Actinomycin D Inhibition of Intestinal Transport of Calcium and of Vitamin D Action.* (30767)

HAROLD E. HARRISON AND HELEN C. HARRISON
Departments of Pediatrics, Baltimore City Hospitals and Johns Hopkins University School of Medicine, Baltimore, Md.

Actinomycin D inhibits the action of parathyroid hormone in raising the concentration of serum calcium in parathyroidectomized rats(1,2,3) and partially blocks the hypercalcemic effect of large doses of vitamin D (4). The action of vit D on intestinal absorption of calcium is also inhibited by actinomycin D given with or prior to the vit D(5, 6). Actinomycin D does not prevent the effect of parathyroid hormone on renal tubular transport of phosphate, however(2). The accepted concept of the mode of action of actinomycin D is that it blocks DNA directed synthesis of mRNA(7). The inhibition of the calcium mobilizing action of parathyroid hormone and of vit D by actinomycin has been regarded as evidence that protein synthesis dependent on new mRNA formation is required for the action of these molecules. The question remains whether either parathyroid hormone or vit D directly activates new mRNA production. There is a known interaction between vit D and parathyroid hormone since the latter agent is without effect when given to vit D deficient rats in doses which elevate the serum calcium (8,9) and increase urine phosphate output in vit D primed rats(8). Inhibition of both vit D and parathyroid hormone effect by actinomycin D could be directed at a common site of action of these two separate molecules. An action of vit D which can readily be measured with *in vitro* preparations is the increase of permeability to calcium of intestinal mucosa (10) and also of the concentrative transport of calcium across the intestinal wall of rats given vit D(11). Experiments were done, therefore, to determine the time relationship of actinomycin D inhibition of vit D action on calcium transport by rat intestine *in vitro* and on calcium mobilization in hypocalcemic rats; and also to examine the effect of actinomycin D on the intestinal transport of calcium in the absence of vit D.

Weanling male rats of the Sprague-Dawley strain were fed a vit D-free diet which provided an intake of both calcium and phosphate adequate for the growing rat(12). After 2 to 3 weeks on this diet, the rats are hpyocalcemic although serum phosphorus levels are normal and histologic or analytic changes of rickets in the bone are absent. Body weight and bone weight are, however, significantly reduced in comparison with vit D-fed controls. At this time intestinal preparations studied under *in vitro* conditions show reduced permeability of intestinal mucosa to calcium(10) and decreased concentrative transport of calcium(11). The following groups of experiments were made.

Series 1. Rats fed the vit D-free diet for 3 weeks were divided into 2 groups. Animals of one group were injected subcutaneously with actinomycin D dissolved in 0.85% NaCl solution in the amount of 1 μg per gram body weight. The control group received the same volume of 0.85% NaCl. Two hours after the actinomycin injection, half of the rats of each group were given 50,000 units vit D_2 in peanut oil orally while the other half were given the solvent alone. The rats were fasted and 18 hours later were bled from the aorta under sodium pentobarbital anesthesia and the small intestine removed for preparation of everted intestinal loops by the technique which has been described(11). The duodenal segment was used for measurement of concentrative transport of calcium(11) while the jejunal and ileal portions were employed for measurement of the rate of diffusion of Ca^{45} as an index of intestinal mucosal permeability to calcium(10). The concentrative transport is expressed as Cs/Cm, namely, the ratio of

* This work was supported by Nat. Inst. Health, U.S.P.H.S. Research Grant AM-00668.

We are indebted to Norman G. Brink, Merck Sharp and Dohme, for the supply of actinomycin D needed in these experiments.

TABLE I. Blocking of Vitamin D Effect by Actinomycin D.

Treatment		Serum	Intestinal permeability		
Vit D	Actin	Calcium	Jejunal	Ileal	Conc transport
		(mg/100 ml)	(μM/loop/hr)		(Cs/Cm)
0	0	6.2 ± .25 (10)	.175 ± .012 (17)	.262 ± .016 (9)	1.1 ± .11 (14)
0	+	4.2 ± .22 (9)	.241 ± .017 (9)	.265 ± .022 (5)	.73 ± .07 (5)
+	0	8.2 ± .22 (10)	.477 ± .019 (14)	.629 ± .011 (7)	4.1 ± .41 (5)
+	+	4.8 ± .22 (8)	.353 ± .022 (8)	.459 ± .017 (4)	1.7 ± .17 (6)

Rats treated with Vitamin D received 50,000 U orally 2 hours after subcutaneous injection of actinomycin D, 1 μg/g or of an equivalent volume of saline. Blood samples were obtained and intestines removed 18 hours after actinomycin injection. The definitions of intestinal permeability to calcium and concentrative transport of calcium are given in the text. The values are means ± standard error of mean and the number of samples are indicated in ( ). The values from rats treated with actinomycin D are compared with those from animals of the same vitamin D status not given actinomycin. The values from the vitamin D treated rats are also compared with those of the vitamin D-deficient animals. The underlined values are significantly different from those with which they are compared (student's t test).

Ca^{45} in the serosal fluid to that in the solution bathing the mucosal surface at the end of the incubation. The rate of diffusion of calcium is expressed as μmols calcium transferred from the mucosal fluid into the serosal solution under conditions which block bulk flow of solution. The blood serum was analyzed for calcium and phosphorus by standard methods(12).

Series 2. In these experiments vit D_2, 50,000 units, was given at either 2, 6, 18 or 72 hours before the actinomycin injection and the studies of intestinal transport and serum calcium and phosphorus concentrations made as above 18 hours after the actinomycin injection. No food was available to the animals following the actinomycin injection. Control rats given the vit D_2 alone were studied at the same time intervals after vit D as the rats injected with actinomycin.

Series 3. Vit D-deficient rats were injected with actinomycin D or saline as in *Series 1*, and 3 hours after the injection the animals were either bled and intestinal preparations made or the rats were given 50,000 units of vit D_2 at this time and blood samples and intestinal preparations taken 4 hours after the vit D_2 and therefore 7 hours following actinomycin D. Vit D-deficient rats were also given vit D_2, 50,000 units, 2 hours before they were injected with either actinomycin D 1 μg/gram body weight, or saline, and blood and intestine obtained 3 hours after the actinomycin or saline injection for study as above.

Table I summarizes results of the experiments of *Series 1* in which actinomycin D was injected 2 hours before administration of vit D. The actinomycin treated vit D-deficient rats showed a reduction of serum calcium concentration below the already low levels found in the control vit D-deficient rats. Actinomycin treatment also blocked the response of the serum calcium concentration to vit D demonstrated by the saline controls. The vit D-induced increase in rate of diffusion of Ca^{45} across the intestinal wall and the correlated increase in concentrative transport of calcium were both partly inhibited 18 hours after actinomycin injection but not completely blocked. Actinomycin alone slightly increased the diffusion of Ca^{45} in the jejunal loop but not in the ileal loop. There was, however, a depression of concentrative transport of calcium by the duodenal loops of the actinomycin D-treated rats which is of borderline significance ($p<0.1$).

Table II presents the results of the experiments in which vit D was given at varying intervals prior to the actinomycin injection. Pretreatment with vit D as little as 2 hours before actinomycin prevented the inhibitory

TABLE II. Effect of Pretreatment with Vitamin D on Actinomycin Inhibition.

Time* (hr)	Serum calcium (mg/100 ml)	Intestinal conc transport (Cs/Cm)
−2†	4.8 ± .22 (8)	1.7 ± .17
2	4.9 ± .21 (6)	3.4 ± .44
6	4.8 ± .20 (6)	4.5 ± 1.14
18	7.2 ± .48 (7)	3.8 ± .52
72	9.7 ± .15 (4)	4.3 ± .43

* Interval between time of oral administration of 50,000 U vit D and subsequent injection of actinomycin D, 1 μg/g.

† These animals received vit D 2 hr post actinomycin D.

In all experiments blood samples were obtained and intestines removed 18 hours following actinomycin injection. Values are means ± standard error of mean. Values from animals given vit D at various intervals prior to actinomycin D are compared with those from rats which received vit D following actinomycin D. The underlined values are significantly different from those with which they are compared.

effect of actinomycin on concentrative transport of calcium when the intestinal preparations were made 18 hours after actinomycin. However, pretreatment with vit D did not obviate the inhibition by actinomycin of the serum calcium raising effect of vit D unless the vit D was given 6 to 18 hours before the actinomycin injection. This relationship is shown graphically in Fig. 1. Actinomycin depressed serum calcium concentrations further in the hypocalcemic vit D-deficient rat and also reduced serum calcium levels of animals given vit D 72 hours before actinomycin injection.

The possibility that actinomycin directly injures the system for concentrative transport of calcium across the intestinal wall prompted the group of experiments of *Series 3* in which studies of intestinal transport of calcium were made 3 or 7 hours after actinomycin D injection rather than 18 hours as in *Series 4*. In control studies the effect of vit D alone was tested at 4 and 5 hours after its feeding. The results of the experiments of this series are shown in Table III. The data indicate that actinomycin D had an inhibitory effect on calcium transport in the vit D-deficient as well as in the vit D-treated rat. The reduction of the Cs/Cm ratio to considerably less than 1 in the vit D-deficient rats injected with actinomycin represents an inhibition of the already low rate of calcium transport in vit D-deficient rats. At the start of incubation the ratio is unity but if calcium transport from the mucosal solution is blocked the concentration of Ca^{45} in this phase is unchanged while that in the serosal fluid decreases below the initial value due to diffusion of Ca^{45} into the intestinal wall, either by exchange with the stable Ca^{40} of the tissue or by actual movement of total calcium into the intestinal wall through the serosal surface. The very low Cs/Cm values in the actinomycin-treated rats suggest that calcium transport across the mucosal phase was prevented by this agent and that vit D was in-

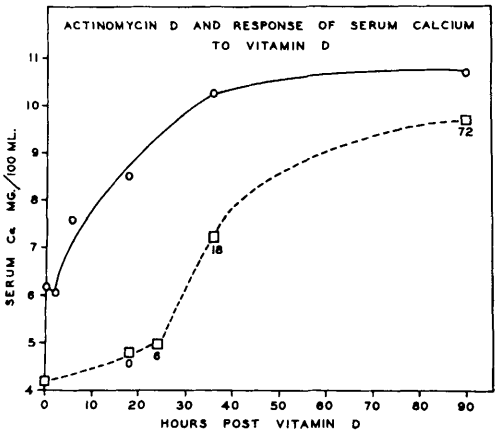

FIG. 1. Time relationship between vit. D administration and subsequent injection of actinomycin D with respect to elevation of serum calcium concentrations of hypocalcemic vit. D-deficient rats. All animals received 50,000 units vit. D orally at 0 time. The actinomycin D-treated rats were injected subcutaneously with 1 μg/g either at the same time as vit. D or 6, 18 or 72 hr following vit. D. The actinomycin D-treated rats and simultaneous vit. D controls were bled 18 hr after injection of actinomycin and serum calcium determined. ○——○ vit. D alone, □- - -□ vit. D and actinomycin D. Abscissa—hr between vit. D feeding and blood serum sampling. Numbers under the □ in the actinomycin D series represent time interval between vit. D feeding and subsequent actinomycin injection. Each point represents average of determinations in 4 to 10 animals.

TABLE III. Inhibition of Intestinal Transport of Calcium by Actinomycin D.

Group	Treatment	Intestinal conc transport of calcium (Cs/Cm)
1	None	1.1 ± .11 (14)
2	Actinomycin D—3 hr	.69 ± .13 (13)
3	Actinomycin D—7 hr; vit D—4 hr	.54 ± .04 (5)
4	" " " "	2.1 ± .05 (9)
5	Vit D—5 hr; actinomycin D—3 hr	.96 ± .16 (12)
6	" " " "	3.4 ± .03 (13)

Intestinal transport of calcium measured *in vitro* with duodenal loops from vit D-deficient rats incubated in K-H buffer containing Ca^{45}. Concentration of total calcium and Ca^{45} was the same in serosal and mucosal fluids at start of incubation. Cs/Cm represents the ratio of concentration of Ca^{45} in serosal fluid to that in mucosal fluid at the end of incubation. Values are means ± standard error of mean. Numbers of intestinal loops given in ( ). Groups: 1—no treatment, 2—actinomycin D 3 hr before experiment, 3—actinomycin D 7 hr and vit D 4 hr before experiment, 4—vit D 4 hr before experiment, 5—vit D 5 hr and actinomycin D 3 hr before experiment, 6—vit D 5 hr before experiment. Vit D 50,000 units was given orally, actinomycin D 1 μg/g injected subcutaneously.

effective in accelerating calcium transport in the presence of inhibitory concentrations of actinomycin. Seven hours after its injection the inhibitory effect of actinomycin was still maximal so that Cs/Cm ratios much below 1 were found despite administration of vit D 4 hours before the intestinal preparation was made. When vit D was given 2 hours before the actinomycin D injection and the intestinal loops made 3 hours after actinomycin treatment, the inhibitory effect of actinomycin on calcium transport blocked the vit D action. This action of actinomycin was not a non-specific injury to all intestinal functions, since determinations of concentrative transport of l-tyrosine in the same everted loop preparations 3 hours following actinomycin D injection showed no injurious effect of actinomycin on this transport system (Table IV).

The inhibition by actinomycin of both the vit D effect on calcium transport across the intestinal wall and its calcium mobilizing action is evident. The latter action of vit D is probably independent of the rate of absorption of calcium from the intestine since the animals received no food during the interval after the actinomycin or control saline injections. This separation of the intestinal and calcium mobilizing effects of vit D is emphasized in the studies in which vit D was given 2 or 6 hours before the actinomycin and the studies made 18 hours post actinomycin. The vit D effect on intestinal transport of calcium was present as shown by the calcium transport studies in the *in vitro* preparation but no rise of serum calcium occurred. This increase of serum calcium in the fasted rat represents redistribution of calcium in the body, probably the result of dissolution of bone mineral with increase of the calcium concentration in solution in the extracellular phase. The action of vit D in enhancing the solution of bone mineral in these experiments may depend in part on its facilitation of response to endogenous parathyroid hormone since these animals have intact parathyroids which are capable of being stimulated by the low concentration of calcium ion in the ambient fluid.

If the action of actinomycin D were only on the DNA mediated synthesis of mRNA the data would suggest that an intact DNA, mRNA and microsomal protein synthesizing system is required for the specific effect of vit D and also of parathyroid hormone. In the case of the latter at least one action can

TABLE IV. Effect of Actinomycin D on Intestinal Transport of l-Tyrosine.

Treatment	Intestinal transport of l-tyrosine			
	Jejunal	Ileal	Jejunal	Ileal
	(Cs/Cm)		(μmols transferred)	
None	5.4 ± .32 (11)	9.6 ± 1.12 (4)	1.80 ± .073 (11)	2.44 ± .085 (4)
Actinomycin D	5.4 ± .52 (10)	8.7 ± .13 (5)	1.77 ± .037 (10)	2.28 ± .174 (5)

Actinomycin D, 1 μg/g given subcutaneously 3 hr before animals were bled and intestines removed for *in vitro* measurement of l-tyrosine transport. Values given are means ± standard error of mean, as before. Both final concentration, Cs/Cm, and actual amount of tyrosine added to the serosal phase, μmols transferred, are given as indices of concentrative transport of l-tyrosine by the *in vitro* preparation.

occur without intervention of new protein synthesis since the parathyroid hormone action on renal tubular transport of phosphate occurs within a few minutes of its injection and is not blocked by actinomycin D(2). The action of parathyroid hormone and of vit D on mobilization of calcium from bone into extracellular fluid probably does require protein synthesis. This effect manifests itself only after an interval of 6 to 12 hours and is blocked not only by actinomycin D(1,2) but also by the puromycin inhibition of protein synthesis(13). We have found that puromycin also prevents the vit D induced rise of serum calcium in hypocalcemic, vit D-deficient rats.

The inhibitory effect of actinomycin D on the intestinal calcium transport system in the vit D-deficient rat indicates that the action of this agent is on the transport system itself rather than on the enhancing effect of vit D on this process. This inhibition develops rapidly, is marked within 3 hours after subcutaneous injection, but is diminishing at 18 hours as indicated by the actinomycin inhibition of vit D effect at 3 hours, but lack of inhibition at 18 hours in experiments in which vit D was given 2 hours before the actinomycin. The rapidity of the development and dissipation of the actinomycin D inhibition of calcium transport across the intestinal wall suggests an action other than the blocking of mRNA synthesis. There is no direct evidence that the action of vit D is exerted through induction of new protein *via* a DNA mediated mechanism as has been adduced for certain steroid hormones(14). Blocking of the effect of a hormone or other regulatory factor by actinomycin D has been interpreted as indirect evidence that the active molecule acts through DNA mediated mRNA synthesis and induction of enzyme or other protein molecule formation. Such an interpretation must be viewed with caution since actinomycin may have injurious effects upon cell systems such as the presently demonstrated inhibition of the intestinal calcium transport mechanism in the rat intestine which would alter the response to agents which regulate the system.

Summary. Actinomycin D injection inhibits the actions of vitamin D in increasing concentrative transport of calcium across the intestinal wall *in vitro* and in raising serum calcium concentrations of fasted vitamin D-deficient rats. Actinomycin D blocks the transport of calcium across the mucosal surface of vitamin D-deficient as well as vitamin D-treated rats so that its action is on the calcium transport system rather than on the stimulatory effect of vitamin D. This inhibitory effect of actinomycin D on calcium transport is present within 3 hours of its subcutaneous injection and is less marked at 18 hours which suggests a direct interaction with the calcium transport system. Under the same conditions actinomycin D does not influence the transport of l-tyrosine across the intestinal wall *in vitro*.

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Received October 19, 1965. P.S.E.B.M., 1966, v121.