mg of crystals for each 1 g of leaf tissue (fresh weight). Tobacco leaves were supplied with a mixture of tritiated amino acids for 6 hours before they were harvested for isolation of fraction I protein. The specific radioactivity of fraction I protein remained constant after the second recrystallization as did also the specific ribulose diphosphate carboxylase activity. Since 80 percent recovery of the protein is achieved after each recrystallization, we recrystallize twice as a routine. Crystals of thrice-crystallized fraction I protein from all species and hybrids of *Nicotiana* investigated have the appearance shown in Fig. 2.

The purification procedure is so simple and the yields so large that it is perfectly feasible to use crystalline protein as a carrier for isolating radioactive fraction I protein from minute quantities of leaf tissue.

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1,25-Dihydroxycholecalciferol: Metabolite of Vitamin D_3 Active on Bone in Anephric Rats

Abstract. *Nephrectomy prevents completely the bone calcium mobilization response to 25-hydroxycholecalciferol. In contrast it does not prevent this response to 1,25-dihydroxycholecalciferol. Because it is known that the kidney is the site of 1,25-dihydroxycholecalciferol formation, these results provide evidence that 1,25-dihydroxycholecalciferol or a further metabolite thereof and not 25-hydroxycholecalciferol is the metabolically active form of vitamin D_3 responsible for bone calcium mobilization.*

The observation by Fraser and Kodicek (*1*) and by Gray *et al.* (*2*) that the kidney is the sole site for the biosynthesis of 1,25-dihydroxycholecalciferol [1,-25-$(OH)_2D_3$] has generated many exciting new ideas relating to the role of the kidney in calcium metabolism. The importance of this vitamin D_3 metabolite, or a further metabolite thereof, as the functional hormonal form of vitamin D_3 responsible for stimulating intestinal calcium transport has been demonstrated many times. It not only acts more rapidly than 25-hydroxycholecalciferol (25-OHD_3) (*3*, *4*) in stimulating intestinal calcium transport, but it also functions in anephric (*5*) and actinomycin D–treated (*6*) rats, whereas 25-OHD_3 does not. Similarly, Omdahl and DeLuca (*7*) have demonstrated that strontium feeding blocks intestinal calcium transport in chickens given 25-OHD_3 by preventing the synthesis of 1,25-$(OH)_2D_3$. This inhibition was reversed in animals treated with 1,25-$(OH)_2D_3$.

The role of various vitamin D metabolites in bone calcium mobilization has not been as clear as it is for the transport of calcium in the intestine. Although it was shown that 25-OHD_3 will induce bone resorption in fetal rat bone tissue culture (*8*), 1,25-$(OH)_2D_3$ is about 100 times more potent on a weight basis (*9*). While 1,25-$(OH)_2D_3$ initiates bone resorption more rapidly than does 25-OHD_3 it does not function in the bone in the presence of actinomycin D (*10*).

The present study was undertaken to examine whether the hydroxylation of 25-OHD_3 at C-1 by the kidney is a necessary requirement for vitamin D's action in bone calcium mobilization.

Male weanling Holtzman rats were fed a vitamin D–deficient diet for 2 weeks (*11*) and then dosed intraperitoneally with 100 μc of ^{45}Ca. The rats were continued on the same diet for another week and then fed a low calcium (0.02 percent) diet (*12*) for 4 days (this was to ensure that any rise in serum calcium was not due to an increase in intestinal calcium absorption).

Rats were divided into groups and either had their ureters ligated or were bilaterally nephrectomized (*2*); immediately after surgery they were injected via the jugular vein with either 625 pmole of 25-OHD_3, 62.5 nmole of 25-OHD_3, or 625 pmole of 1,25-$(OH)_2D_3$ dissolved in 50 μl of 95 percent ethanol. The controls received the ethanol alone. The animals were killed by decapitation 21 hours after administration of the dose, and the blood serum was collected. Determinations of ^{45}Ca were made by spotting 0.1 ml of serum on circular glass fiber filter disks (2.4 cm in diameter), and the radioactivity was counted (*4*). Serum calcium was determined with an atomic absorption spectrophotometer (*10*).

As much as 62.5 nmole of 25-OHD_3 will not produce its characteristic bone calcium mobilization response (*13*) in rats that had their kidneys removed. Uremia or other problems associated with kidney dysfunction by themselves cannot account for this block since 25-OHD_3 is active in bone calcium mobilization in animals with ligated ureters. The fact that the 1-hydroxy derivative of 25-OHD_3 does stimulate bone resorption in anephric rats further demonstrates that 25-OHD_3 must be hydroxylated at C-1 in the kidney before it acts in bone calcium mobilization. The data in Table 1 also show that the specific activity of the ^{45}Ca in the blood serum remained the same in all groups, even though the serum calcium only increased in rats that received 1,25-$(OH)_2D_3$. These results support the belief that the rise in serum calcium was at the expense of bone rather than from

Table 1. Bone calcium mobilization response to 25-OHD_3 and 1,25-$(OH)_2D_3$ in anephric rats. Results are given as means ± standard error of the mean.

Dose	Animals (No.)	Ca in serum (mg/100 ml)	^{45}Ca/mg Ca per 100 ml of serum (count/min)
	Anephric		
50 μl of 95% ethanol	4	4.6 ± 0.1	260 ± 20
625 pmole of 25-OHD_3	6	4.5 ± 0.1	259 ± 10
62.5 nmole of 25-OHD_3	5	4.2 ± 0.1	
625 pmole of 1,25-$(OH)_2D_3$	6	7.1 ± 0.2	268 ± 20
	Ureter ligated		
50 μl of 95% ethanol	7	3.7 ± 0.1	
625 pmole of 25-OHD_3	5	5.4 ± 0.1	
	Normal		
625 pmole of 25-OHD_3	4	7.5 ± 0.2	

an unlabeled pool of calcium such as intestinal contents. The serum calcium concentration for the 25-OHD_3–treated anephric rats was not significantly different from that of the control group.

The results in this report add to the body of evidence that vitamin D must be "activated" by hydroxylation first in the liver at C-25 (*14*) and then in the kidney (*1, 2*) at C-1 before it can produce its physiological functions in the intestine and bone. Frolik and DeLuca (*15*) have provided evidence that 1,25-$(OH)_2D_3$ is not metabolized further before it acts on intestine. Our results show that 1,25-$(OH)_2D_3$ or a further metabolite thereof is the metabolically active form of vitamin D responsible for bone calcium mobilization.

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Cytochalasin B Inhibits Lymphotoxin Production by Antigen-Stimulated Lymphocytes

Abstract. *Lymph node cells of rats sensitized with hen ovalbumin produced lymphotoxin after 6 to 12 hours of exposure to specific antigen. Lymphotoxin was assayed by its cytotoxicity for fibroblasts from syngeneic embryos during a 72-hour incubation. Cytochalasin B inhibited lymphotoxin production, as well as later DNA synthesis, at concentrations (0.1 to 5.0 micrograms per milliliter) comparable to those which affect microfilament function and cell motility in other systems, and this inhibition was reversible. Binding of antigen was not affected.*

Suitably primed T lymphocytes (thymus-dependent or thymus-processed cells), when stimulated by antigen, release a variety of physiologically active factors (*1*). Prominent among these are "lymphotoxin" (*2–4*), "proliferation inhibitory factor" (*5*), and an "inhibitor of DNA synthesis" (*6*). Release may also be triggered by nonspecific lymphocyte mitogens such as phytohemagglutinin and concanavalin A. Release precedes and is not necessarily correlated with increased synthesis of lymphocyte DNA and with blast transformation. The B lymphocytes (thymus-independent) can be stimulated by antigen (*7, 8*), antiserum to immunoglobulin (*9*), or mitogens such as lipopolysaccharide endotoxin (*10*) and appear to produce and release specific antibody (*8, 11*), but there is no information to show whether or not they also release active factors like those mentioned above.

The detailed mechanism of lymphocyte triggering has been extensively studied. The generally recognized sequence of RNA, protein, and DNA synthesis (*12–14*) is preceded by one or more steps initiated by the binding of antigen or mitogen to the cell membrane. The antigen-antibody aggregate

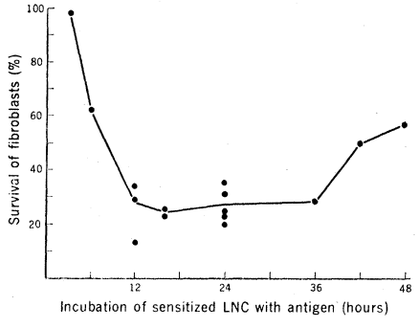

Fig. 1. Time course of lymphotoxin production. Sensitized lymph node cells (8×10^7) were incubated with ovalbumin (50 μg/ml) for varying lengths of time, and supernatants were tested. Results of four separate experiments are shown.

or mitogen-receptor complex moves toward the uropod of the cell, and "cap formation" (*15–17*) and pinocytosis (*13, 16–18*) occur within the first minutes, followed by discharge of lysosomal hydrolases (*18, 19*) and gene activation (*13, 14*). The synthesis of new proteins, including lymphotoxin, begins 2 to 4 hours after addition of antigen.

Taylor *et al.* (*17*) have shown that cap formation, pinocytic ingestion of antigen-antibody aggregates, and the selective disappearance of the latter (*17, 20*) are inhibited by cytochalasin B, a fungal product that interferes with microfilament function (*21, 22*) and inhibits many types of cell movement, including phagocytosis and pinocytosis (*17, 23*). It seemed of interest to determine the effect of this substance on cell triggering, as measured by the production and release of lymphotoxin (*2, 24*).

Young adult DA rats of both sexes were sensitized with 100 μg of hen ovalbumin (*25*) in complete Freund adjuvant, which was injected into the two hind footpads. Suspensions of sensitized lymph node cells (LNC) were prepared, 9 days after sensitization, from the inguinal and iliac lymph nodes. These and comparable normal LNC, usually 8 to 12×10^7, were incubated for various lengths of time with or without specific antigen (50 μg/ml) in 2 ml of "medium"—Ham's F10 medium (*26*) containing 10 percent fetal calf serum (*27*) plus penicillin (100 unit/ml) and streptomycin (100 μg/ml). Supernatants were cleared by centrifuging for 7 minutes at 800 rev/min and again for 15 minutes at 2400 rev/min and assayed for cytotoxic activity on monolayers of fibroblasts from syngeneic rat embryos (*2, 24*).

Fibroblasts giving a uniform size distribution after primary culture and three to five weekly transfers were plated in petri dishes measuring 60 by 15 mm (*28*), 1 to 2×10^5 cells in 4 ml of medium. After 48 hours, the medium was decanted and replaced with 2 ml of test supernatant plus 2 ml of fresh medium, and the cultures were incubated at 37°C in 5 percent CO_2 for 72 hours. At this time detached cells, shown earlier to consist almost entirely of dead cells, were removed by washing the culture twice with Hanks balanced salt solution. The remaining cells were treated with 2.5 ml of 0.25 percent Viokase solution (*27*), and the resulting homogenous suspension was counted in a Coulter model A electronic particle counter. All values shown in Table 1