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 report to present 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" (600 µm 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

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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 mg per ml vitamin B12.
VITAMIN B₁₂ OXIDATION PRODUCT

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>
<thead>
<tr>
<th>VITAMIN B₁₂</th>
<th>B₁₂ OXIDATION PRODUCT, µg per tube</th>
<th>µg per tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>87</td>
<td>86</td>
</tr>
<tr>
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<td>21</td>
</tr>
<tr>
<td>0.005</td>
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<td>17</td>
</tr>
<tr>
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<td>17</td>
</tr>
<tr>
<td>5.0</td>
<td>15</td>
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<table>
<thead>
<tr>
<th>NaCl equivalence, mg per tube</th>
<th>µg per tube</th>
</tr>
</thead>
<tbody>
<tr>
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<td>85</td>
</tr>
<tr>
<td>0.001</td>
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<tr>
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<tr>
<td>0.05</td>
<td>16</td>
</tr>
<tr>
<td>5.0</td>
<td>16</td>
</tr>
</tbody>
</table>

* 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. O—O No NaCl; X—X 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 *B*12 requiring microorganism, high levels of vitamin *B*12 had no reversing effect.

Recently, Villela and Abreu (1952) demonstrated that vitamin *B*12 oxidation mixtures were toxic to *Euglena gracilis*. They were unable to overcome such toxicity with low levels of vitamin *B*12 (1 mg 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 *B*12 from solution by the lactobacilli. Davis and Chow (1952) in studies with *L. leichmannii* noted that the adsorption of vitamin *B*12 was affected markedly by salt concentration. They noted that NaCl at levels of 1.7 per cent and above caused a decrease in vitamin *B*12 adsorption from broth. It is apparent that the equilibrium between bound and free vitamin *B*12 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 *B*12.

**SUMMARY**

The toxicity ascribed to hydrogen peroxide oxidation products of vitamin *B*12 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 *B*12.

**REFERENCES**


**ADDENDUM**

Since preparing this paper for publication, Bardos and Gordon (J. Am. Chem. Soc., 76, 1919, 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 *B*12. Our data and conclusions are consistent with those presented by them.