

MECHANISM OF PRODUCTION OF VITAMIN K DEFICIENCY IN RATS BY SULFONAMIDES

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(Received for publication, May 29, 1944)

The use of sulfonamides in purified diets of rats has resulted in the production of vitamin deficiencies. The first identified deficiency thus induced was that of vitamin K produced by sulfaguanidine and succinyl sulfathiazole and was reported by Black *et al.* (1). It was plausible to postulate that this deficiency was a result of the inhibition by the sulfonamides of bacterial synthesis of vitamin K in the intestinal tract. It is known that coliform organisms produce vitamin K *in vitro* (2) and that the feces of rats on a vitamin K-free ration contain vitamin K (3). Sulfaguanidine and succinyl sulfathiazole reduce the coliform count in the feces of rats (4). *p*-Aminobenzoic acid, which antagonizes sulfonamide bacteriostasis, was also shown to prevent the production of vitamin K deficiency by sulfaguanidine (1).

Recently other data (5, 6) have been reported which support this hypothesis of sulfonamide inhibition of intestinal bacterial synthesis of vitamin K. We (5) have shown that sulfapyrazine, sulfadiazine, and sulfathiazole are more effective than sulfaguanidine, succinyl sulfathiazole, and sulfanilamide in producing vitamin K deficiency and that this order of effectiveness of these sulfonamides approximates the order of their bacteriostatic potency against intestinal coliform organisms as reported by White (7). In addition it was observed (5) that factors such as absorption, utilization, and alteration of requirements of vitamin K did not appear to be significant elements in the production of vitamin K deficiency by these sulfonamides. Day *et al.* (6) found that cecectomy increased the incidence of vitamin K deficiency in rats fed diets containing succinyl sulfathiazole.

Certain evidence has been presented by Black *et al.* (1) which they interpret to be inconsistent with the hypothesis limiting the sulfonamide action to the intestinal tract. These workers found that *p*-aminobenzoic acid even when administered parenterally antagonized the vitamin K deficiency produced by sulfaguanidine. They reasoned, therefore, that this sulfaguanidine action "cannot be explained on the basis of changes in intestinal flora alone, but may be due to a toxic action . . . on certain tissues of the rat, which is counteracted by *p*-aminobenzoic acid." They concluded that the exact mode of action of sulfaguanidine is, therefore, obscure.

The present study was undertaken to gather more evidence concerning the mechanism of production of vitamin K deficiency by sulfonamides. We have confirmed the findings of Black *et al.* (1) that parenterally administered *p*-aminobenzoic acid prevents the development of vitamin K deficiency but in addition we have found that *p*-aminobenzoic acid so administered appears in high concentrations in cecal contents. Thus the findings of Black *et al.* do not necessarily conflict with the hypothesis of sulfonamide inhibition of intestinal bacterial synthesis of vitamin K. In addition new and more direct evidence is presented here in support of this hypothesis.

Methods

The techniques and diets used here were generally the same as those previously described (5). Weanling, albino rats were fed a purified control or experimental diet. The diets were identical except that, in the experimental, 1 per cent of the dextrose was replaced by an equal weight of the sulfonamide drug.

Prothrombin time was determined by a micromethod on whole blood from the tail. The "prothrombin level" was derived by dividing the average prothrombin time of a group of control rats by the prothrombin time of the experimental rat; the result was multiplied by 100 and expressed as a per cent. The term *hypoprothrombinemia* was applied only to those rats whose "prothrombin levels" had fallen below 30 per cent.

Determinations of sulfadiazine were by the method of Bratton and Marshall (8) with a photoelectric colorimeter. Blood determinations were made on blood obtained by decapitation or by a micromethod (9) on 0.02 cc. of tail blood. Cecal contents were homogenized with 200 cc. of water in a Waring blender for 5 minutes and then made up with vigorous shaking to a volume of 500 cc. containing 100 cc. of 15 per cent trichloroacetic acid. Determinations were made on the clear filtrates and concentrations were expressed on a wet weight basis. The water fraction of cecal contents was generally from 75 to 85 per cent.

p-Aminobenzoic acid was assayed by the microbiological method of Landy and Dicken (10) on cecal contents dried for 10 hours at 80° and powdered.¹ The test organism was *Acetobacter suboxydans*. Only free *p*-aminobenzoic acid was determined. The dried cecal contents were extracted with hot water in preparation for the assay. The microbiological method was preferred to diazo chemical methods because the latter were not sufficiently sensitive for the entire range of values.

The technique for the assay of vitamin K activity of crude material has

¹ Lieutenant M. Landy, Army Medical School, performed the *p*-aminobenzoic acid assays.

been described previously (5). The responses of hypoprothrombinemic rats to a sample of feces or cecal contents were compared with responses of similar rats to doses of pure vitamin K. "Increases" over the pretreatment "prothrombin levels" of 60 per cent or more, of 20 to 60 per cent, and of less than 20 per cent were considered to represent the following respective degrees of vitamin K (2-methyl-1,4-naphthohydroquinone diacetate) activity: 5 γ or greater, 2 to 4 γ , and less than 2 γ . Aliquots of a suspension of feces or cecal contents gave similar values in different rats. Feces were collected and frozen daily. Cecal contents were removed from a rat sacrificed by decapitation and used immediately for assay purposes; in a few instances they were frozen and used later. The materials were suspended in water and administered by stomach tube. Prothrombin determinations were made immediately before and 24 hours after administration of the assayed material.

Sodium sulfadiazine in an aqueous solution containing 10 gm. per 100 cc. was administered subcutaneously each day at a dose level of 0.5 mg. per gm. of body weight. *p*-Aminobenzoic acid in a neutralized solution containing 0.1 or 1.0 gm. per 100 cc. was administered subcutaneously each day at levels of 5 or 50 γ per gm. of body weight.

Results

Hypoprothrombinemia and hemorrhage were produced by the daily subcutaneous administration of sodium sulfadiazine and this effect was prevented by *p*-aminobenzoic acid also administered subcutaneously (Table I). *p*-Aminobenzoic acid at a dose level of 50 γ per gm. of body weight almost completely antagonized the development of the vitamin K deficiency.

The production and prevention of vitamin K deficiency by parenterally administered agents does not exclude the intestinal tract as a locus of these actions. This is shown by a study of the concentrations of sulfadiazine and *p*-aminobenzoic acid in the cecal contents of rats injected subcutaneously with these agents.

Sulfadiazine determinations (Table II) were made of the cecal contents of rats injected daily with sulfadiazine (0.5 mg. per gm. of body weight) and of rats ingesting diets containing from 0.25 to 1.0 per cent sulfadiazine. It was found that sulfadiazine concentrations in the cecal contents of the injected rats were of the same general order as those found in rats ingesting a diet containing 0.5 per cent sulfadiazine and the incidence of vitamin K deficiency was similar in both groups. In rats with low concentrations of sulfadiazine in the cecum, vitamin K deficiency did not appear, but in rats with high concentrations of sulfadiazine in the cecum, vitamin K deficiency was noted in most cases. Thus in a group of seven rats with cecal sulfadiazine concentrations under 110 mg. per 100 gm., none developed hypo-

TABLE I
Production of Vitamin K Deficiency by Sodium Sulfadiazine Administered Subcutaneously and Prevention by p-Aminobenzoic Acid Administered Subcutaneously

Type of diet	No. of rats	Lowest "prothrombin levels"* reached by individual rats	Average of lowest "prothrombin levels"
		<i>per cent</i>	<i>per cent</i>
Control diet + sodium sulfadiazine, 0.5 mg. per gm. body weight	10	4, 4, 4, 16, 25, 38, 39, 53, 67, 97	35
Control diet + sodium sulfadiazine, 0.5 mg. per gm. body weight + 5.0 γ p-aminobenzoic acid per gm. body weight	5	20, 83, 85, 88, 96	75
Control diet + sodium sulfadiazine, 0.5 mg. per gm. body weight + 50.0 γ p-aminobenzoic acid per gm. body weight	5	81, 83, 88, 96, 97	89
Experimental diet containing 1% sulfadiazine	5	8, 11, 12, 14, 19	13
Experimental " " 1% sodium sulfadiazine	5	4, 4, 10, 13, 26	11

* Determinations were made at 1, 2, and 3 weeks. Litter mates were used in this experiment.

TABLE II
Concentrations of Sulfadiazine in Cecal Contents and Blood

Type of diet	No. of rats	Sulfadiazine (free)* concentrations in cecal contents (wet weight basis)		Sulfadiazine (free)* concentrations in blood		No. of rats with hypoprothrombinemia ("prothrombin level" = <30 per cent)
		Average	Range	Average	Range	
		mg. per 100 gm.	mg. per 100 gm.	mg. per cent	mg. per cent	
0.25% sulfadiazine	5	76	64-103	16	16-16	0
0.50% "	4	138	43-245	32	27-34	1
0.75% "	3	704	522-868	43	41-44	2
1.0% "	10	1609	1160-1850	48	34-54	10
Control diet + sodium sulfadiazine subcutaneously (daily), 0.5 mg. per gm. body weight	5	126	104-155	35	30-46	2

* Determinations were made from 2 to 4 weeks after the start of the experiment.

prothrombinemia. Of twenty-five rats, with sulfadiazine concentrations over 110 mg. per 100 gm. twenty developed hypoprothrombinemia. The other five rats had the following concentrations of sulfadiazine in their cecal contents: 118, 129, 147, 245, and 723 mg. per 100 gm.

The concentration of *p*-aminobenzoic acid (subcutaneously administered) in the cecal contents was determined in rats on a control diet

TABLE III

Estimation of Free p-Aminobenzoic Acid in Cecal Contents

The values are given in micrograms per gm. (dry weight).*

Control rats†	Rats injected daily for 10 days with <i>p</i> -aminobenzoic acid, 5 γ per gm. body weight	Rats injected daily for 10 days with <i>p</i> -aminobenzoic acid, 50 γ per gm. body weight
0.144	7.1	31.2
0.408	6.8	20.2
0.216	7.3	17.0
0.168	7.5	19.8

* Stool moisture content varied from 77 to 84 per cent.

† Four groups of three litter mates were used in this experiment. Lieutenant M. Landy, Army Medical School, performed the *p*-aminobenzoic acid assays.

TABLE IV

Vitamin K Activity of Feces and Cecal Contents

Type of diet	Material assayed	Amount of material assayed	No. of rats	"Prothrombin levels" of assay rats before and after administration of test material*		Approximate vitamin K† activity of material assayed
				Before	After	
Control	Feces	1/10 of 5 day sample	3	Before	8, 12, 12	>50 γ (for total 5 day sample)
				After	94, 100, 86	
Experimental‡	"	1/20 of 5 day sample	2	Before	11, 25	<2 γ (for total 5 day sample)
				After	78, 108	
Control	Cecal contents	Total 5 day sample	8	Before	19, 19, 20, 5, 6, 6, 7, 21	<2 γ (for total 5 day sample)
				After	37, 26, 53, 15, 6, 14, 31, 11	
Control	Cecal contents	1/4 of total	4	Before	15, 19, 27, 27	>12 γ (for total cecal contents)
				After	51, 51, 78, 84	
Experimental‡	Cecal contents	Total	5	Before	25, 20, 23, 10, 17	<2 γ (for total cecal contents)
				After	27, 15, 27, 12, 8	

* Values from the same individual rats are tabulated in the same order on the "Before" and "After" lines.

† 2-Methyl-1,4-naphthohydroquinone diacetate.

‡ The experimental diet contained either 1 per cent sulfapyrazine or 1 per cent sulfadiazine.

(Table III). A daily parenteral dose of 5 γ per gm. of body weight resulted in a 30-fold increase in the concentration of *p*-aminobenzoic acid in the

cecal contents, while a dose of 50 γ per gm. of body weight increased the *p*-aminobenzoic acid concentration in the cecum about 100-fold.

Vitamin K activity of feces and cecal contents was assayed on hypoprothrombinemic rats. The responses of these rats were compared with those of rats given known amounts of pure vitamin K. A large difference was found between the vitamin K activity of feces of rats on sulfonamide diets and those of rats, usually litter mates, on the control diets (Table IV). The total feces for 5 days obtained from each of eight rats on experimental diets gave responses of less than 2 γ of vitamin K activity in seven cases and a response of 2 to 4 γ in one case. Similar values were obtained from two rats with hypoprothrombinemia due to subcutaneously injected sulfadiazine. When the total feces for 5 days of these two rats were fed to hypoprothrombinemic rats with "prothrombin levels" of 13 and 11 per cent, responses to levels of 56 and 17 per cent respectively were obtained. This indicates 2 to 4 γ of vitamin K activity in one rat and less than 2 γ in the other. 5 day fecal samples from control rats of similar age and weight were found to contain more than 50 γ of vitamin K activity. Wet and dry weights of fecal samples from experimental and control rats were of the same order of magnitude.

Since vitamin K synthesis may occur in feces at room temperature during intervals between daily collections and refrigeration, vitamin K assays were made of cecal contents. Comparable rats on experimental and control diets were sacrificed. Cecal contents of four control rats each showed about 12 γ of vitamin K activity; those of five rats on a deficiency-producing, sulfonamide diet each possessed no demonstrable vitamin K activity or less than 2 γ . One rat injected subcutaneously with sulfadiazine also had less than 2 γ of vitamin K activity in its cecal contents.

DISCUSSION

The vitamin K activity of cecal contents (or collected feces) was very low in rats with vitamin K deficiency produced by the oral or parenteral administration of sulfonamides. The results obtained show that the cecal contents of such vitamin K-deficient rats possess no demonstrable vitamin K activity or less than 2 γ , while the cecal contents of control rats possess 12 γ or more of vitamin K activity. This finding points to the inhibition of intestinal bacterial synthesis of vitamin K as an important factor in the production of vitamin K deficiency by sulfonamides.

This hypothesis for the mechanism of production of vitamin K deficiency by sulfonamides is further supported by other data presented here. The production of vitamin K deficiency by orally or parenterally administered sulfadiazine was found to be definitely related to the sulfadiazine concentration in the cecal contents. It was also observed that *p*-aminobenzoic

acid injected subcutaneously appeared in significant amounts in the cecum and prevented the production of a vitamin K deficiency by subcutaneously administered sulfadiazine. The known antagonism of sulfadiazine bacteriostasis by *p*-aminobenzoic acid provides a plausible basis for the action of *p*-aminobenzoic acid in preventing vitamin K deficiency.

We have reported previously on the parallelism between the order of bacterial potency of a series of sulfonamides and the order of their effectiveness in production of a vitamin K deficiency (5). It was also reported by us that factors of absorption, utilization, and altered requirements of vitamin K did not appear to be significant elements in the production of a vitamin K deficiency by sulfonamides. Day *et al.* (6) noted that cecectomy facilitated the development of a vitamin K deficiency in rats fed succinyl sulfathiazole diets. These findings are all in keeping with those presented at this time.

It should be pointed out that the mechanism of production of other vitamin deficiencies by sulfonamides may not be the same as that considered here for the production of a deficiency of vitamin K.

SUMMARY

1. The cecal contents and collected feces of rats with vitamin K deficiency produced by sulfonamides showed either very slight or no vitamin K activity. The cecal contents and collected feces of control rats possessed much greater vitamin K activity.

2. Sulfadiazine administered subcutaneously resulted in a vitamin K deficiency. The production of the deficiency by orally or parenterally administered sulfadiazine was closely related to the concentration of sulfadiazine in the cecal contents.

3. *p*-Aminobenzoic acid administered subcutaneously antagonized the vitamin K deficiency produced by subcutaneously administered sulfadiazine. *p*-Aminobenzoic acid injected into rats on a control diet appeared in the cecum in significant concentrations.

4. These findings together with other available data indicate that the inhibition of intestinal bacterial synthesis of vitamin K is the dominant factor in the production of vitamin K deficiency in rats by the sulfonamides used in these studies.

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