the products gave the previtamin D derivative which was equilibrated to the desired vitamin D metabolite **1**, by warming to 70° in 95% ethanol. Synthetic **1** gave the expected ultraviolet spectrum ($\lambda_{max}$ 265 nm, $\lambda_{min}$ 228 nm), mass spectrum (Figure 3) and nmr spectrum.

*Biological Activity.* In agreement with our results on the natural metabolite (Boyle *et al.*, 1973), synthetic 24,25-$(OH)_2D_3$ (**1**) significantly stimulated intestinal calcium transport of animals maintained on a vitamin deficient, low calcium diet, but had no significant effect on serum calcium concentration (bone calcium mobilization). Typical results for both assays conducted over a 96-hr period are illustrated in Figure 4. In a similar experiment involving six animals per group, but conducted for a more limited (50-hr) period, entirely analogous results were obtained: the serosal/mucosal calcium ratio (I/O ratio) was 3.5 ± 0.4 at 25 hr after dosing with 0.25 μg of **1**, and 5.0 ± 0.5 at 50 hr while the controls gave a value of 1.5 ± 0.3. Within the error limits, stimulation of intestinal calcium transport observed for the synthetic product corresponds exactly to that found for the natural metabolite, both in terms of magnitude and long-term duration of the response.

Bilateral nephrectomy completely abolished the intestinal transport response of synthetic **1** as previously reported for the natural metabolite (Boyle *et al.*, 1973). Anephric rats given a dose of 0.25 μg of synthetic 24,25-$(OH)_2D_3$ (in 50 μl of 95% ethanol) 24 hr before sacrifice gave a calcium I/O ratio of 1.5 ± 0.2 (four animals), identical within the error limits to the ratio of 1.7 ± 0.3 observed for anephric controls.

## Discussion

With 25-keto-27-norcholesterol acetate (**4**) as starting material, the synthesis outlined here presents an unambiguous and fairly convenient route to 24,25-$(OH)_2D_3$ (**1**). Other equally facile syntheses are, of course, possible among which the hydroxylation of desmosterol, which can be prepared efficiently from fucosterol (Ohtaka *et al.*, 1973), is perhaps the most attractive and this route has indeed been used for the synthesis of the 24,25-dihydroxy metabolite.[3] These syntheses leave the question of the stereochemistry at C-24 unresolved.

[3] Personal communication from Professor Ikekawa; results of his work have also been presented at the 165th American Chemical Society Meeting, Dallas, Texas, April 1973, Abstract BIOL.

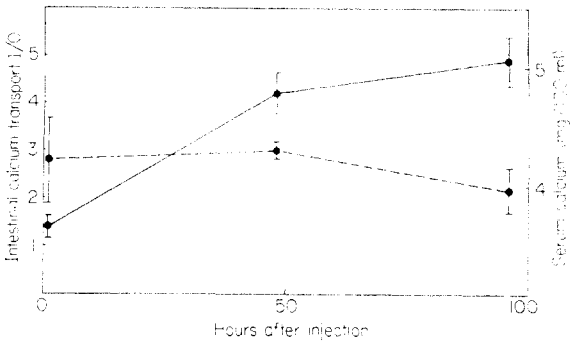



FIGURE 4: Biological activity of chemically synthesized 24,25-$(OH)_2D_3$. Rats were fed the vitamin D deficient, low calcium diet (Suda *et al.*, 1970) for 3.5 weeks. At this time they were given an intrajugular injection of 0.05 ml of 95% ethanol containing 0.25 μg of 24,25-$(OH)_2D_3$. Controls received ethanol alone. The rats were killed at various times thereafter for the determination of intestinal calcium transport (●—●) and serum calcium (bone calcium mobilization (●---●). There were usually six and not less than four rats in each group. The vertical bars represent standard error of the mean. Control rats which received ethanol alone show an intestinal calcium transport ratio of 1.4 ± 0.3 and a serum calcium value of 4.2 ± 0.2.

From our sequence a mixture of α and β C-24 epimers could be expected, but the isolated intermediates as well as the final product appeared homogeneous on tlc and glc. Although these techniques may not distinguish between the two isomers, the synthesis of a single epimer is also a possibility. It has been shown, for example, that equilibration of 24,25-dibromides of lanosterol and derivatives results in preferential (*ca.* 5:1) formation of the 24(*S*) isomer (Barton *et al.*, 1972). In our synthesis, the iodination step would seem to allow ample time (45 hr) for equilibration to the thermodynamically preferred epimer and chromatography and crystallization of the acetoxy ketone product could amplify this preference. Correlation of one of our intermediates with 24-hydroxy steroids of known configuration is necessary to resolve this ambiguity, and the synthesis of both epimers of the 24,25-dihydroxyvitamin appears desirable to provide clear-cut information on the importance of C-24 stereochemistry for the expression of biological activity of this metabolite.

## Acknowledgment

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# Interaction of a Fluorescent Ligand with Membrane-Bound Cholinergic Receptor from *Torpedo marmorata*†

Jonathan B. Cohen‡ and Jean-Pierre Changeux*

ABSTRACT: Studies are presented of the interaction in a physiological ionic environment of the fluorescent probe 1-(5-dimethylaminonaphthalene-1-sulfonamido)propane-3-trimethylammonium iodide (Dns-chol) with membrane fragments rich in cholinergic receptor protein that are purified from the electric organ of *Torpedo marmorata*. Energy transfer permits the preferential excitation of probe molecules interacting with the membrane, and two classes of Dns-chol binding sites are revealed. The effect of cholinergic ligands on Dns-chol fluorescence as well as binding studies with tritiated acetylcholine and tritiated $\alpha$-toxin from the venom of *Naja nigricollis* show that one of these classes of sites is the physiologically important receptor site of acetylcholine. The second class of Dns-chol sites is associated with spectral emission properties ($\lambda_{max}$) that depend upon whether a cholinergic agonist (depolarizing agent) or a cholinergic antagonist (depolarizing blocking agent) is bound to the cholinergic receptor. On the basis of this result, it is concluded that, upon the binding of cholinergic agonists, some change of membrane structure occurs which can be reversed by antagonists and, hence, is associated with the physiological response of the membrane.

It is now well established that the response of an excitable membrane to acetylcholine and to cholinergic agonists involves a selective increase of permeability to cations (Katz, 1966) which is blocked by a class of related compounds, the cholinergic antagonists (Nachmansohn, 1959). Acetylcholine acts as a regulatory ligand controlling membrane permeability. A minimum of two distinct structural elements have, therefore, been postulated: a "receptor" protein which recognizes cholinergic agonists and an "ionophore" which accounts for the selective translocation of ions and which could be part of the receptor protein or constitute a distinct but tightly coupled entity. The hypothesis was proposed that a conformational transition (Nachmansohn, 1959) mediates the interaction between receptor and ionophore (Changeux *et al.*, 1970).

One step toward the analysis of this mechanism was to demonstrate that the permeability response to cholinergic agonists persists *in vitro* in membrane fragments (or microsacs) isolated from the electric organ of *Electrophorus electricus* (Kasai and Changeux, 1971). The amplitude of the response was directly related to the amount of agonist bound to the cholinergic receptor site present in the membrane fragments.

Fluorescent probes have been used extensively to study biological membranes and model systems (Radda and Vanderkooi, 1972; Waggoner and Stryer, 1970), but previous use of fluorescence techniques to study the cholinergic microsacs (Kasai *et al.*, 1969; Wahl *et al.*, 1971) did not provide information of physiological interest. The recent development (Cohen *et al.*, 1972) of a method of preparation from *Torpedo marmorata* electric organ of membrane fragments rich in cholinergic receptor protein makes possible the use of fluorescence techniques to study this particular protein in the membrane environment.

1-(5-Dimethylaminonaphthalene-1-sulfonamido)propane-3-trimethylammonium iodide, (Dns-chol),[1] was introduced by Weber *et al.* (1971) as a fluorescent ligand possessing a high affinity for a proteolipid binding acetylcholine in an organic phase (De Robertis, 1971) and for horse serum cholinesterase in aqueous solution (Mayer and Himel, 1972). We report here studies showing that in *physiological* saline solution Dns-chol interacts strongly with receptor-rich membrane fragments from *Torpedo marmorata*. A strong enhancement of Dns-chol fluorescence occurs upon binding, and the effect can be partially reversed by cholinergic effectors and a snake venom $\alpha$-toxin in the concentration range expected from the value of their dissociation constants for the cholinergic re-

† From the Departement de Biologie Moléculaire, Institut Pasteur, 75 Paris, France. *Received July 17, 1973*. This work was supported by funds from the Centre National de la Recherche Scientifique, the Délégation Générale à la Recherche Scientifique et Technique, the Collège de France, The Commissariat à l'Energie Atomique, and the National Institutes of Health. A first presentation of this work was given at the fourth annual meeting of the American Society for Neurochemistry (Columbus, Ohio, March 1973).

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[1] Abbreviations used are: Dns-chol, 1-(5-dimethylaminonaphthalene-1-sulfonamido)propane-3-trimethylammonium iodide; physiological saline, 250 mM NaCl–5 mM KCl–4 mM $CaCl_2$–2 mM $MgCl_2$–5 mM sodium phosphate buffer (pH 7.0).