devised as follows: To 1.7 mmole of *l*-cysteine-*N*-acetate in 5 ml of distilled water 1.5 mmole of nonradioactive phenylmercuric acetate in 40 ml of ethanol was added. After the solution was mixed thoroughly, it was acidified with 10 ml of 1*N* H_2SO_4 and extracted with 100 ml of benzene. Upon purification on a Florisil column the major reaction product was crystallized in *n*-hexane. This compound matched the major microbial metabolic product in six different thin layer chromatographic systems (the spot corresponding to an R_F value of 0.52 in the chromatogram in Fig. 3). The product was a white crystalline material which melted at 123° to 124°C. Elementary analyses showed that the material contained 42.13 percent carbon, 3.19 percent hydrogen, and no nitrogen (the mercury content was not analyzed). The mass spectrum of this compound showed the presence of two phenyl rings in addition to one mercury atom per molecule. Comparison of the infrared spectra of this material with that of an authentic sample of diphenylmercury unequivocally established that the crystalline material was diphenylmercury. On the average, diphenylmercury was responsible for 31.9 percent (for seven aquatic microorganisms) and 46.6 percent (for nine soil microorganisms) of the total solvent-extractable radioactivity. The corresponding figures for the second major metabolic product ($R_F = 0.46$, Fig. 3) were 20.0 and 15.3 percent, respectively. The chemical identity of this second major metabolite, which could be observed in most of microbial incubation products, could not be established.

Since it is known that methylmercury and dimethylmercury are produced from various mercury sources, it was surprising that these microorganisms did not convert phenylmercuric acetate into methylmercury compounds. It is possible that a direct conversion of phenylmercury to methylmercury is not a common process in microorganisms, at least not under aerobic conditions. However, at least one species of microorganism is known (*14*) to be capable of directly converting phenylmercury to metallic mercury, a process which could eventually lead to the formation of methylmercury through the conversion of Hg^0 to Hg^{2+}.

Diphenylmercury is the most apolar (and probably the most lipophilic) component of the metabolic products from phenylmercury. It has been suggested (*1, 15*) that the rate of absorption of mercury compounds by biological materials in water is related to the apolarity-lipophilicity of the compound. In view of the possibility that diphenylmercury can form in nature by the action of microorganisms, the toxicological implications of such a form of mercury in relation to its impact on various biospheres must be carefully examined.

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1,25-Dihydroxycholecalciferol: Identification of the Proposed Active Form of Vitamin D_3 in the Intestine

Abstract. *The major polar metabolite of cholecalciferol (vitamin D_3) present in chick intestinal mucosa has been chemically characterized by mass spectrometric analysis to have a molecular formula of $C_{27}H_{44}O_3$ and a structure of 1,25-dihydroxycholecalciferol. This compound, which is produced in the kidney from 25-hydroxycholecalciferol, has been previously shown to be from 4 to 13 times as active as cholecalciferol in stimulating intestinal calcium transport. 1,25-Dihydroxycholecalciferol (previously designated metabolite 4B in this laboratory) probably represents the biologically active form of cholecalciferol in the intestine.*

The concept has been developed in a number of laboratories that cholecalciferol (CC) (vitamin D_3) must first be metabolized to a more polar form prior to the stimulation of intestinal calcium transport (*1–3*). It has been conclusively shown that administration of radioactive CC to rachitic chicks or rats results in the production of two major polar metabolites and a number of other minor metabolites. The predominant metabolite present in the blood has been shown by Blunt *et al.* to be 25-hydroxycholecalciferol (25-OH-CC) (*4*). It is known to be produced by the liver (*5*). The major metabolite of CC present in the target intestine was shown by Norman and co-workers to be chemically different from both the parent vitamin and 25-OH-CC (*1, 2*). We have previously designated this substance metabolite 4B. The highly selective nature of the binding of metabolite 4B to the target intestine and the kinetics of appearance of this metabolite in relation to the well-known lag in action of vitamin D suggested to us that metabolite 4B played a prominent role in the development of this physiological response (*1, 6*). Lower concentrations of metabolite 4B were also found in bone and kidney of chicks, in the eggshell gland of the laying hen, other known sites of intensive calcium metabolism, and in the intestinal mucosa of the frog, rat, rabbit, monkey, and man (*7*).

We have carried out extensive studies on the relative biological activities of CC, 25-OH-CC, and highly purified metabolite 4B. Myrtle and Norman (*8*) reported that metabolite 4B is four to five times as effective as CC and over two times as effective as 25-OH-CC in stimulating intestinal calcium transport 24 hours after administration. After a considerable lag, CC and its 25-hydroxy derivative produce a maxi-

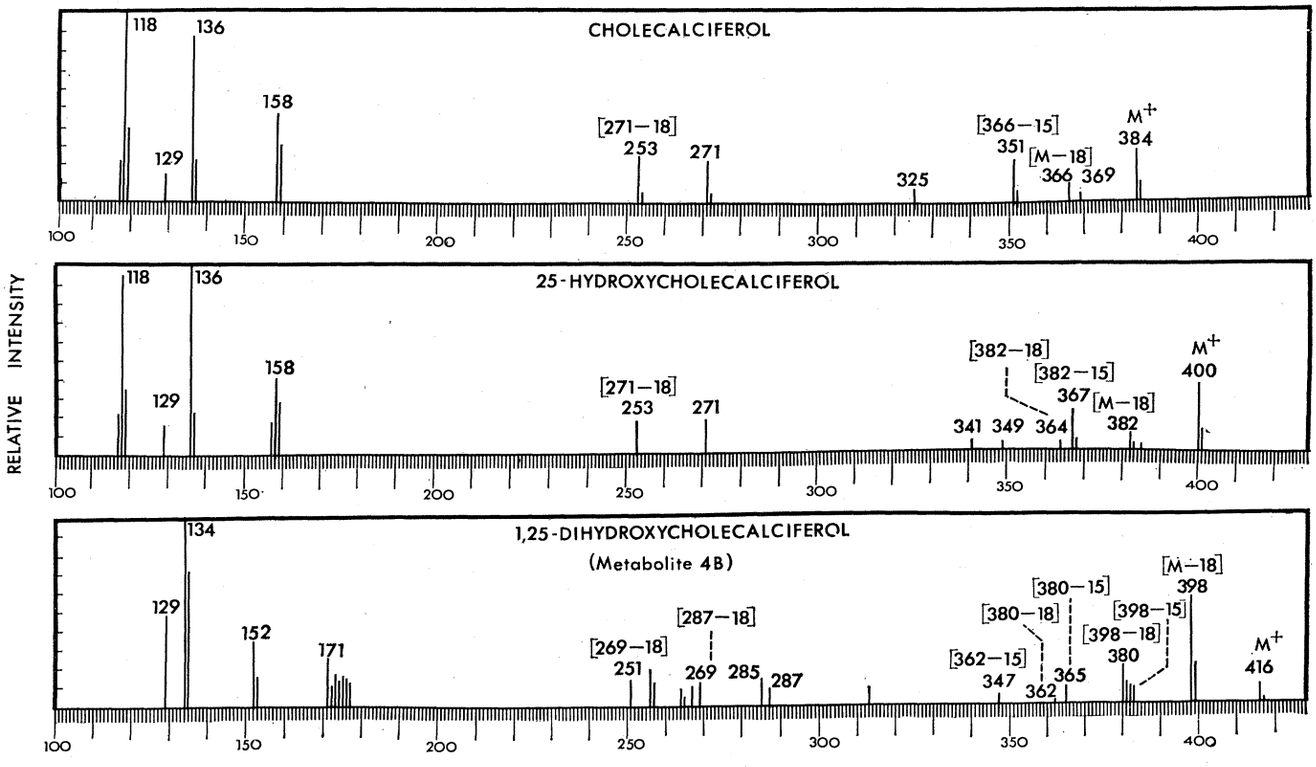


Fig. 1. Mass spectra of cholecalciferol, 25-hydroxycholecalciferol, and 1,25-dihydroxycholecalciferol.

mum stimulation of the transport response at 24 to 48 hours. Most significantly, metabolite 4B greatly shortens this lag, stimulating maximum calcium transport by 9 hours. At 9 hours this metabolite is at least 13 times as active as the parent vitamin (*8*). Similar results were also obtained by Haussler *et al.* (*9*).

Although the intestine is by far the richest source of metabolite 4B, owing to the low absolute amounts of the metabolite present (*10*) it has proved difficult to isolate enough metabolite 4B from the intestine to permit its chemical characterization. Accordingly a search was initiated for tissues which might be capable of producing metabolite 4B in an in vitro incubation. Thus Norman *et al.* (*11*) and Fraser and Kodicek (*12*) have recently reported that the kidney is the major if not exclusive site of production of metabolite 4B when 25-OH-CC is present as a substrate. It had previously been shown (*2*) that 25-OH-CC was an intermediate in the production, in vivo, of metabolite 4B. Furthermore, Norman *et al.* demonstrated that metabolite 4B produced from a kidney homogenate incubation is both chromatographically identical with and has biological activity equivalent to metabolite 4B obtained from chick intestinal mucosa (*11*).

This finding has permitted the production in vitro of large enough quantities of metabolite 4B to permit its chemical characterization. We now report that metabolite 4B has been identified to be 1,25-dihydroxycholecalciferol. While this report was in preparation, identical results were reported by two other laboratories (*13*).

Microgram quantities of metabolite 4B were prepared for mass spectrometric analysis in the following manner. Kidneys were obtained from groups of approximately 20 rachitic white Leghorn cockerels (24 days old), and 10 percent homogenates were made in 0.25*M* sucrose. With this homogenate a series of 50 incubations each containing 2.5 nmole of [26,27-^{3}H]25-OH-CC (specific activity 196 mc/mmole) and a reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system in 12 ml was performed as described previously (*11*). After incubation at 37°C for 2 hours, 45 ml of methanol-chloroform (2 : 1) was added to each incubation to obtain total lipids. This lipid extract was subjected to extensive chromatography (*14*) to obtain essentially pure metabolite 4B. The yield of metabolite 4B from such a series of incubations followed by chromatography has varied from 4 to 8 μg.

Mass spectra were obtained with an AEI MS-902 high-resolution mass spectrometer. The samples were introduced into the ion source on a direct insertion probe at source temperatures between 150° and 230°C above ambient. The mass spectra of metabolite 4B samples were obtained by increasing the ion source temperature up to 230°C so as to fractionate the sample while at the same time continuously scanning the molecular ion region for the appearance of the *m/e* 416 (molecular ion 416). High-resolution spectra of metabolite 4B were taken at a resolving power of 1 : 10,000. Typical mass spectra of CC and 25-OH-CC and metabolite 4B (1,25-diOH-CC) are shown in Fig. 1. The data for 1,25-diOH-CC is the observed spectrum from which contributing unknown contaminants have been omitted. In Fig. 2 are presented the structures of the three compounds and a tabulation of the important fragments produced in the mass spectrometer.

It was known that CC and 25-OH-CC have one and two oxygen atoms, respectively. Thus the finding that the parent molecular ions of CC, 25-OH-CC, and metabolite 4B were at *m/e* of 384, 400, and 416, respectively, suggested that metabolite 4B had only one more oxygen atom than 25-OH-CC and two more oxygen atoms than CC. The presence of three oxygen atoms was

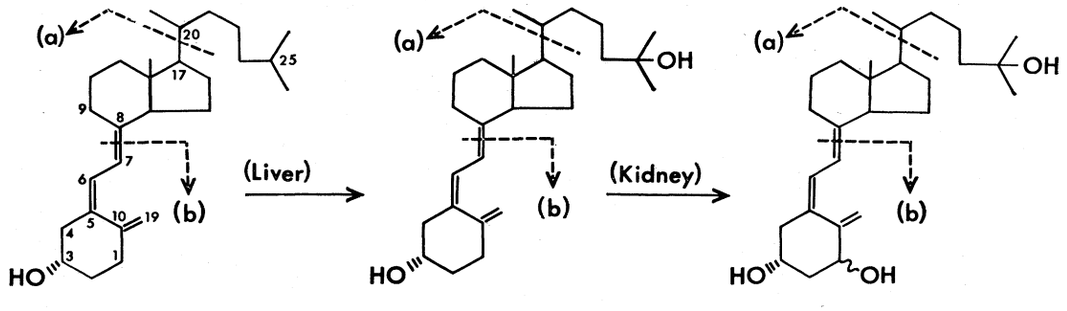
confirmed by high-resolution mass spectra determination of metabolite 4B. The molecular weight of metabolite 4B was found experimentally to be 416.32900, which is in good agreement with the expected mass of 416.32903 for $C_{27}H_{44}O_3$.

Since 25-OH-CC ($C_{27}H_{44}O_2$) had served as a substrate in the homogenate incubation for the production of metabolite 4B and since it was believed that metabolite 4B contained only one additional functional group which made it more polar than 25-OH-CC (*2, 7*), it seemed probable that two of the three oxygen atoms of metabolite 4B were still present at their original carbon-3 and carbon-25 positions (*15*). Furthermore we have reported (*7*) that the metabolism, in vivo, of mixed doses of [1,2-^{3}H]CC or [1-^{3}H]CC and [4-^{14}C]CC results in the stereospecific loss of tritium from the carbon-1 position in the formation of metabolite 4B. These results strongly suggest that the additional oxygen atom present in metabolite 4B is introduced at carbon-1. Since the molecular formula of metabolite 4B is $C_{27}H_{44}O_3$, this means that the additional oxygen atom had to be present at carbon-1 as a hydroxyl group rather than as a ketone. These suggestions were confirmed by comparison of the fragments of the three compounds.

The mass spectra of CC and 25-OH-CC both give fragments at *m/e* 271 which results from loss of the side chain due to cleavage of the bond between carbon 17 and 20, while metabolite 4B gives a fragment at *m/e* 287 (271 + 16). Thus the additional oxygen atom in metabolite 4B is not associated with the side chain. Both CC and 25-OH-CC give intense peaks at *m/e* 136. All molecules so far examined in this and other laboratories (*4, 13*) which have an intact triene structure with Δ 5-6, 7-8, 10-19 double bonds give this characteristic fragment which results from cleavage of the bonds between carbons 7 and 8. Both CC and 25-OH-CC gave a peak at *m/e* 118 (136—18) associated with the loss of water from this fragment containing ring A of the steroid nucleus. Metabolite 4B gave intense peaks at *m/e* of 152 (136 + 16) and 134 ($152 - H_2O$). Thus the additional oxygen atom known to be associated with metabolite 4B is unequivocably present in the ring A fragment, since the *m/e* 136 ion is shifted by 16 mass units to *m/e* 152. Also, the presence of the *m/e* 152 and 134 fragments is consistent with retention of the triene structure characteristic of CC (vitamin D) present in metabolite 4B (*4, 13*). Carbons 1, 2, and 4 are the only possible positions in the ring A fragment for the position of the additional hydroxyl. Consideration of the stereospecific loss of tritium from the α-position of carbon-1 in the production of metabolite 4B (*7*) indicates that the additional hydroxyl group of metabolite 4B is located at carbon-1. Thus the structure of metabolite 4B has been established to be 1,25-dihydroxycholecalciferol. This result confirms the previous prediction of Myrtle and Norman (*2, 7*).

Additional data of the mass spectrum of metabolite 4B which are consistent with this structure assignment include the ions at *m/e* of 398, 380, and 362 which correspond to the loss of one, two, and three molecules of H_2O from the molecular ion *m/e* 416 and the peaks at *m/e* of 269 and 251 resulting from the loss of one and two molecules of H_2O from the *m/e* 287 fragment. In a similar fashion CC had a fragment at *m/e* 366 ($384 - H_2O$), and 25-OH-CC had peaks at *m/e* 382 ($400 - H_2O$) and 364 ($382 - H_2O$).

The identification of 1,25-dihydroxycholecalciferol, the most biologically active form of vitamin D yet found, promises to provide many new avenues of experimentation. Myrtle and Norman have previously proposed that metabolite 4B (1,25-dihydroxycholecalciferol) is the biologically active form of vitamin D in the intestine. It now should be possible to synthesize this compound chemically and to examine directly its action in a number of systems. Several important problems that can now possibly be examined include determination of whether 1,25-dihydroxycholecalciferol (i) is further metabolized in the intestine, (ii) is biologically "active" as a result of its interaction with the genome of the mucosa or whether it mediates calcium transport directly via some membrane interaction, (iii) is biologically active in other CC-dependent systems, particularly bone deposition and resorption, (iv) is capable of acting in



	CHOLECALCIFEROL (VITAMIN D_3)	25-HYDROXYCHOLECALCIFEROL (25-OH-VITAMIN D_3)	1,25-DIHYDROXYCHOLECALCIFEROL (1,25-diOH-VITAMIN D_3)
Emp. Formula	$C_{27}H_{44}O$	$C_{27}H_{44}O_2$	$C_{27}H_{44}O_3$
Molecular Wt.	384	400	416
(a)	271	271	287
(b)	136	136	152

Fig. 2. Structures of cholecalciferol (vitamin D_3) and its two metabolites with a tabular evaluation of their fragmentation patterns.

concert with the other known prime regulators of calcium homeostasis, parathyroid hormone and calcitonin, and (v) is effective in alleviating some of the pathological conditions of vitamin D–resistant rickets, osteomalacia, sarcoidosis, and uremia, all diseases which have been postulated to be due to abnormalities in CC metabolism.

It is possible that 1,25-dihydroxycholecalciferol should be reclassified as a steroid hormone. Certainly its secretion by the kidney followed by its selective accumulation in the target intestinal mucosa where it exerts its characteristic physiological effect on calcium metabolism satisfies the minimal definitions of a hormone. Norman has previously commented on the analogies in the mode of action of CC to that of several other steroid hormones, particularly aldosterone, testosterone, and estrogen (*16*).

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10. Only some 4 to 6 pmole or 2 to 3 ng of metabolite 4B are present in the intestine obtained from a 110-g chick (*1*).
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14. The lipid was applied in diethyl ether to a 30-g (1 by 80 cm) column of silicic acid. The column was batch eluted sequentially with 200 ml of 100 percent diethyl ether (25-hydroxycholecalciferol), 300 ml of 50 percent diethyl ether in dichloroethane (vol/vol) (metabolite 4B), and 200 ml of 100 percent methanol (other polar metabolites). The metabolite 4B fractions were concentrated under nitrogen and applied to a 1 by 80 cm column of Sephadex LH-20 eluted with chloroform-petroleum ether (65 : 35). Metabolite 4B emerged between 275 and 325 ml. These fractions were concentrated under nitrogen and applied to a 20-g (1 by 80 cm) Celite liquid-liquid partition column which was eluted with a mobile phase of 20 percent (vol/vol) 1,2-dichloroethane and 80 percent petroleum ether saturated with a stationary phase of methanol-water (90 : 10 by volume) as described previously (*2*). The metabolite 4B was eluted after 700 to 800 ml, concentrated under nitrogen, and subjected to silica gel thin-layer chromatography. The silica gel had been prewashed with 100 percent acetone, followed by 100 percent diethyl ether, and dried, and the plates were preeluted with 100 percent ethyl acetate to remove trace lipids. After application of the metabolite 4B sample to the plate, it was developed with ethyl acetate : petroleum ether (93 : 7). In this system cholecalciferol, 25-hydroxycholecalciferol, and metabolite 4B have R_F's of 0.89, 0.76, and 0.45, respectively. The metabolite 4B was eluted from the gel with redistilled 100 percent methanol, and concentrated under nitrogen prior to mass spectrometric analysis.
15. The kidney homogenate incubations employing 25-hydroxycholecalciferol (25-OH-CC) were found to be one-step metabolic transformations. That is, only one major compound more polar than 25-OH-CC was found in an extensive chromatographic analysis of the incubation media (*11*).
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Fertilization of Rabbit Ova in vitro by Sperm with Adsorbed Sendai Virus

Abstract. *Fertilization occurs when rabbit ova are cultured in vitro with epididymal sperm to which Sendai virus is adsorbed. These sperm do not require capacitation in vivo in order to fertilize. Evidence for fertilization is penetration of sperm, the appearance of two polar bodies and pronuclei, and cleavage through the eight-blastomere stage. The viruses attach almost exclusively to the sperm acrosome, with resultant head-to-head agglutination of the sperm.*

Chang (*1*) in 1959 submitted incontestable evidence for fertilization in vitro; live young were born after transplantation of cleaved rabbit ova to foster mothers. Others have confirmed and extended the technique (*2, 3*). Common to these studies was the use of sperm which have undergone a physiological change (capacitation) within the female tract. Several workers have attempted, with partial success, to capacitate rabbit sperm in vitro (*4*), but all successful studies of fertilization of rabbit ova in vitro have used sperm capacitated beforehand in vivo. We report here the fertilization of rabbit ova in vitro with sperm that have not been capacitated in vivo. Originally we mixed Sendai virus with washed ejaculated sperm for a short incubation with ova. Some of these ova underwent cleavage when transplanted 3 to 5 hours later to recipient does. In this work we used epididymal sperm, because more agglutinate with the same amount of virus; improved equipment permitted longer incubation of ova. Criteria for fertilization are penetration of sperm, production of the second polar body, formation of pronuclei, and cleavage (*5*). The experiments are reproducible with approximately 50 percent of ova cleaving (Table 1).

Dutch-belted and New Zealand does were bred to vasectomized males or injected intravenously with 125 I.U. of chorionic gonadotrophin (National Biochemical Company). Their freshly ovulated ova were recovered by flushing of the oviducts 12 hours later with culture medium TC-199 kept at 37°C (flushing was carried out at room temperature). Gametes were incubated at 37°C in a 35 by 10 mm petri dish with 1.5 to 2.0 ml of TC-199 (*p*H 7.6) and 100 units of penicillin and 100 μg

Table 1. In vitro fertilization of rabbit ova by epididymal sperm with adsorbed Sendai virus. From these and other experiments an additional 159 (sperm with virus), 22 (sperm control), and 26 (virus control) ova were observed before cleavage for evidence of fertilization 3 to 12 hours after culture. In the table, those ova which had cleaved had reached the two- to eight-cell stage.

Experiment	Sperm with virus		Sperm control	
	Ova cultured (No.)	Ova cleaved (No.)	Ova cultured (No.)	Ova cleaved (No.)
1	8	3		
2	31	8	12	0
3	15	4	12	0
4	22	16		
5	15	5		
6	38	28		
7	15	9	25	0
8	25	9		
9			36	0
10	16	9		