Catalytic reduction of S-p-nitrobenzyl-L-cysteine. S-p-nitrobenzyl-L-cysteine H_2O (0.548 g.) was dissolved in ethanol (40 ml.) and 1*N* HCl (20 ml.). The compound was hydrogenated for 3 hr. at room temperature and atmospheric pressure over 10% palladium on carbon (138 mg.). The catalyst was separated by filtration. The filtate gave the positive nitroprusside test for sulfhydryl. The product was precipitated as mercaptide with Hopkin's reagent. After 24 hr., the mercaptide was filtered and washed with cold water. The product was suspended in water (20 ml.) then stirred and saturated with H_2S, and the mercury sulfide was separated by filtration. The filtrate was made alkaline with sodium hydroxide and a small crystal of copper sulfate was added. Air was bubbled through the solution until the violet color disappeared (2 hr.). The solution was decolorized with charcoal, filtered, and neutralized with HCl. Crystallization soon began. After standing for 2 hr. at room temperature, the product was filtered, washed with cold water, alcohol and ether. For recrystallization the product was dissolved in 1*N* NaOH, then neutralized with 1*N* HCl. Yield: 96 mg. (40%), m.p. 255–260° (dec.) $[\alpha]_D^{25}$ − 225° (*c*, 1.04 in 1*N* HCl).

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MONTREAL, QUE.
CANADA

Isolation of Vitamin Dm and Vitamin D_3 from the Irradiation Products Obtained from Sterols of the Mussel, *Modiolus Demissus*, Dillwyn[1]

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A new vitamin D named vitamin Dm has been isolated from the irradiation products of the sterols derived from the ribbed mussel, *modiolus demissus*, Dillwyn. It has been characterized by the physical and chemical properties of the crystalline dinitrobenzoate. Its biological efficacy has been shown to be about 30,000,000 U.S.P. or A.O.A.C. units per g. of resin, and to be equal for rats and chicks.

The new compound is distinct from the known purified and crystalline vitamins D. It exists side by side in the ultraviolet irradiation products of the mussel sterols with vitamin D_3. A method for separating these two compounds as the dinitrobenzoates is described.

According to available reports only three compounds having vitamin D activity have been obtained in crystalline form or as crystalline derivatives. Calciferol, or vitamin D_2, was first obtained by Askew *et al.*,[3] and later also by Windaus and coworkers.[4] Vitamin D_3 was first isolated by Brockmann[5,6] from fish liver oils as the crystalline dinitrobenzoate, from which Brockmann and Busse[7,8] prepared the crystalline vitamin. Later the same compound was obtained by Windaus, Schenck, and von Werder[9] and by Schenck[10] from the irradiation products of 7-dehydrocholesterol both as crystalline esters and as the free alcohol. Finally, vitamin D_4 was obtained in pure form as the free alcohol and as an ester by Windaus and Trautmann[11] from the irradiation products of 22,23-dihydroergosterol.

The biological efficacy of these three vitamins D are established with reasonable accuracy because of the availability of the pure compounds. The existence of other vitamins D from time to time has been reported, but in every instance the evidence rests on comparative biological assay and little actually is known of the structure of these compounds or of their real efficacy and physiological function.

This report deals with the isolation of a fourth vitamin D, vitamin Dm, from the irradiation products of the sterols obtained from the mussel, *modiolus demissus*, Dillwyn, and also describes the separation of this compound from vitamin D_3 which is also found in the irradiation products of the mussel sterols.

Petering and Waddell[12] recently described the isolation and characterization of a new provitamin Dm which was isolated from the same ribbed mussel, *modiolus demissus*, Dillwyn, and which appears to be a C_{29} sterol. In the early stages of that investigation of the sterols of the ribbed mussel, it did not seen likely that a sufficient sample of the purified provitamin Dm would be available to permit a careful study of its properties and also allow for the irradiation of a portion for the isolation of the corresponding vitamin D. Therefore, it was de-

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cided to attempt the isolation and separation of the vitamins D produced commercially by the irradiation of the crude mussel sterols, a sufficient quantity of which had been made available to me, even though it was realized that such a study might be complicated by the presence of a multiplicity of active compounds.

This investigation resulted in the separation of the total vitamin D fraction from the other irradiation products and unchanged sterols as crude dinitrobenzoates, and the subsequent purification of this fraction and its separation into two distinct compounds, one being vitamin D_3 dinitrobenzoate and the other a new compound named vitamin Dm dinitrobenzoate. There was no evidence for the existence of vitamin D_2 in the crude vitamin fraction, and other unpublished work shows that the vitamin fraction contained no compounds having low chick-activity.

The data presented here indicate that vitamin Dm constitutes a large portion of the vitamin D formed by the irradiation of mussel sterols, and that it has a biological efficacy of about 30,000,000 U.S.P. and A.O.A.C. units per gram. A gram of the crystalline dinitrobenzoate contains about 20,000,000 U.S.P. and A.O.A.C. units of vitamin D activity. These values indicate that vitamin Dm is definitely of the D_3-type in its biological action, but is only about two-thirds as active as vitamin D_3.

Vitamin Dm dinitrobenzoate crystallizes readily from acetone in light yellow needles which tend to form rosettes, and is hardly distinguished from the high melting form of vitamin D_3 dinitrobenzoate obtained from the same solvent. No evidence of polymorphism of vitamin Dm dinitrobenzoate was obtained during this investigation.

The physical properties, like the biological activity, of vitamin Dm are distinctly different from those of any other known vitamin D. The absorption spectra of vitamin Dm and vitamin D_3 dinitrobenzoates are qualitatively similar, but the specific absorption of the former ester is considerably less than is that of the latter. At 2650 Å the ratio of the specific constants of vitamin Dm and vitamin D_3 dinitrobenzoates is 1.07, and the ratio of specific rotation constants is also 1.07, which probably reflect the larger molecular weight of the vitamin Dm moiety rather than any peculiar difference in structure. The mixed melting point of the dinitrobenzoates of vitamins Dm and D_3 is lower than that of either, and has a wide range as well as a clearing point not observed with either compound alone. These facts together with the isolation of vitamin D_3 dinitrobenzoate from the same crude irradiation fraction indicate the distinctiveness of vitamin Dm.

The vitamin D_3 dinitrobenzoate obtained from crude mussel vitamin D esters has been characterized by its physical and chemical properties, all of which are identical with those reported in the literature[14,15] and which are identical with similar compounds prepared from the irradiation products of 7-dehydrochholesterol. The mixed melting point of the isolated ester and an authentic sample of vitamin D_3 dinitrobenzoate shows no lowering of the value which identifies the latter compound and no lack of sharpness. As further evidence of the identity of the compound obtained from the mussel vitamin fraction, the dinitrobenzoate has been obtained in two crystalline forms, the melting points of which correspond with those of vitamin D_3 dinitrobenzoate.

The fact that vitamin D_3 dinitrobenzoate has been obtained in good yield from the crude irradiation products of mussel sterols indicates that 7-dehydrocholesterol must constitute an appreciable portion of the provitamins D present in *modiolus demissus*, Dillwyn.

EXPERIMENTAL

Isolation of vitamin Dm dinitrobenzoate (I). Two hundred milliliters of ethanolic solution of the activation products of crude sterols obtained from *modiolus demissus*, Dillwyn by the method of Rosenberg and Waddell[13] and containing about 25 g. of transformed provitamin D was used as the starting product. The bulk of the sterols had been previously removed by crystallization at 5°. The alcohol was removed by distillation under nitrogen at reduced pressure. The residual resin was dissolved in 200 ml. of benzene and the solution was again concentrated under nitrogen at reduced pressure to 60 ml. to remove alcohol and traces of water. Sixty milliliters of dry pyridine were added to the benzene solution and this was followed with the slow addition under constant agitation of 33 g. of 3,5-dinitrobenzoyl chloride in 80 ml. of dry benzene. The mixture, which had warmed to 60° during the addition of the acid chloride, was allowed to stand at room temperature for 16 hr., after which the pyridinium chloride was removed by filtration and washed with benzene.

The filtrate and benzene washings were transferred to a separatory funnel and washed successively with several volumes of 10% acetic acid, 5% sodium carbonate solution, and finally water to remove the excesses of pyridine and acid chloride which remained. The benzene layer was dried over anhydrous sodium sulfate and passed through a large chromatographic column containing 225 g. of 60–80 mesh adsorptive grade alumina. The vitamins D dinitrobenzoates were washed through the column with benzene, the washing being continued until the filtrates were clear, leaving a brown impurity remaining on the column.

The benzene filtrate was concentrated under nitrogen at reduced pressure and at 65° bath temperature until the resin containing only a trace of benzene was obtained. The resin, which weighed 36 g., was dissolved in 35 ml. of acetone. This solution deposited a crystal crop of 3.4 g. on standing 24 hr. at 5°; the crystals were high-melting mixed sterol esters and were discarded. The filtrate from this crop and the acetone washings were combined and methanol was added until a permanent turbidity remained at room temperature. The mixture was allowed to stand at 5° until two layers separated. The upper methanolic layer was removed

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and an amount of acetone equal to the remaining lower layer was added. Ethanol was added dropwise until a slight turbidity appeared and then acetone was added dropwise until this turbidity just disappeared. This solution was set aside at 5° for 24 hr., after which a crop of crystals (A) weighing 5.1 g. was removed and washed with cold acetone.

The mother liquor and washings were concentrated by distillation under nitrogen and at reduced pressure until a thick resin remained. The resin was taken up in an amount of acetone equal to its volume, ethanol was added until a slight turbidity resulted, and the mixture was cleared with acetone. Crystallization was again allowed to proceed at 5°, with the formation of 1.7 g. of crop B. Repetition of this procedure produced a C crop of 2.8 g.

The A and B crops (6.8 g.) were combined and dissolved in 60 ml. of boiling acetone. The hot solution was filtered, and after cooling to room temperature the solution was allowed to stand at 5° for several days. The first crop of light yellow crystals consisting of rosettes of needles was filtered and carefully washed with cold acetone. It amounted to 5.0 g. and had a melting point of 127–128°, and an $[\alpha]^{25}_{5461}$ + 100 (1.14% in $CHCl_3$). A second crop of crystals amounting to 0.70 g. and having a m.p. of 124–125° was obtained from the mother liquor.

The dinitrobenzoate having a m.p. of 127–128° (4.7 g.) was recrystallized from 35 ml. of acetone at room temperature to give 3.5 g. of vitamin Dm dinitrobenzoate (I) having a m.p. of 128.0–128.5°, and $[\alpha]^{25}_{D}$ + 90.8 (1.60% in $CHCl_3$) and $[\alpha]^{25}_{5461}$ + 106.0 (1.16% in $CHCl_3$).

The mother liquor from this recrystallization step as well as the C-crop and second crystal crops described above were worked up to yield 2.6 g. of a similar preparation of vitamin Dm dinitrobenzoate with a m.p. of 128–129° and $[\alpha]^{25}_{D}$ +90.8 (1.6% in $CHCl_3$). Thus a total of 6.1 g. of vitamin Dm dinitrobenzoate of highest purity was obtained from the starting material, which represents about 40% of the estimated amount of vitamin D available.

Further attempts to raise the purity of these preparations by crystallization from acetone and other solvents such as methyl ethyl ketone or by a combination of these with chromatographic methods failed to change the physical constants given above. $E^{g/l}_{1\,cm.}$ (2650 Å) = 38.0 ± 0.5 (solvent 1% $CHCl_3$- 99% ethanol). This value is significantly lower than that of vitamin D_3 dinitrobenzoate found to be 40.6 ± 0.5.

Anal. Found: C, 71.25, 71.52; H, 8.24, 8.30; N, 5.50, 5.22.

Isolation of vitamin D_3 dinitrobenzoate (II) *from the irradiation products of mussel sterols.* During the course of the work described above it was observed that a small amount of a vitamin D ester could be obtained from one of the crops of crude dinitrobenzoate by crystallization from methyl ethyl ketone, which on purification appeared to be vitamin D_3 dinitrobenzoate. The amount obtained was too small for extensive analyses, and therefore an attempt was made to fractionate a larger amount of crude vitamins D dinitrobenzoates obtained from the irradiation products of ribbed mussel sterols. Esterification and separation of the vitamins D dinitrobenzoates from unchanged sterol esters and impurities was accomplished as described for (I) above.

Thirty-eight grams of purified crude esters, labeled Fraction I, were dissolved in 150 ml. of warm methyl ethyl ketone, and the solution allowed to stand at room temperature for deposition of crystals. Fraction IA, amounting to 18.8 g., had $[\alpha]^{25}_{D}$ +93.4 (1.00% in $CHCl_3$). A second crop of crystals, Fraction IB, was obtained from the mother liquor by adding an equal volume of methanol and allowing the crystallization to go on at 5°. Fraction IB amounted to 13.1 g. and had an $[\alpha]^{25}_{D}$ +85.4 (1.00% in $CHCl_3$). A small third crop of crystals with low specific rotation constant and the mother liquor were discarded.

In a similar manner 32 g. of Fraction II was dissolved in 200 ml. of warm methyl ethyl ketone and allowed to deposit crystals at 5°. When no crystals formed, an equal volume of methanol was added and the mixture was allowed to stand overnight at room temperature. Fraction IIA crystals (13.1 g.) were removed from this mixture. Fraction IIA had $[\alpha]^{25}_{D}$ +92.5 (1.00% in $CHCl_3$). The addition of 150 ml. of methanol to the mother liquor and washings permitted the deposition of 13.6 g. of Fraction IIB crystals at 5°. This fraction had an $[\alpha]^{25}_{D}$ +73.2 (1.00% in $CHCl_3$).

Fraction IA and IIA were combined to give 31.8 g. of composite I and IIA, which was recrystallized at room temperature from 180 ml. of methyl ethyl ketone. Fraction IIIA amounted to 4.6 g. and had an $[\alpha]^{25}_{D}$ +96.3 (1.00% in $CHCl_3$). Fraction IIIB was obtained by adding an equal volume of methanol to the mother liquor and crystallizing at 5°. It amounted to 17.8 g., and had $[\alpha]^{25}_{D}$ +96.2 (1.00% in $CHCl_3$). A third crop of crystals having $[\alpha]^{25}_{D}$ +78.6 (1.00% in $CHCl_3$) and amounted to 5.8 g. It was not used in subsequent fractionations.

A second composite I and IIB, from fractions IB and IIB, amounting to 26.7 g. was recrystallized from 130 ml. of methyl ethyl ketone at 5°. The crystals (0.8 g.) which formed were discarded, and the filtrate was mixed with an equal volume of methanol and allowed to stand at room temperature overnight. Fraction IVA (15.5 g.) had $[\alpha]^{25}_{D}$ +86.7 (1.00% in $CHCl_3$). A third crop of crystals, amounting to 5.2 g., and having $[\alpha]^{25}_{D}$ +69.7 was discarded.

Fractions IIIA and IIIB, amounting to 22.2 g., and having nearly identical specific rotations were combined and allowed to deposit crystals from 130 ml. of methyl ethyl ketone at 5°. Fraction VA, weighing 9.9 g., was obtained. It melted at 134.2–135.2° and had $[\alpha]^{25}_{D}$ +96.2 (1.00% in $CHCl_3$). This compound showed no depression of the melting point when mixed with an authentic sample of vitamin D_3 dinitrobenzoate. It is considered to be the high-melting form of vitamin D_3 dinitrobenzoate, which crystallizes in lemon yellow needles which form rosettes. Its extinction coefficient is given in Table I.

Anal. Found: N, 4.96, 5.02; Theory: N, 4.85.

TABLE I

Preparation	M.P.[a]	$[\alpha]^{25}_{D}$ (in $CHCl_3$)	$E^{g/l}_{1\,cm.}$ (2650Å.) (in Ethanol Containing 1% $CHCl_3$)
Vitamin Dm dinitrobenzoate (I)	128–128.5	+90.8	38.0
Vitamin D_3 dinitrobenzoate (II)[b]	134.5–135.5	+96, +98	40.6
(II) from mussels Fraction V-A	134.2–135.2	+96.2	40.7
(II) from mussels Fraction V-B	127.5–128.5	+98.8	40.0
(I) from mussels Fraction VI-A	128–129	+91.2	37.5

[a] *All melting points determined on an electrically heated* microscopic stage, rate of heating being 1° per min. Sample placed on stage about 15° below the melting point. [b] Data reported are the author's. Some variation in these constants has been reported by Huber *et al.*[14,15]

A second crop of (II) of the low-melting form was obtained from the mother liquor of Fraction VA by adding an equal volume of methanol and allowing crystallization to proceed

at 5°. Fraction VB (8.2 g.) had m.p. 127.5–128.5° and $[\alpha]_D^{25}$ +98.8 (1.00% in $CHCl_3$). The crystals of this fraction were long orange colored needles which did not tend to form rosettes. They were indistinguishable from vitamin D_3 dinitrobenzoate crystals obtained from crystallization from benzene-methanol. The extinction coefficient of Fraction VB was identical with that of Fraction VA (cf. Table I).

Fraction IVA (15.5 g.) was dissolved in 80 ml. of methyl ethyl ketone and allowed to stand overnight at 5°. The small crop of crystals which formed was discarded, and an equal volume of methanol was added. Crystallization at 5° yielded 14.7 g. of vitamin Dm dinitrobenzoate (I) as Fraction VIA. This fraction had m.p. 128.5–129° and $[\alpha]_D^{25}$ (+91.2 1.00% in $CHCl_3$). Mixed melting point with authentic vitamin D_3 dinitrobenzoate showed a marked lowering, a wide range of melting and a high clearing point, it being 127–131–137°. The extinction coefficient given in Table I for this fraction is identical with that described above for (I).

Anal. Found: N, 5.00, 4.87.

Biological efficacy of vitamin Dm. Since vitamin Dm (III) has not yet been obtained in crystalline form as the free alcohol, the biological efficacy of the vitamin was determined by using a resin obtained from highly purified dinitrobenzoate (I), or by using the vitamin in solution obtained from a given amount of highly purified ester and calculating the efficacy on the basis of the ester.

The dinitrobenzoate I (0.600 g.) was saponified according to the procedure of Petering and Waddell[12] with the exception of the use of hexane instead of benzene. The hexane solution, containing the free vitamin Dm, was concentrated under nitrogen and reduced pressure until a resin resulted. The resin was then subjected to alternate periods of dry ice bath temperature and warming to 25° while being evacuated under high vacuum. In the end a brittle resin free of all solvent, which could be weighed readily, was obtained.

This resin (0.1000 g.) was dissolved in hexane (250 ml.) and an aliquot of 2.50 ml. was transferred to 100.0 g. of corn oil. The corn oil mixture was evacuated under nitrogen at 50° to remove the hexane. This sample was then used for biological assays.

The corn oil solution, containing 10.00 γ of vitamin Dm resin per g., was tested against Standard U.S.P. Reference Cod Liver Oil in the accepted U.S.P. assay for rat activity and A.O.A.C. test for chick vitamin D activity. It was found that the oil contained 335 U.S.P. units per g. and 300 A.O.A.C. units per g. These data indicate that the resin itself then has a biological efficacy of 33,000,000 U.S.P. units per g. and 30,000,000 A.O.A.C. units per g. This indicates a rat-chick ratio of activity of about 1.0, which identifies the vitamin as of the D_3-type.

In another experiment in which the resin was not isolated, it was found that the ester (I) contains 22,500,000 U.S.P. units per g. and 19,500,000 A.O.A.C. units per g., which agrees well with the above data. If one assumes a molecular weight of 425 for the free vitamin Dm, which is indicated by the spectroscopic and specific rotation data, then the values obtained above for free resin and (I) are self-consistent.

On the other hand, although vitamin Dm is of the D_3-type insofar as its physiological function is concerned, yet a comparison of the efficacy of vitamin D_3, which has an activity of about 45,000,000 USP or AOAC units per gram, with vitamin Dm, which contains about 30,000,000 units per gram, indicates that the latter is a less active compound. The lower efficacy of vitamin Dm over that of vitamin D_3 cannot be explained on the basis of the higher molecular weight of the former compound. These facts indicate the need for careful work with purified compounds rather than the reliance only on comparative biological assay for the determination of structural relationships in the area of the vitamins D.

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KALAMAZOO, MICH.

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY OF THE UNIVERSITY OF MINNESOTA]

On the Color of Diaminopyromellitic Esters and Related Compounds*

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The tetramethyl and tetraethyl esters of diaminopyromellitic acid (I, II) are inherently colored compounds, and the colors do not arise from the presence of diimino compounds or from molecular compounds (quinhydrones) formed from diimino and diamino compounds. Several other diaminobenzenecarboxylic acids and their derivatives have been reported in the literature as being colored, and the color of these substances is discussed in terms of resonance among the functional groups present. The principal frequencies in the ultraviolet, visible, and infrared spectra of compounds I, II, XII, XIII and XIV are given in tabular form.

Many years ago, it was reported by Nef that tetramethyl diaminopyromellitate (I) and the corresponding ethyl ester (II) were colored orange-red and red, respectively.[1–3] These are not the only diaminobenzenepolycarboxylic acids that show color of themselves or in their derivatives. 3,6-Diaminophthalic acid (III) forms brown needles which become black when heated above 200°;[4] 4,6-diaminoisophthalic acid (IV) and its ethyl ester (V) are colored pink and yellow, respectively;[5] diethyl 2,5-diaminoterephthalate (VI) is

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