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The Effect of Heat on the Microbiological and Anti-anaemic Properties of Human Gastric Juice mixed with Vitamin B_{12}

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Ternberg & Eakin (1949) found in normal human gastric juice a substance which combined with vitamin B_{12} to make the vitamin unavailable as a growth factor in microbiological assays. The gastric juice of normal people was reported to contain more of the substance than the juice of patients with pernicious anaemia in relapse. It was suggested that this substance was the intrinsic factor of Castle. This suggestion aroused much interest, for, if confirmed, it could provide a relatively simple method for detecting intrinsic factor. Clinical observations by Hall *et al.* (1950) indicated that the complex formed between the intrinsic factor, extracted from hog stomach or duodenum, and vitamin B_{12}, was more stable to heat than the intrinsic factor alone. Ross (1950) found that substances having similar growth-promoting activity to that of vitamin B_{12} for *Euglena gracilis* were released on heating human serum. Heating at 100° for 30 min. released more activity than heating at 70° for the same time, so that serum apparently contains a complex of vitamin B_{12} which is fairly stable to heat.

During experiments to see whether certain proteins other than the substance in gastric juice could bind vitamin B_{12}, i.e. make it unavailable to bacteria, indications were obtained that the factor in gastric juice was more stable to heat in neutral than in acid solution. In view of present interest in the role of intrinsic factor in the metabolism of vitamin B_{12}, it seemed worth while to investigate the phenomenon further, using both microbiological and clinical methods.

EXPERIMENTAL AND RESULTS

The effect of acidity on the stability of the vitamin B_{12}-binding factor to heat

Treatment of gastric juice. The samples of gastric juice were residues from fractional test meals, obtained through the kindness of the Biochemistry Department of this hospital. Only samples containing free acid were used. The juice was filtered and stored in sterile flasks at 0° for at least 24 hr. After this treatment there was no evidence of bacterial growth when 0·5 ml. portions of the juice were added aseptically to broth and incubated for several days at 37°. The juice was neutralized just before use by adding sterile 2 N-NaOH drop by drop, and the pH was checked on a meter to between 7·2 and 7·6.

Microbiological assays. Vitamin B_{12} was assayed with *Lactobacillus leichmanii* in a medium containing acid-hydrolysed casein with the usual supplements. A solution of crystalline vitamin B_{12} (10 μg./ml., 'Cobione', Merck) was diluted for use as standard, and for addition to the gastric juice.

Sterile vitamin B_{12} solution was added to gastric juice in sterile graduated test tubes to give concentrations of 0·25 μg. or 0·5 μg./100 ml. of juice. After receiving the treatment indicated in Tables 1–3, the solutions were diluted with sterile water to give apparent vitamin B_{12} concentrations of 0·5 mμg./ml., and 1 ml. portions were added to the culture medium for assay. The amount of bacterial growth was read turbidimetrically after incubating at 37° for 18 hr.

In agreement with the results of Ternberg & Eakin (1949), gastric juice was shown to bind vitamin B_{12} (Table 1). The vitamin was released from its complex with the substance in gastric juice by heating the acid solution at 100° for 5 min. It was not necessary to incubate the juice and the vitamin together before adding them to the culture medium, in fact when vitamin B_{12} and gastric juice were added separately to the medium before incubation, very little bacterial growth occurred.

When gastric juice was neutralized before adding the vitamin B_{12}, heating at 100° for 5 min. no longer released all the vitamin from its bound form (Table 2). Neutralized gastric juice retained some capacity to bind vitamin B_{12} after heating at 100° for 15 min., but autoclaving at 20 lb. pressure for 30 min. destroyed the binding substance. This treatment also released the vitamin from its compound.

Microbiological activity was not released by heating a mixture of vitamin B_{12} and neutralized gastric juice, even when it was re-acidified with hydrochloric acid to the same pH as the original gastric juice after incubation. Autoclaving a re-acidified sample, however, released a considerable part of the vitamin B_{12}, although some was apparently destroyed. This would be expected, since experiments showed that over 95% of the pure vitamin was destroyed by autoclaving in acid solution. The stability to heat after re-acidification was not shown by mixtures which were stored at 0°

Table 1. *Effect of normal human gastric juice on the utilization of vitamin* B_{12} *by* Lactobacillus leichmanii

(Each reading of optical density is the mean of the values for four separate tubes containing identical samples. The optical densities of the cultures are related to the quantities of vitamin B_{12} added to the tubes by a curve, the slope of which decreases fairly rapidly in the range 0·0–1·0 mμg. vitamin B_{12}. Larger amounts of vitamin B_{12} only produce very small further increases in opacity.)

No.	Supplements present in 5 ml. culture medium	Optical density × 100 Exp. 1	Exp. 2	Exp. 4
1	Nothing	10	7	11
2	0·5 mμg. vitamin B_{12}	58	60	43
3	0·5 mμg. vitamin B_{12} + 0·1 ml. gastric juice, incubated 22 hr. at 37° before adding to medium	14	9	12
4	As no. 3 but with 0·2 ml. juice	—	12	10
5	As nos. 3 and 4, but heated 5 min. at 100° after incubation and before adding to medium	64	66	57
6		—	64	66
7	0·5 mμg. vitamin B_{12} + 0·05 ml. gastric juice added separately to medium just before inoculation	10	—	—

Table 2. *Effect of acidity on the breakdown by heat of the compound formed by vitamin* B_{12} *with a substance in gastric juice*

(All tubes in nos. 3–12 were incubated 22 hr. at 37° and heated 5 min. at 100° before adding to culture medium. Nos. 13 and 14 were autoclaved 30 min. at 20 lb. after incubating 22 hr. at 37°.)

No.	Supplements present in 5 ml. culture medium	Optical density × 100 Exp. 3	Exp. 4	Exp. 5	Exp. 6
1	Nothing	7	11	9	7
2	0·5 mμg. vitamin B_{12}	42	43	47	43
3	0·5 mμg. vitamin B_{12} + 0·1 ml. acid gastric juice	56	57	66	52
4	0·5 mμg. vitamin B_{12} + 0·2 ml. acid gastric juice	64	66	70	56
5	0·5 mμg. vitamin B_{12} + 0·1 ml. neutralized gastric juice	10	10	18	10
6	0·5 mμg. vitamin B_{12} + 0·2 ml. neutralized gastric juice	8	7	14	8
7	As nos. 5 and 6, but re-acidified after incubation	—	10	—	10
8		—	6	—	10
9	As nos. 5 and 6, but neutralized juice heated 15 min. at 100° before incubation	10	10	36	8
10		10	—	30	8
11	As nos. 5 and 6, but neutralized juice autoclaved 30 min. at 20 lb. before incubation	54	—	62	48
12		56	—	68	56
13	0·5 mμg. vitamin B_{12} + 0·1 ml. neutralized gastric juice	—	50	61	44
14	0·5 mμg. vitamin B_{12} + 0·2 ml. neutralized gastric juice	—	61	70	48
15	As nos. 13 and 14, but re-acidified before autoclaving	—	39	—	26
16		—	42	—	28

Table 3. *Effect of incubation on the stability to heat in acid solution of the compound of vitamin* B_{12} *with a substance in gastric juice*

(*A*, vitamin B_{12} and gastric juice mixed and stored 22 hr. at 0° and then heated 5 min. at 100°; *B*, vitamin B_{12} and gastric juice mixed and incubated 22 hr. at 37° and then heated 5 min. at 100°.)

No.	Supplements present in 5 ml. culture medium	Optical density × 100 Exp. 7		Exp. 8	
1	Nothing	27		4	
2	0·5 mμg. vitamin B_{12}	57		43	
		A	*B*	*A*	*B*
3	0·5 mμg. vitamin B_{12} + 0·1 ml. acid gastric juice	66	66	54	53
4	0·5 mμg. vitamin B_{12} + 0·2 ml. acid gastric juice	70	68	58	61
5	0·5 mμg. vitamin B_{12} + 0·1 ml. neutralized gastric juice	19	22	4	4
6	0·5 mμg. vitamin B_{12} + 0·2 ml. neutralized gastric juice	18	20	4	3
7	As nos. 5 and 6, but re-acidified with HCl after incubation or storage at 0°	66	51	47	8
8		70	42	42	5

instead of being incubated, although incubation was not essential for the greater stability in neutral solution (Table 3). Perhaps, therefore, incubation produces some change in the vitamin B_{12}-binding factor which makes it more stable to heat in acid solution.

The somewhat higher growth responses shown in the tables for mixtures of gastric juice and vitamin B_{12}, as compared with those due to the vitamin alone, are due to the small amounts of growth-promoting substances present in the gastric juice itself.

Thus the substance in gastric juice which combines with vitamin B_{12}, and the compound so formed, appear to be more stable to heat in neutral solution than in solutions at the pH of normal human gastric juice. If neutralized gastric juice and vitamin B_{12} are first incubated together, the compound appears to retain its stability to heat, even on re-acidification to the original pH of the gastric juice.

Failure of a microbiologically inactive compound of vitamin B_{12} *to show haemopoietic acitivity in pernicious anaemia*

It has been suggested (Ungley, 1950) that Castle's intrinsic factor may act by combining with vitamin B_{12} to make it unavailable for the growth of intestinal bacteria; otherwise the bacteria would use up the vitamin so that it could not be absorbed by the host. Failure to secrete the intrinsic factor, such as occurs in patients with pernicious anaemia, is thus supposed to cause a deficiency of vitamin B_{12} in the host. On this hypothesis, a mixture of neutralized gastric juice and vitamin B_{12}, which has been incubated at 37° and then heated at 100°, should retain anti-anaemic activity when given by mouth to a patient with pernicious anaemia in relapse, since most of the vitamin B_{12} would be in a form unavailable to micro-organisms.

Accordingly, a number of samples of gastric juice from normal subjects, either resting samples or those secreted under histamine stimulation, and all containing free acid, were pooled and stored at 0° and pH 2·0. A patient with classical Addisonian pernicious anaemia in relapse was given 5 μg. of vitamin B_{12} ('Cytamen', Glaxo) in 100 ml. water, daily by mouth for 10 days. For the following 11 days he received 100 ml. of neutralized gastric juice, which had been incubated 22 hr. at 37° with 5 μg. vitamin B_{12} and then heated at 95° for 20 min. In a third period of 10 days he was given a similar mixture of gastric juice and vitamin B_{12} which had been incubated but not heated at 95°. All the samples of gastric juice were portions of the pooled specimen, and they were neutralized with 10N-NaOH just before starting incubation.

Table 4 shows that a considerable proportion of the vitamin B_{12} remained in a bound form after heating at 95° for 20 min. The patient did not respond either to 5 μg. vitamin B_{12} per day alone, or to a mixture of the vitamin and gastric juice which had been incubated and heated. There was a significant response to the vitamin incubated with unheated gastric juice (Table 5). Similar results were obtained by Hall (1950), who found that intrinsic factor in gastric juice was destroyed by incubating neutralized juice at 25° for 24 hr., followed by heating at 70° for 1 hr.; incubating at 25° without heating did not destroy the factor.

DISCUSSION

The results show that when neutralized human gastric juice is mixed with vitamin B_{12} and heated at 100°, a considerable part of the vitamin remains in a form which cannot be used as a growth factor by bacteria, whereas the power of the juice to potentiate the haemopoietic action of vitamin B_{12} by mouth is lost. Thus Castle's intrinsic factor seems to be destroyed by a degree of heating which is resisted by the substance which reacts with vitamin B_{12}, indicating that the two factors in gastric juice

Table 4. *Effect of heat on mixtures of neutralized gastric juice and vitamin* B_{12}

Additional supplements	Vitamin B_{12} in 5 ml. culture medium (mμg.) 1·0	2·0	3·0	4·0
	Optical densities (× 100) with the vitamin B_{12} and the additional supplements			
Nothing	64	64	66	65
0·1 ml. neutralized gastric juice, incubated 22 hr. at 37° with the vitamin B_{12} before adding to medium	30	47	54	62
0·1 ml. neutralized gastric juice, incubated 22 hr. at 37° with the vitamin B_{12} and then heated 20 min. at 95° before adding to medium	19	26	40	48
0·1 ml. 0·067 M-phosphate buffer pH 7·4, incubated 22 hr. at 37° with the vitamin B_{12} and then heated 20 min. at 95° before adding to medium	71	73	74	76

Table 5. *Response of an untreated patient with pernicious anaemia to mixtures of vitamin* B_{12} *and gastric juice by mouth*

Period no.	Daily treatment	No. of days observed	Red blood cell count (millions/cu.mm.) Beginning	End	Haemoglobin (g./100 ml.) Beginning	End	Maximum reticulocytosis (%)	Day observed
1	5 μg. Vitamin B_{12} in 100 ml. water	10	1·29	1·08	6·4	5·4	2·2	5
2	5 μg. Vitamin B_{12} in 100 ml. neutralized gastric juice, incubated 22 hr. at 37° and then heated 20 min. at 95°	11	1·08	1·07	5·4	4·6	3·2	4
3	5 μg. Vitamin B_{12} in 100 ml. neutralized gastric juice, incubated 22 hr. at 37°	14	1·07	2·03	4·6	8·0	10	11

are not identical. These experiments do not preclude the possibility that the substance which binds vitamin B_{12} may be one of several factors involved in the absorption of vitamin B_{12} from the gastrointestinal tract, and which together constitute the intrinsic factor.

SUMMARY

1. The factor in normal human gastric juice which combines with vitamin B_{12} so that the latter is not available as a growth factor for microorganisms, is shown to be more stable to heat in neutral solution than in acid solution.

2. Heating mixtures of gastric juice and vitamin B_{12} under conditions which left a considerable part of the vitamin in a form unavailable to bacteria, destroyed the ability of the juice to potentiate the action of orally administered vitamin B_{12} in a patient with untreated pernicious anaemia.

3. These results suggest that the vitamin B_{12}-binding factor is not identical with the intrinsic factor of Castle.

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