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Some Factors Affecting the Vitamin B_{12}-binding Properties of Hog Mucosal Extracts

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Extracts of hog gastric mucosa (Ventriculin) contain a substance, probably protein in nature, which inhibits the growth promoting activity of vitamin B_{12} for *Lactobacillus leichmannii*. Several investigators have claimed that heating destroyed the vitamin B_{12}-binding factor. Ternberg and Eakin (7), Shaw (5), and Burkholder (2) heated extracts of hog gastric mucosa to release vitamin B_{12} in order to make it available to bacteria. Shaw (5) and Spray (6) reported that not all of the vitamin B_{12} was released by heating. Gregory, Ford and Kon (3), however, found a vitamin B_{12}-binding substance in sow's milk which could withstand heating at 100° C. for 30 minutes. Several workers (3,6) reported that the binding factor is destroyed by heating at alkaline pH, but that it is relatively stable at neutral and acid pH values.

In this work the effect of heat on the vitamin B_{12}-inhibitory factor and the possible physiological significance of vitamin B_{12}-binding was studied.

EXPERIMENTAL

Cultures of the test organism *L. leichmannii* 7830 were carried in a peptonized milk agar and transferred weekly. The assay medium used was a modification (8) of that used by Peeler, Yacowitz and Norris (4). Growth of *L. leichmannii* in the assay medium was measured by determination of the turbidity (per cent transmission) with a KWSZ photometer using filter 7 (650 mμ) after incubation at 37°C. for 22 hours.

An extract of Ventriculin (Parke, Davis and Co.) was prepared by extracting it with five times its weight of distilled water. This extract was saturated with ammonium sulfate to precipitate the proteins. The precipitate was collected by centrifugation, dialyzed against distilled water and diluted to the volume of the original extract. Ammonium sulfate then was added to 25 per cent saturation; the precipitate was removed by centrifugation and discarded. This procedure removed a part of the growth-promoting substances but did not affect the vitamin B_{12}-inhibitory activity. The protein material in the supernatant was precipitated by saturation with ammonium sulfate, collected by centrifugation and dialyzed against distilled water to remove ammonium sulfate.

In determining vitamin B_{12}-inhibitory activity 1 ml. of appropriate dilutions of the dialyzed material was added to each assay tube containing 5 ml. of the assay medium, 0.15 mμg vitamin B_{12} and water to make 9 ml. Diluted extracts were heated at pH 5.5 to 6.0 by (a) autoclaving for 5 minutes at 15 lb. pressure both with and without the assay medium, and (b) heating at 100°C. for 10 minutes in a water bath.

Results

Preliminary experiments on the effect of heat on the inhibitory factor showed that this factor could withstand heating at 100°C. for 10 minutes at neutral and acid pH values (up to and including pH 2.5), but was destroyed by the same heat treatment at alkaline pH. In these experiments extracts and assay medium were heated separately.

The effect of heating extract and assay medium together as compared with heating them separately on the inhibitory activity was studied in subsequent experiments (table 1). When the extract and the basal medium were autoclaved separately, no appreciable inactivation of inhibitory factor occurred, except in the case of extract No. 1 in the 1:10 dilution, which probably was caused by some other substance in the extract. Autoclaving extract and assay medium together destroyed almost all of the inhibitory factor. Autoclaving extracts for 5 minutes at 15 lb. pressure was equivalent to heating at 100°C. for 10 minutes.

Table 1

Comparison of effect upon inhibitory factor of autoclaving 5 minutes at 15 pounds pressure with boiling for 10 minutes.

		Per cent transmission[a]				
Sample	Dilution	Not heated	Autoclaved with medium		Autoclaved separately	Heated in boiling water
Extract 1	1:10	87	16	16[b]	21	21
Extract 1	1:100	100	54	....	70	86
Extract 2	1:10	75	35	15[b]	65	65
Extract 2	1:100	100	65	....	100	100

[a] 0.06 mμg. vitamin B_{12} = 56.

[b] No vitamin B_{12}.

Constituents of the assay medium (amino acids, vitamins, salts, etc.) were tested for their abilities to inactivate the vitamin B_{12}-inhibitory factor. One ml. of these constituents in the concentration used in the assay medium (8) were heated at 100°C. for 10 minutes with 4 ml. of diluted extract. These extracts then were

tested for vitamin B_{12}-inhibitory factor in the usual manner. The only significant inactivation of vitamin B_{12}-inhibitory activity was caused by *L*-histidine with iron (II) or manganese (II) (table 2).

Table 2

Effect of heating metal ions and imidazoles with vitamin B_{12}-inhibitory principle.

Substances heated with extract	Transmission[a]
Extract alone	90
L-histidine and Fe^{II}	57
L-histidine and Fe^{II} [b]	83
L-histidine and Co^{II}	59
L-histidine and Co^{II} [b]	98
Benzimidazole and Fe^{II}	54
Benzimidazole and Co II	41

[a]0.15 mμg. vitamin $B_{12} = 36$.
[b]Substances were mixed before adding inhibitory principle.

Because of the structural similarities between L-histidine and 5,6-dimethylbenzimidazole, a degradation product of vitamin B_{12}, and the presence in the vitamin B_{12} molecule of cobalt which forms complexes similar to iron and manganese, benzimidazole and cobalt (II) were tested for their effects upon the inhibitory factor. The concentration of L-histidine was 0.05 *M*, benzimidazole 0.01 *M* and salt solutions 0.025 *M*. Representative results are presented in table 2. Inhibition was reversed by L-histidine, benzimidazole and the metal ion when each substance was added separately to the extract. The anomalies shown by L-histidine and iron (II) or cobalt (II) occurred when this amino acid and the metal ion were mixed and allowed to stand before being added to the extract. Histidine and the metal ion were mixed in stoichiometric amounts, 1 mole of metal ion to 2 moles L-histidine, and the conditions were favorable for the formation of complexes which take up oxygen irreversibly (1). It is apparent that the products formed from histidine and cobalt (II) or iron (II) cannot react with the vitamin B_{12}-inhibitory factor under the conditions described, but when they are added separately to the inhibitory factor reversal of inhibition is possible. It may be that a reaction between the inhibitory factor and L-histidine or cobalt (II) is not possible because a bond required is being used in a cobalt-L-histidine linkage.

Benzimidazole with iron (II) or cobalt (II) neither promoted nor inhibited the growth of *L. leichmannii*.

The inactivation of inhibitory factor in the presence of these compounds would be of physiological significance if the same reaction occurred at 37°C. L-histidine and benzimidazole were incubat-

ed at 37°C. for 2 hours with iron (II) and cobalt (II) in the presence of the inhibitory factor. No reaction occurred at this temperature.

Conclusions and Summary

Heating experiments in this laboratory showed that the vitamin B_{12}-inhibitory factor is not inactivated by heat in dilute solutions when heated separately. Heating in a water bath at 100°C. for 10 minutes, and autoclaving for 5 minutes at pH 5.5 to 6.0 resulted in no significant loss in activity whereas boiling at alkaline pH destroyed the vitamin B_{12}-inhibitory principle. From the differences observed by heating the vitamin B_{12}-inhibitory principle in the assay medium and heating it separately, it appeared that heat was not the destructive agent, but that other substances were responsible for the inactivation when the mixture was heated. The constituents of the assay medium and the results showed that among these substances only L-histidine in the presence of iron (II) or manganese (II) caused inactivation of the vitamin B_{12}-inhibitory factor upon heating. The similarity of these compounds to components of the vitamin B_{12} structure led to the testing of benzimidazoles and cobalt (II) which are found in the vitamin B_{12} molecule. Cobalt (II) and benzimidazoles were found to be even more effective than iron (II) and L-histidine. These results were obtained at acid pH at 100°C., but not at 37°C.

The following hypothesis for the action of these compounds is suggested. The inhibitory substance unites with vitamin B_{12} at two points, with the cobalt and the 5,6-dimethylbenzimidazole, thus making the vitamin unavailable to the test organism and resulting in inhibition of growth. In the presence of both cobalt (II) and benzimidazole the two reactive sites of the inhibitory factor are tied up, so that it can no longer react with vitamin B_{12}. However, neither cobalt (II) nor benzimidazole alone can inactivate the inhibitory factor and prevent the inhibition of vitamin B_{12}.

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