RELATIONSHIP BETWEEN VITAMIN B₁₂ OXIDATION PRODUCT AND SODIUM CHLORIDE TOXICITY FOR LACTOBACILLI

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Beiler *et al.* (1951) reported that oxidation of vitamin B₁₂ by hydrogen peroxide under strongly acid conditions resulted in the production of a colorless product which inhibited the vitamin B₁₂ requiring microorganism, *Lactobacillus leichmannii* (ATCC 4797). Such inhibition was antagonized competitively by vitamin B₁₂. Since we had observed that hydrogen peroxide itself competitively inhibited growth of another B₁₂ requiring microorganism, *Lactobacillus lactis* Dorner (Koditschek *et al.*, 1949), we reinvestigated the role of hydrogen peroxide in the formation of toxic oxidation products.

It became apparent, upon examination of the oxidation procedure used, that neutralization of reaction mixtures resulted in extremely high levels of NaCl, levels which have been found to be toxic to many lactobacilli.

It is the purpose of this paper to report the data obtained from such a study and to show that the inhibitory effect observed with an oxidation product of vitamin B₁₂ can be ascribed to NaCl toxicity and that with *L. leichmannii* (ATCC 7830) such toxicity is overcome by high levels of vitamin B₁₂.

EXPERIMENTAL METHODS

Formation of the vitamin B₁₂ oxidation product was carried out as outlined by Beiler and co-workers (1951). To 10 ml of a vitamin B₁₂ solution (100 µg per ml), 5 ml 12 N HCl and 7 drops of "superoxal" (Merck) (30 per cent H₂O₂) were added with stirring at room temperature. The reaction mixture was allowed to stand for one hour, after which time it was neutralized with 10 N NaOH and diluted to 100 ml. The effect of various constituents of the reaction mixture was tested by excluding each singly from the above procedure and then testing the resultant solutions at the levels prescribed by Beiler *et al.* (1951). The following reaction mixtures thus were tested: (a) B₁₂ + H₂O₂ + HCl + NaOH, (b) H₂O₂ + HCl + NaOH, and (c) HCl + NaOH.

In addition, vitamin B₁₂ dosage-response curves were run in media supplemented with varying concentrations of NaCl.

Assay procedure. The U.S.P. vitamin B₁₂ procedure (1952) was used with *L. leichmannii*, strain 7830.¹ *L. leichmannii*, strain 4797, studies were performed by the method of Skeggs *et al.* (1948) with the following modifications: (1) the medium was adjusted to pH 6.8 prior to sterilization and (2) a total volume of 10 ml per tube was employed.

For *L. lactis* (ATCC 8000) the method of Caswell *et al.* (1949) was used.²

Growth of both strains of *L. leichmannii* was determined turbidimetrically after 40 hours' incubation at 37 C with a "lumetron" (660 mµ filter). Titration with 0.05 N NaOH after 40 hours' incubation at 37 C was used to measure *L. lactis* growth.

RESULTS AND DISCUSSION

Table 1 shows the results obtained with the vitamin B₁₂ oxidation procedure and the same procedure in which vitamin B₁₂ and hydrogen peroxide were eliminated. Both reaction mixtures were equally toxic to *L. leichmannii*, strain 7830. Under our experimental conditions, the toxicity obtained appears to be due solely to NaCl and not to an oxidation product of vitamin B₁₂. Such toxicity was antagonized effectively by high levels of vitamin B₁₂. A more detailed study of this toxicity and its reversal was carried out with NaCl. Figure 1 demonstrates that NaCl, at levels of 10 mg per ml and above, markedly increased

¹ The inoculum was diluted 10-fold in our experiments.

² The medium was supplemented with "tween 80" to give a final concentration of 250 µg per tube, and the stock culture was carried in a modified medium (Difco skim milk, 3.7 per cent; Difco yeast extract, 0.5 per cent; glucose, 0.5 per cent; and Difco bacto agar, 0.1 per cent). Inoculum was grown in basal medium supplemented with 0.2 mµg per ml vitamin B₁₂.

the vitamin B₁₂ requirement of *L. leichmannii*, strain 7830. Similar observations were reported recently by Corbett (1952) who noted a 16 to 40-fold decrease in sensitivity to vitamin B₁₂ when the assay medium was supplemented with 15 mg NaCl per ml. Sodium chloride is not unique in this respect. We have obtained identical results with KCl, Na acetate, and CaCl₂.

While essentially similar data were noted with *L. leichmannii*, strain 4797, the results varied from experiment to experiment. In all cases, however, toxicity was observed, but in many cases reversal of such toxicity with increased vitamin B₁₂ was not apparent. Prolonged incubation (60 to 72 hr) in these cases resulted in reversal of NaCl toxicity.

When *L. leichmannii*, strain 7830, was grown in the medium used for *L. leichmannii*, strain 4797, reversal of the NaCl toxicity by low levels of vitamin B₁₂ was not obtained. These results indicate that the basal medium may be responsible for the inconsistency observed with *L. leichmannii*, strain 4797.

TABLE 1

The effect of B₁₂ oxidation product and NaCl on B₁₂ response with *Lactobacillus leichmannii*, strain 7830

VITAMIN B ₁₂ , μG PER TUBE	B ₁₂ OXIDATION PRODUCT, μG PER TUBE*				
	0	10	25	35	50
0.0	87	86	99	94	90
0.001	20	21	65	83	90
0.005	17	18	41	67	84
0.05	17	17	31	38	48
5.0	15	17	29	29	36

	NaCl EQUIVALENCE, MG PER TUBE†				
	0	35	87.5	122.5	175
0.0	85	89	88	85	90
0.001	20	21	48	78	91
0.005	17	18	31	57	78
0.05	16	17	24	25	45
5.0	16	16	22	23	36

* Procedure a: B₁₂ + H₂O₂ + HCl + NaOH.

† Procedure c: HCl + NaOH.

Figure 1. The reversal of NaCl toxicity by vitamin B₁₂ for *Lactobacillus leichmannii*, strain 7830. ○—○ No NaCl; ×—× 15 mg NaCl per ml; ●—● 20 mg NaCl per ml; ◇—◇ 25 mg NaCl per ml.

The effect of NaCl on *L. lactis* Dorner also was studied. While salts proved toxic to this B₁₂ requiring microorganism, high levels of vitamin B₁₂ had no reversing effect.

Recently, Villela and Abreu (1952) demonstrated that vitamin B₁₂ oxidation mixtures were toxic to *Euglena gracilis*. They were unable to overcome such toxicity with low levels of vitamin B₁₂ (1 µg per 10 ml); higher levels were not tested. No doubt the inhibition noted with this protozoan also can be attributed to NaCl toxicity.

The effect of NaCl might well be on the adsorption of vitamin B₁₂ from solution by the lactobacilli. Davis and Chow (1952) in studies with *L. leichmannii* noted that the adsorption of vitamin B₁₂ was affected markedly by salt concentration. They noted that NaCl at levels of 1.7 per cent and above caused a decrease in vitamin B₁₂ adsorption from broth. It is apparent that the equilibrium between bound and free vitamin B₁₂ is sensitive to salt concentration. For this reason, the addition of NaCl to the basal medium causes marked inhibition of growth which, under certain conditions, can be reversed by reestablishing the equilibrium with additional vitamin B₁₂.

SUMMARY

The toxicity ascribed to hydrogen peroxide oxidation products of vitamin B₁₂ has been found to be due to NaCl toxicity. The presence of relatively high levels of salts in microbiological media results in inhibition of growth of several lactobacilli, and, in some instances, such inhibition is antagonized by vitamin B₁₂.

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ADDENDUM

Since preparing this paper for publication, Bardos and Gordon (*J. Am. Chem. Soc.*, **75**, 2019, 1953) have reported that hypertonic solutions of various inorganic salts inhibit the growth of *Lactobacillus leichmannii*, strain 313, and that such inhibition is reversed by addition of excess vitamin B₁₂. Our data and conclusions are consistent with those presented by them.