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Spermine Reversal of Microbial Growth Inhibition by Thioesters of *p*-Aminosalicylic Acid*

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Natural extracts were found to reverse partially the toxicity of the thiobenzyl ester of *p* aminosalicylic acid. Properties of the active factor(s) indicated that spermine is the major principle present in hot water extract of beef liver, which reverses this inhibition.

The incorporation of a reactive thioester group into an analog of a metabolite has resulted in derivatives which bind and interact with certain enzymes utilizing the metabolite (Ravel *et al.*, 1958; Skinner *et al.*, 1958). Thus, *S*-carbamoyl-L-cysteine inhibits certain enzymatic processes involving glutamine by inactivating the enzymes. Recently, other thioester analogs of various metabolites were prepared and shown to be inhibitory to the growth of certain lactic acid bacteria (Hayashi *et al.*, 1961; Skinner *et al.*, 1961). The corresponding metabolite as anticipated exerted very little effect upon reversing the toxicity of the antagonists. For example, the growth inhibitory effects of the thiobenzylester of *p*-aminosalicylic acid for *Streptococcus lactis* is not overcome to any appreciable extent by *p*-aminobenzoic acid.

In the present investigation it was observed that supplements of natural extracts such as hot water extract of beef liver caused a two-fold increase in the amount of thioester required for inhibition of growth. The identity of the reversing agent(s) was desired in order to determine whether or not it might be related to a biochemical role of *p*-aminobenzoic acid. In the course of this work, an improved technique for the synthesis of thioesters of *p*-aminosalicylic acid was utilized and several new analogs were prepared.

Preliminary chemical evidence concerning the properties of the naturally occurring reversing agent(s) for these thioesters suggests that the compound(s) were probably strongly basic polyamines. Thus, a number of naturally occurring amines of this type were examined, and spermine, and to a lesser extent spermidine, were found to reverse the toxicity of the thiobenzyl ester of *p*-aminosalicylic acid.

Experimental[1]

Microbiological Assays

A previously described assay medium (Lansford *et al.*, 1958) which had been utilized for an "*L. arabinosus-*

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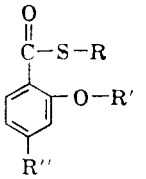
† Predoctoral Fellow (CF-12,189) National Cancer Institute, U. S. Public Health Service.

TABLE I

p-AMINOSALICYLIC ACID THIOESTER ANALOGS



R	R′	R″	Yield (%)	M.p. (°C)	Recryst. Solvent	Empirical Formula	% Nitrogen (Calcd.)	% Nitrogen (Found)
$O_2N—C_6H_4—CH_2—$	$C_6H_5CH_2—$	$—NO_2$	78	143–144	Acetone-Skelly-solve B	$C_{21}H_{16}N_2O_6S$	6.57	6.57
$H_2N—C_6H_4—CH_2-$ ·HCl	—H	$—NH_2$·HCl	41	160–162 dec.	Ethanol-ether	$C_{14}H_{14}N_2O_2S$·2HCl	8.07	7.96
$C_6H_5—CH_2—$	$C_6H_5CH_2—$	$—NO_2$	77	94–95	Ether-Skelly-solve B	$C_{21}H_{17}NO_4S$[a]	3.69	3.94
$C_6H_5—CH_2-$	—H	$—NH_2$·HCl	50	118–119[b] dec.	Ethanol-ether	$C_{14}H_{13}NO_2S$·HCl	4.45	4.63
n-C_5H_{11}	$C_6H_5CH_2—$	$—NO_2$	65	47–48	Ether-Skelly-solve B	$C_{19}H_{21}NO_4S$[c]	3.89	3.91
n-$C_5H_{11}—$	—H	$—NH_2$	75	89–91	Ether-Skelly-solve B	$C_{12}H_{17}NO_2S$[d]	5.85	5.71
$C_2H_5OOC—CH_2-CH_2—$	$C_6H_5CH_2—$	$—NO_2$	82	57–58	Acetone-Skelly-solve B	$C_{19}H_{19}NO_6S$[e]	3.59	3.67
$HOOC—CH_2—$	$C_6H_5CH_2—$	$—NO_2$	66	138–140	Ether-Skelly-solve B	$C_{13}H_{17}NO_6S$[f]	4.03	4.17

[a] Calcd.: C, 66.47; H, 4.52. Found: C, 66.74; H, 5.00. [b] The corresponding free base has been reported (Hayashi *et al.*, 1961). [c] Calcd.: C, 63.49; H, 5.89. Found: C, 63.16; H, 6.03. [d] The residue from the hydrogenolysis was taken up in benzene, dried, reduced to dryness *in vacuo*, and crystallized directly from the indicated solvent mixture. [e] Calcd.: C, 58.60; H, 4.92. Found: C, 58.67; H, 5.09. [f] Calcd.: C, 55.32; H, 3.77. Found: C, 55.56; H, 4.13.

pteridine" assay was modified as indicated below for the present assays with *Streptococcus lactis* 8039. Pteridine, formate, and thymine were omitted; the concentrations of L-tryptophan, L-cysteine, and L-asparagine were changed to 100, 200, and 10 μg/ml, respectively; and Sheffield N-Z-Case, pyridoxamine, and pantethine were added at levels of 200, 0.2, and 0.02 μg/ml, respectively. The medium was adjusted to *p*H 6.8 and sterilized by steaming for 20 minutes. The resulting medium was added to previously sterilized assay tubes containing the samples to be tested, following which the tubes were inoculated and the assays were incubated at 30° for about 20 hours.

Because of the poor solubility as well as the instability of the thioesters upon being heated in aqueous solution, the derivatives were prepared fresh daily at a concentration of 10 mg/ml in ethanol, and subsequently diluted 1:100 with sterile distilled water just prior to being serially added to the sterile assay tubes. For those assays which contained a constant amount of inhibitor, an appropriate quantity of the alcohol solution was added directly to the sterile medium which was subsequently added to sterile assay tubes. In some experiments, natural extracts and/or metabolites were dissolved in sterile water and added without heating to the assay tubes so that any heat-labile component would not be destroyed.

The effect of the various reversing agents on the toxicity of the thioester analogs of *p*-aminosalicylic acid were determined by growth studies, and the amount of growth of the microorganism was determined turbidimetrically with a previously described turbidimeter (Williams *et al.*, 1929). The instrument was so adjusted that distilled water read zero and an opaque object 100.

Organic Syntheses

Intermediates.—2-Benzyloxy-4-nitrobenzoic acid and the corresponding acid chloride were prepared by previously reported procedures, m.p. 170–171° and 120–121°, respectively (Jensen and Ingvorsen, 1952). The mercaptans were purchased through the normal commercial sources.

2-Benzyloxy-4-nitrobenzoic Acid Thioesters.—All of these analogs were synthesized in approximately the same manner, and the pertinent data for the individual compounds are recorded in Table I. The preparation of the *p*-nitrobenzylthioester of *p*-nitrosalicylic acid is described as a representative procedure. To a solntion of 1 g of 2-benzyloxy-4-nitrobenzoyl chloride in 10 ml of ethanol was added 1 g of *p*-nitrobenzylthiol, and the reaction mixture was heated gently for about one-half hour, after which about 0.5 g of pyridine was added and the reaction mixture was heated under reflux overnight. After cooling, 35 ml of Skellysolve B (b.p. 60–70°) was added, and the resulting precipitate was filtered and recrystallized from acetone–Skellysolve B to yield 1.7 g of product which gave the anticipated elemental analysis, m.p. 143–144° (dec).

4-Amino-2-hydroxybenzoic Acid Thioesters (*p-Aminosalicylic Acid Thioesters*).—The various reaction products from the above condensations were each hydrogenolyzed in essentially the same fashion and are tabulated in Table I. The preparation of the *p*-aminobenzylthioester of *p*-aminosalicylic acid will be described as an illustrative example. A solution of 0.6 g of 2-benzyloxy-4-nitrobenzoic acid *p*-nitrobenzylthioester in 30 ml of ethanol containing about 0.1 g of platinum oxide was treated with hydrogen gas in a Paar low-pressure hydrogenator at about 50 p.s.i.

[1] All melting points are uncorrected. The authors are indebted to Mrs. Sandra R. Lax for assistance with the microbial assays and to J. D. Glass for the elemental analyses.

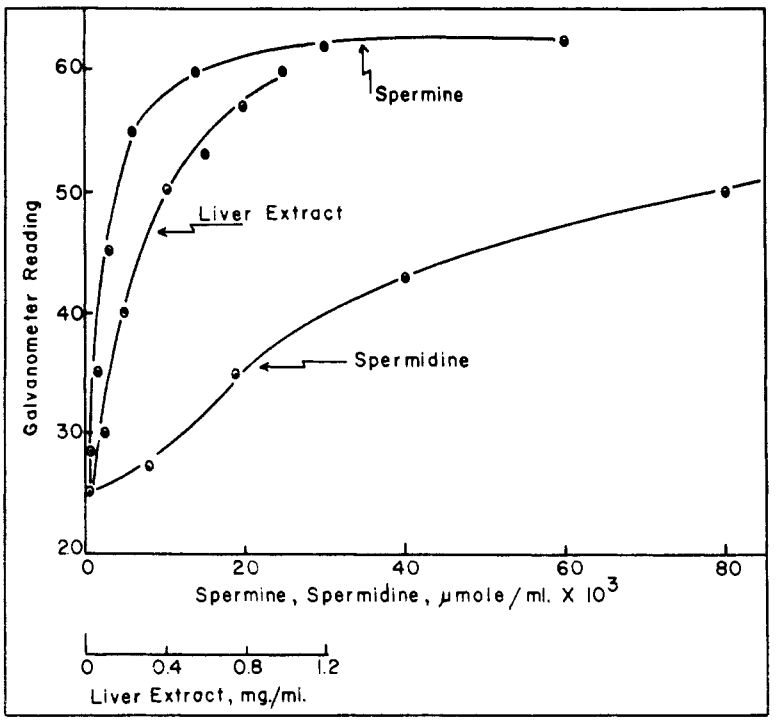


FIG. 1.—Reversal of toxicity of thiobenzylester of *p*-aminosalicylic acid for *Streptococcus lactis* by spermine, spermidine, and liver extract. The basal medium was supplemented with 10 μg/ml of inhibitor, and the assays were incubated at 30° for 18 hours.

pressure until 7 molecular-equivalents of hydrogen had been absorbed. The catalyst was filtered and the solvent was removed *in vacuo* to yield a residue which was then crystallized by dissolving it in ether and precipitating the product as a hydrochloride salt with anhydrous hydrogen chloride. There was obtained 0.2 g of material which gave the anticipated elemental analysis, m.p. 160–162° (dec.).

O - (2 - Benzyloxy - 4 - nitrobenzoyl) - N - corbobenzoxyserine Benzyl Ester.—A mixture of 1.46 g of 2-benzyloxy-4-nitrobenzoyl chloride and 1.65 g of *N*-carbobenzoxyserine benzyl ester (Skinner *et al.*, 1956) in 20 ml of benzene was heated for about one hour, and then 10 ml of pyridine was added and the reaction mixture was heated at about 60–70° for 2 days. The solvent was removed *in vacuo*, the oily residue was taken up in ether and filtered, Skellysolve B was added to induce turbidity, and the solution was left in the refrigerator overnight. There was recovered 2.1 g of product, m.p. 97–99°.

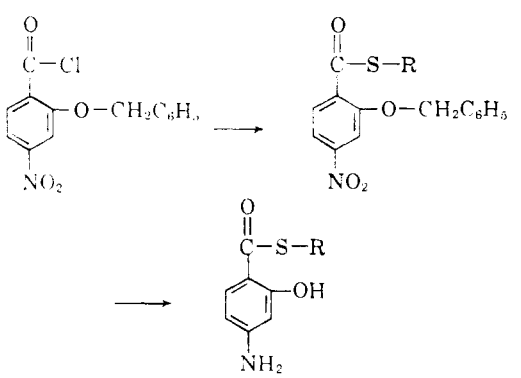
Anal. Calcd. for $C_{33}H_{28}N_2O_9$: C, 65.75; H, 4.83; N, 4.79. Found: C, 66.12; H, 5.19; N, 4.44.

O - (4 - Amino - 2 - hydroxybenzoyl)serine.—A solution of 0.7 g of *O*-(2-benzyloxy-4-nitrobenzoyl)-*N*-carbobenzoxyserine benzyl ester in 50 ml of ethanol containing 0.1 g of platinum oxide was treated with hydrogen under about 50 p.s.i. pressure until no further uptake was observed. The catalyst was filtered, the filtrate was reduced to dryness *in vacuo*, and the residue was crystallized from ethanol-ether to yield 0.25 g of material, m.p. 145–149° (dec).

Anal. Calcd. for $C_{19}H_{12}N_2O_5$: N, 11.66. Found: N, 11.55.

Results and Discussion

The previously described thioesters of *p*-aminosalicylic acid (Hayashi *et al.*, 1961) were prepared from the intermediate *p*-nitrosalicyloyl chloride, which is quite unstable. An alternate preparative approach was accordingly devised for the preparation of benzylthio *p*-aminosalicylate, and it was also utilized for the preparation of additional thioester derivatives in an attempt to find an active microbial growth inhibitor with greater solubility than the thiobenzylester. 2-Benzyloxy-4-nitrobenzoic acid, which forms a relatively stable acyl chloride (Jensen and Ingvorsen, 1952), was utilized as indicated in the accompanying equations for the preparation of the new thioester derivatives. The additional new analogs herein described were not sufficiently inhibitory toward lactic acid bacteria and



Escherichia coli at the limits of their solubilities in the assay medium to warrant extensive biological studies in place of the previously reported thiobenzylester.

As previously indicated (Hayashi *et al.*, 1961), various metabolites related to *p*-aminobenzoic acid, as well as *p*-aminobenzoic acid itself, failed to affect appreciably the toxicity of the benzylthioester of *p*-aminosalicylic acid for *S. lactis;* however, hot water extract of beef liver exerted a reversing effect which gave a reasonable dose response curve in preventing the toxicity of 10 μg/ml of the thioester (Fig. 1). With this assay, the active principle at *p*H 7.5 was found to be absorbed in one hour at 0° on a weight of charcoal

equal to the dry weight of liver solids. The activity was subsequently eluted from the charcoal, with a recovery of 50% of the total reversing effect, by eluting the charcoal with 1 N hydrochloric acid in ethanol, whereas it was not eluted from charcoal with either non-polar solvents or alkaline solutions. The factor was also found to be absorbed upon Amberlite IRC 50 (H^+) form resin. These and other properties suggested that the factor was a polyamine, and the effects of trimethylenediamine, putrescine, cadaverine, hexamethylenediamine, spermine, spermidine, and a number of related compounds on reversing the toxicity of the analog were accordingly determined. Of the compounds studied, spermine, and to a lesser extent spermidine, were effective in partially reversing the toxicity of the analog in a manner similar to that of liver extract as indicated in Figure 1 and Table II. Other polyamines were essentially inactive up to a concentration of 0.5 μmole/ml.

TABLE II

REVERSAL OF TOXICITY OF THIOBENZYL ESTER OF *p*-AMINOSALICYLIC ACID BY SPERMINE AND/OR BEEF LIVER EXTRACT[a]

Thiobenzyl Ester of *p*-Aminosalicylic Acid (μg/ml)	Supplement (μg/ml)[b]			
	None	Spermine *tetra*-HCl,[c] 4 μg	Beef Liver Extract, 800 μg	Beef Liver Extract, 800 μg + Spermine *tetra*-HCl, 4 μg
	Galvanometer Reading			
0	57	55	79	78
6	42			
8	27		67	
10	27	59	49	78
12	11	57	45	50
14		19	31	13
16		0	4	8

[a] Test organism: *S. lactis*, incubated at 30° for 18 hours. [b] Both spermine and the liver extract at one-half and twice the indicated concentrations gave essentially identical growth response data as that of the reported concentration levels. [c] Spermidine at a concentration of 4 μg/ml gave a response equal to about 30% of that of 800 μg/ml of beef liver extract.

A time-course study of growth of *S. lactis* indicated that spermine is not stimulatory to growth in the absence of the inhibitor. Figure 2 shows the effect of a supplement of 0.01 μmole of spermine per ml upon growth in the presence of the inhibitor, and also shows the absence of any effect of the supplement in the absence of the inhibitor.

The conclusion that spermine is a major principle present in the purified beef extract which reversed the inhibitory effects of the thioesters of *p*-aminosalicylic acid is based upon (*a*) the comparative chemical properties of the active principle and spermine, (*b*) a correlation of the spermine content of beef liver[2] (Fischer and Bohn, 1957) with the observed biological

[2] The concentration of spermine in beef liver is reported to be 12.7 mg per 100 g of fresh liver, and, since a hot water extraction of beef liver yields between 5 and 10% by weight of dry soluble solids, the concentration of spermine anticipated in 1 mg of this latter material would be about 1.2 to 2.4 μg. The interpolated data from Figure 1 indicate that 1 mg of the liver extract is equivalent to about 2.3 μg of spermine in reversing the toxicity of the thiobenzyl-ester of *p*-aminosalicylic acid.

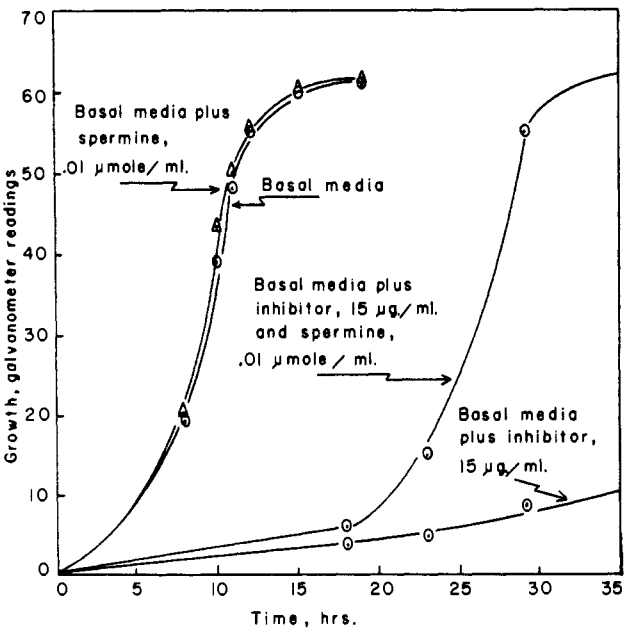


FIG. 2.—Effect of spermine on the rate of growth of *Streptococcus lactis* in both the presence and absence of *p*-aminosalicylic acid thiobenzyl ester.

response, (*c*) the failure to obtain any appreciable reversal of growth inhibition by beef liver extract in the presence of a supplement of spermine, and (*d*) similar R_F values of the active principle and spermine in chromatographic systems.[3]

Other compounds which have been observed to inhibit growth of *E. coli* and to be reversed by spermine or spermidine include quinacrine (atabrine) (Miller and Peters, 1945; Silverman and Evans, 1944) and propamidine (Miller and Peters, 1945; Snell, 1944); also, the inhibition of respiration of yeast cells caused by trypaflavin, methylene blue, quinine, crystal violet, and protamine was reversed by spermine (Massart, 1948). In addition, high concentrations of polyamines have been found to reverse the inhibitory effects of stilbamidine and pentamidine for *E. coli* and *Staphylococcus aureus* (Bichowsky-Slomnitzki, 1948) and to overcome the proflavine inhibition of an *E. coli* phage (Kay, 1959).

A complete reversal of the microbial toxicity of the thiobenzyl ester of *p*-aminosalicylic acid was not observed with any natural extract or known metabolite. This may be the result of an irreversible interaction of the inhibitor with active enzymic sites. Since a direct relationship of the thioester toxicity to an inhibition involving folic acid was not demonstrated, a separate study was undertaken to determine whether spermine itself could affect systems in which single carbon unit metabolism is limiting. Spermine was found, as a result of this latter study, to decrease the requirement of *Pediococcus cerevisiae* for folinic acid (5-formyltetrahydrofolic acid) under appropriate conditions (Shive, 1961), to decrease the requirement for thymidine necessary for reversal of the toxicity of 2,4-diaminodiphenylpteridine for *Lactobacillus arabinosus*, and to exert similar effects in other lactic acid bacterial systems (Turner *et al.*, 1963). Thus, the thioesters of *p*-

[3] R_F values for spermine and spermidine were diffuse in alkaline and neutral solvents; the acidic solvent, however, gave more definite data, *e.g.*, spermine gave R_F values of 0.66 and 0.68 by the ascending technique in methanol-hydrochloric acid (80 ml 95% methanol–20 ml 0.1 N hydrochloric acid) and isobutyric acid–ammonium hydroxide–water (66 ml isobutyric acid–1 ml ammonium hydroxide–33 ml water), respectively.

aminosalicylic acid appear to exert inhibitory effects which are related to some degree to functions of folic acid; however, these data do not as yet demonstrate an enzymic interaction of the analog at the site of the *p*-aminobenzoic acid moiety.

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A Metabolic Relationship of Spermine to Folinic Acid and Thymidine*

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Spermine reduces the amount of thymidine required for reversal of inhibition of growth of *Lactobacillus arabinosus* 17-5 caused by 2,4-diamino-6,7-diphenylpteridine, aminopterin, or sulfanilamide, under conditions of growth-limiting thymidine biosynthesis. Spermine also decreases the growth requirement of *Pediococcus cerevisiae* for folinic acid, and that of *L. leichmannii* 313 for folinic acid, thymidine, or thymine. Cells of *L. arabinosus* grown in the presence of diaminodiphenylpteridine show after approximately 20–24 hours' growth, if spermine is present in the growth medium, enhanced ability to incorporate formate into acid-insoluble material, and decreased utilization of exogenous thymidine. These and related results suggest that spermine is involved directly or indirectly within some area of single carbon unit metabolism.

The ability of spermine to prevent partially the toxicity of a benzyl thioester of *p*-aminobenzoic acid (Smith *et al.*, 1963) suggested the possibility of a biochemical relationship of spermine to coenzymes of the folic acid group, but the inability, as indicated in the same report, of *p*-aminobenzoic acid or its coenzymatic conjugates to affect the toxicity of the thioester analog precluded the use of that system for demonstration of a relationship of spermine to single carbon unit metabolism. Previously, a synergistic effect of certain natural extracts in combination with thymidine in preventing bacterial growth inhibition caused by diaminodiphenylpteridine was reported (Lansford *et al.*, 1958), and the chemical behavior of the active factors during concentration steps was found to be consistent with a polyamine structure. In the present study, spermine has been found to decrease several fold the amount of thymidine required for reversal of growth inhibition of *Lactobacillus arabinosus* in the presence of an inhibitory concentration of 2,4-diamino-6,7-diphenylpteridine, or of aminopterin. Spermine exerts a similar sparing effect upon the folinic acid (5-formyltetrahydrofolic acid) requirement of *Pediococcus cerevisiae*. These and related results involving a stimulation by spermine of endogenous thymidine biosynthesis and of formate utilization in *L. arabinosus* suggest a direct or indirect role of spermine within some area of single carbon unit metabolism.

* A preliminary report was presented at the Robert A. Welch Foundation Conference, "Molecular Structure and Biochemical Reactions," December, 1961, Houston, Texas.

Experimental Procedures

Culture Media for Bacterial Growth Assays.—A basal medium previously described (Lansford and Shive, 1952) was utilized for experiments with *Lactobacillus arabinosus* 17-5 (ATCC No. 8014) and *Pediococcus cerevisiae* (ATCC No. 8081) with the modification that the casein acid hydrolysate used was the salt-free grade supplied by the Nutritional Biochemicals Corporation (11 g/1000 ml double strength medium). For study of effects upon the growth response of *L. arabinosus* to thymidine (Fig. 1) and to folinic acid, the basal medium was supplemented with vitamins, purines, pyrimidines, formate, and glucose as described for thymidine assay using diaminodiphenylpteridine inhibitor (Lansford *et al.*, 1958) except that aminopterin replaced the pteridine inhibitor where indicated (Fig. 1). For *P. cerevisiae* no folic or folinic acid was included in the vitamin supplement, and to ensure removal of traces of these vitamins the medium was treated with activated charcoal (200 mg Darco G-60 per 100 ml double strength basal medium) before the addition of purine-pyrimidine and vitamin supplements. Growth medium for *Lactobacillus leichmannii* 313 (ATCC No. 7830) (U. S. Pharmacopeia, Vol. 16, Mack Publishing Co., Easton, Pa., p. 888) was supplemented with vitamin