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Crystalline Fraction I Protein: Preparation in Large Yield

Abstract. *About 1 milligram of twice-recrystallized fraction I protein of constant specific ribulose diphosphate carboxylase activity per gram of leaves (fresh weight) has been obtained from each of seven different species of* Nicotiana *and 14 reciprocal, interspecific F_1 hybrids. Crystals are produced from homogenates that have only been centrifuged to remove particulate matter.*

Two curious properties of crystalline fraction I protein (*1*) isolated from *Nicotiana tabacum* leaves have been described. (i) Storage at 0°C caused substantial loss in specific ribulose diphosphate carboxylase activity (*2*), which was entirely regained by heat treatment, whereas the activity remained unchanged during prolonged storage at 25°C. (ii) Protein in the presence of ribulose-1,5-diphosphate was extremely soluble (> 100 mg/ml), whereas in the absence of ribulose-1,5-diphosphate and the presence of Mg^{2+} and HCO_3^-, the enzyme was extremely insoluble (< 1.0 mg/ml) (*3*). The latter property, in particular, provided the basis for the following method which has also been successfully used elsewhere for isolating the protein from *N. tabacum* leaves by crystallization. In our laboratory, the method has been successful with seven additional species of *Nicotiana* as well as 14 reciprocal, interspecific *Nicotiana* F_1 hybrids. The general procedure is as follows.

Grind to a smooth paste 15 g of demidribbed leaves with 15 ml of ice-cold buffer A (0.05*M* tris-HCl, *p*H 7.4; 1.0*M* NaCl; 0.001*M* EDTA; 0.002*M* $MgCl_2$; 0.08*M* β-mercaptoethanol) using a pestle and a mortar surrounded by ice. Filter paste through cheesecloth and Miracloth and squeeze to obtain juice, which is centrifuged at 17,000*g* for 5 minutes. Remove any floating material; decant the supernatant and centrifuge it at 17,000*g* for 30 minutes. The supernatant from *N. tabacum* will still be green; from other species and hybrids, it is usually green to yellow. The volume is about 22 ml.

At room temperature, pass 22 ml of the supernatant through a G-25 Sephadex column (1 by 50 cm) previously equilibrated with buffer B [0.025*M* tris-HCl, *p*H 7.4; 0.20*M* NaCl; 0.0005*M* EDTA (no Mg; no mercaptoethanol)] and elute with this same buffer. Test successive fractions of eluate for the presence of protein by testing for a precipitate when trichloroacetic acid (TCA) is added. Collect the first 12 ml of eluate after the first TCA precipitate appears. Concentrate 12 ml to 4 ml by placing the eluate in collodion dialysis bags and subjecting them to reduced pressure or to pressurized membrane filtration. Remove the solution from the bag or membrane with the use of 0.5 to 1 ml of 0.2*M* NaCl, if necessary, to ensure that the concentrated protein layer on the wall of the bag or membrane is also removed, and centrifuge to remove any undissolved material, particularly green aggregates. Using solutions of 1*M* each, add enough to the supernatant to make the concentration of $NaHCO_3$ 0.02*M* and that of $MgCl_2$, 0.003*M*. Incubate for 15 minutes at 30°C. Transfer to a collodion dialysis bag and submerge the conical end of the bag to a depth of about 5 cm into 200 ml of buffer C (0.025*M* tris-HCl, *p*H 7.4; and 0.0005*M* EDTA) contained in a 250-ml Erlenmeyer flask. Leave undisturbed either at room or ice-box temperature, the latter seeming to accelerate the formation of fraction I protein crystals, which usually appear overnight and which will have the appearance in the bag as shown in Fig. 1. Remove mother liquor by a Pasteur pipette. Scrape the crystals off the wall, suspend them in buffer C, and transfer them to a small centrifuge tube. Collect the crystals by allowing them to settle or by gentle centrifugation; in either case remove the wash liquid.

Wash again with fresh buffer C. Add 1 ml of buffer B to dissolve crystals. Centrifuge again to remove any undissolved material. Transfer solution to a collodion bag and dialyze against buffer C as before. Crystals will appear in a few hours, maximum yield is obtained after about 15 hours and should be around 1

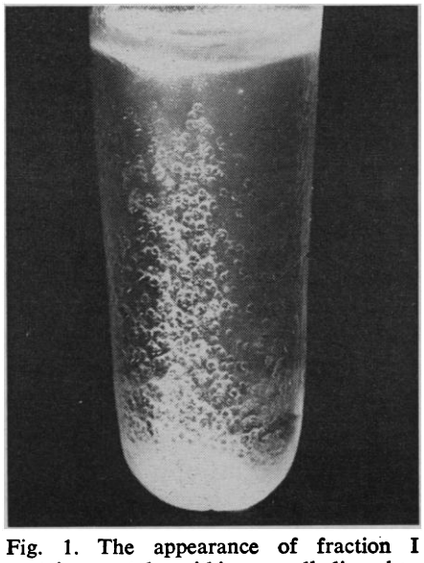

Fig. 1. The appearance of fraction I protein crystals within a collodion bag after dialysis for 16 hours. The maximum diameter of the bag is 1.5 cm.

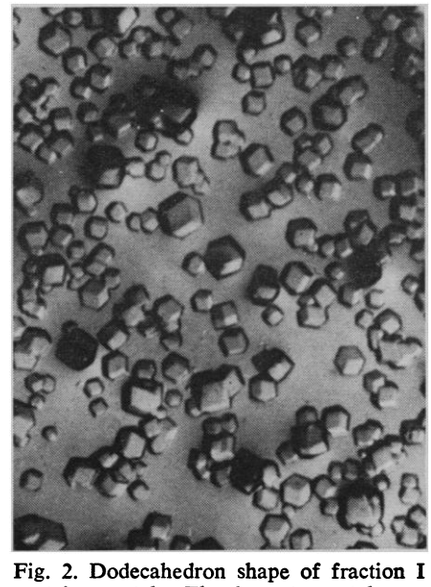

Fig. 2. Dodecahedron shape of fraction I protein crystals. The largest crystals are more than 0.2 mm in diameter.

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.

P. H. CHAN, KATSUHIRO SAKANO
SHALINI SINGH, S. G. WILDMAN
Department of Botanical Sciences and
Molecular Biology Institute,
University of California,
Los Angeles 90024

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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	