ALKALI-STABLE GROWTH-PROMOTING FACTORS FOR CERTAIN LACTOBACILLI IN LIVER EXTRACTS

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The discovery of Vitamin B_{12} and the demonstration of its growth-promoting effect on various organisms has made possible a better standardisation of liver extracts than previously. However, it has become evident that a number of other substances in liver extracts may stimulate the growth of such organisms, a disturbing factor in the determination of vitamin B_{12}. It is known that thymidine and other desoxyribosides and also desoxyribonucleic acids promote the growth of *Lactobacillus lactis* Dorner and *Lactobacillus leichmannii*. Peeler and Norris[1] have shown that a number of liver extracts contain a factor stimulating the early stage of growth of *L. leichmannii*, and that prolonged incubation period eliminates the need of this factor. Robinson *et al.*[2] have found in crude liver extracts an alkali-stable factor capable of stimulating the growth of *L. leichmannii*. In paper chromatography this factor behaves like vitamin B_{12}, but differs from the factor described by Peeler and Norris. Cronheim and Dannenburg[3] and Mulli[4] have found in a number of liver extracts that alkali treatment does not completely destroy the vitamin B_{12} activity on *L. leichmannii*. Similar observations have been made in our laboratory where we have found a factor probably identical with the one described by Robinson *et al.* In addition, we have observed the presence of one or more additional alkali-stable microbiologically active factors in liver extracts. Such factors have been described by Östling[5] in a liver extract and by Nyberg[6] in an extract from the fish tapeworm, prepared in the same way as Östling's liver extract. As it is obvious that such factors may have a disturbing effect on the determination of vitamin B_{12} in liver extracts, a report on the microbiological investigations we have made into alkali-stable factors seems to be called for.

Material

Liver extracts investigated: Crude Liver Extract A, made by Medica Ltd. according to a method worked out by one of the present authors (G. Ö.) and Miss I. Petrell, and the commercial Liver Extracts B, C, D, E and F. The extracts were investigated before and after boiling in a water bath for 1 hour at *p*H 10, in amounts of 5×10^{-1}, 10^{-1}, 10^{-2} 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} ml. per test tube.

Micro-organisms employed: *Lactobacillus leichmannii* ATCC 4797, *Lactobacillus casei* ATCC 7469, *Leuconostoc citrovorum* ATCC 8081, a strain of *Streptococcus fæcalis R*, isolated from human urine, and a flagellate *Euglena gracilis* var. *bacillaris* ATCC 10616.

METHODS

Lactobacillus leichmannii: The tube assay and the bio-autographic method with *L. leichmannii* have been described by Nyberg.[6]

Lactobacillus casei and *Streptococcus fæcalis*: The cultures were maintained by monthly transfers into difco assay culture agar. The assay and inoculum medium was the difco folic acid assay medium. The standard employed was folvite (Lederle). The standard and liver extract dilutions were tested in triplicate. Incubation time 24 hours at 37° C. Turbidimetric reading of growth responses.

Leuconostoc citrovorum: The culture was maintained by monthly transfers into difco assay culture agar. The assay medium has been described by Sauberlich and Baumann.[7] The assay medium was also employed as inoculum medium. The standard used was leucovorin (Lederle). Triplicate series were tested in each assay experiment. Incubation time 24 hours at 37° C.

Euglena gracilis: The assay method with *Euglena gracilis* has been described by Nyberg.[6]

RESULTS

Table I shows the vitamin B_{12} activity for *L. leichmannii* after 72 hours of incubation of the liver extracts studied. It is evident from this table that liver extract F alone loses all B_{12} activity after alkali treatment. The reduction in the activity of the other liver extracts varies between 13 and 60 per cent.

TABLE I

THE MICROBIOLOGICAL VITAMIN B_{12} ACTIVITY FOR *L. leichmannii* IN LIVER EXTRACTS

	Total B_{12} activity µg./ml.	B_{12} activity after alkali treatment µg./ml.	"Pure" B_{12} activity µg./ml.	Reduction of B_{12} activity after alkali treatment per cent.
Liver extract A	5·2	1·7	3·5	33
„ „ B	2·2	0·7	1·5	32
„ „ C	1·0	0·4	0·6	60
„ „ D	0·5	0·06	0·44	13
„ „ E	20·0	9·0	11·0	45
„ „ F	20·0	0·0	20·0	100

Östling[5] has shown that a polyvalent liver extract (= crude liver extract A) in high concentrations contains factors that give heavier growth with *Lactobacillus lactis* Dorner than the maximal growth response to vitamin B_{12}. Similar experiments were carried out by us with liver extract A with *L. leichmannii*. Figure 1 shows the growth response curves for this bacterium. Curve 1 shows the growth response of *L. leichmannii* to liver extract A after 20 hours. In the assay conditions prevailing in our laboratory practically no growth is obtained with pure vitamin B_{12} after an incubation time of 20 hours, as can be seen from Curve 2. Curves 3, 4 and 5 represent the growth responses, respectively, on liver extract A, alkali-treated liver extract A and pure vitamin B_{12} (vibecon, Medica) after 72 hours. The experiments show that an addition of more than

10^{-2} ml. of extract A gives a growth response exceeding the maximal growth of vitamin B_{12}. This increase in growth is obtained after incubation of both 20 and 72 hours. It cannot be due to changes in the redox potential since the E_h in the test tubes is not affected by the liver extract added.

The growth curves of the other liver extracts examined have the same

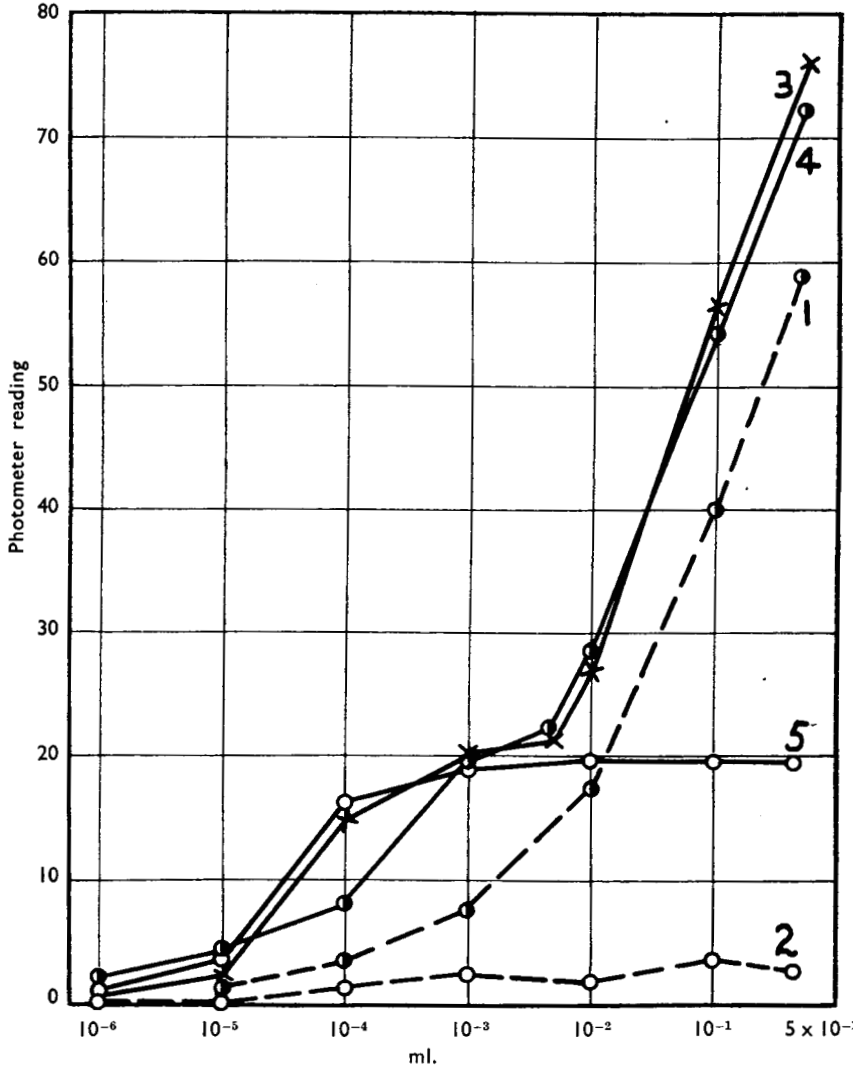

FIG. 1. Growth response curves for *L. leichmannii* to liver extract A.

Incubation time 20 hours.
1. Liver extract A.
2. Vitamin B_{12} (7·5 μg./ml.).

Incubation time 72 hours.
3. Liver extract A.
4. Alkali-treated liver extract A.
5. Vitamin B_{12} (7·5 μg./ml.).

shape as the growth curves for liver extract A, but with growth of varying intensity in the highest concentrations. Liver extract F induces a growth similar to that induced by vitamin B_{12}. The marked increase in growth in the highest concentration is missing (Table II).

TABLE II

GROWTH RESPONSE OF *L. leichmannii* TO UNTREATED AND ALKALI-TREATED LIVER EXTRACTS

Liver extract	Photometer reading											
	A		B		C		D		E		F	
ml./test tube	Untreated	Alkali-treated	Untreated	Alkali-treated	Untreated	Alkali-treated	Untreated	Alkali-treated	Untreated	Alkali-treated	Untreated	Alkali-treated
$5 \times \frac{1}{1}10^{-1}$	77	72	61		52		53		61		32	
10^{-1}	56	54	52	52	46	45	48	47	55	56	32	0
10^{-2}	27	38	35	40	33	31	36	30	41	39	32	0
10^{-3}	21	20	32	31	30	22	18	3	38	33	32	0
10^{-4}	18	8	12	12	3	2	0	0	31	21	32	0
10^{-5}	1	2	1	0	0	0	0	0	12	1	18	0
10^{-6}	0	0	0	0	0	0	0	0	1	0	1	0

From these investigations it seems probable that liver extracts A, B, C, D and E contain two groups of alkali-stable growth factors for *L. leichmannii*. Let us call these groups Factor I and Factor II. Factor I may be shown with quantities of extract less than 10^{-2} ml. per test tube, and to utilise this factor for its growth *L. leichmannii* requires 72 hours incubation. Factor II, which gives growth with larger quantities of extract, can promote a heavier growth of this bacterium than the maximal growth response to vitamin B_{12} after an incubation of only 20 hours.

Bioautographic experiments with liver extract A give only one growth zone for *L. leichmannii*. This zone has an R_F value roughly corresponding to that of pure vitamin B_{12}. A similar growth zone is obtained with the alkali-treated liver extract. If the chromatograms are stained according to Buchanan's method to demonstrate desoxyribosides,[8] red-violet spots appear, as shown in Figure 2. Spot 1 corresponds to the growth zone obtained in the bioautographic experiments.

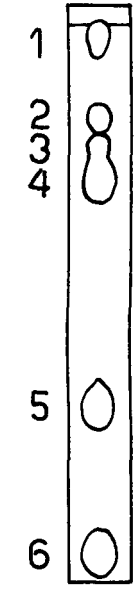

FIG. 2. Paper chromatogram of liver extract A developed with *n*-butanol on Whatman No. 1 filter paper for 24 hours. The chromatograms were stained according to Buchanan,[8] giving red-violet, well marked spots 1, 3, 4, 5 and 6. Spot 2 shows the position of the riboflavine zone. Spot 1 corresponds to the growth zone in the bioautographic experiments. Spot 4 is identical with desoxyriboside of guanine and spot 6 with thymidine. Spots 3 and 5 are unidentified.

Microbiological investigations with the desoxyribosides of cytosine, guanine, hypoxanthine and thymine, separately and all together, in quantities up to 200 μg., give for *L. leichmannii* after 72 hours incubation a growth which does not exceed, or only slightly exceeds, the maximal growth response to vitamin B_{12}. The growth response to these desoxyribosides is at its maximum already after 20 hours, in contrast to vitamin

B_{12}, which fails to induce any growth in this period. The Peeler and Norris factor (liver fraction L) yields a maximal growth response for *L. leichmannii* after 20 hours of incubation, considerably exceeding B_{12} maximal growth after 72 hours, but less than the growth response on liver extract A. No Factor I activity can be shown with liver fraction L.

With *Lactobacillus casei* and *Leuconostoc citrovorum*, growth curves in configuration with Curve 1 in Figure 1 are obtained after 24 hours incubation with liver extract A. The growth responses are approximately 3 times heavier than the maximal effect with folic acid and with leucovorin. 72 hours of culture do not change the appearance of the curves. Alkali-treated liver extract A gives similar results. Bioautographic experiments with *Lactobacillus casei* and *Leuconostoc citrovorum* only yield a growth zone corresponding to spot 1 in Figure 2.

The *Streptococcus fæcalis* strain employed by us does not grow with folic acid or Leucovorin but responds on liver extract A with an increase in growth similar to that of *L. casei* and *Leuconostoc citrovorum.*

With *Euglena gracilis* no growth exceeding the vitamin B_{12} maximal response is obtained with liver extract A. The extract, even after alkali-treatment, promotes the growth of *Euglena.*

Discussion

From the experiments carried out it seems as if crude liver extract A contained two groups of alkali-stable growth factors for *Lactobacillus leichmannii.* These groups have been called Factor I and Factor II. They also exist in varying quantities in a number of the other liver extracts investigated.

The alkali-stable Factor I, like vitamin B_{12}, induces growth only after an incubation of 72 hours, and is utilised by the bacterium in smaller quantities of extract than 10^{-2} ml. per test tube. Chromatographically it behaves like vitamin B_{12}, but stains like desoxyribosides and seems identical with the alkali-stable factor described by Robinson *et al.*[2]

The alkali-stable Factor II, in contrast to Factor I, gives growth only in quantities of extract exceeding 10^{-2} ml. per test tube, after an incubation of only 20 hours. The growth response is considerably higher than that to vitamin B_{12} or pure desoxyribosides. The strongly growth-promoting effect of Factor II, therefore, cannot be entirely attributed to desoxyribosides but does not exclude the possibility that its effect may be due to an interaction between desoxyribosides and other substances. The alkali-stable liver fraction L (Peeler and Norris[1]), after 20 hours' incubation, has a growth-promoting effect greatly reminiscent of the effect of liver extract A, but does not yield equally intense growth in high concentrations. It is therefore possible that the Factor II effect is partially due to the substances contained in liver fraction L. The characteristic Factor II effect on *L. leichmannii* also varies greatly in the other liver extracts investigated. This may be interpreted to mean that there are several factors with this effect, and that, depending on the extraction method, these are present in different proportions in the liver preparations in question.

Growth responses for *L. casei* and *Leuconostoc citrovorum* to alkali-treated

liver extract A were roughly 3 times the maximal growth response to folic acid and to leucovorin. A similar growth response for *Streptococcus fæcalis* was obtained with the alkali-treated liver extract. The alkali-stable factors contained in liver extract A, therefore, also have a growth-promoting effect on these lactobacilli. Demonstrable quantities of folic acid and the citrovorum factor could not be shown. Alkali-treated liver extract A was also able to promote the growth of *Euglena gracilis*. In contrast to the results with the lactobacilli studied, the same maximal growth was obtained with the liver extract and vitamin B_{12}. It is of interest that *Euglena*, which is considered to be specific to vitamin B_{12} (Hutner *et al.*[9]), also grows with alkali-treated liver extract (Nyberg[6]).

That the alkali-stable substances present in liver extract A are not only of theoretical interest or significance for the determination of vitamin B_{12} is shown by the clinical experiments by Östling, Nyberg and Gordin.[10] In 3 patients with pernicious anæmia intramuscular injections of the alkali-treated extract resulted in complete remission; the doses administered were so small that thymidine, folic acid and the citrovorum factor effect cannot enter into question.

Our experiences seem to indicate that vitamin B_{12} determination alone in liver extracts is unsatisfactory as a standardisation method. This is supported by the fact that the vitamin B_{12} content of commercial liver extracts varies greatly.[11,12,13,14] Liver extracts containing vitamin B_{12} in amounts small enough to preclude clinical effect have nevertheless proved of full therapeutic value in the same doses as preparations of a high vitamin B_{12} content. A suitable method of standardising liver preparations would be to compare them microbiologically with a standard liver preparation of good effect, both microbiological and clinical. However, as it is as yet uncertain whether the microbiological and clinical experiments agree, it is still necessary to test the preparations clinically, side by side with the microbiological standardisation.

Summary

1. Two groups of alkali-stable factors for *L. leichmannii* have been demonstrated in a crude liver extract and in some commercial liver preparations. One of the groups, Factor I, gives growth after 72 hours incubation which can be demonstrated in quantities of extract under 10^{-2} ml. per test tube. The other group, Factor II, gives growth already after 20 hours but only in extract quantities exceeding 10^{-2} ml. per test tube.

2. Factor I is probably identical with the alkali-stable growth-promoting factor described by Robinson *et al.*[2] Factor II probably contains several unknown alkali-stable growth factors for *L. leichmannii* and promotes a faster and heavier growth than vitamin B_{12}.

3. The alkali-stable factors in the crude liver extract investigated are also growth-promoting for *Lactobacillus casei*, *Leuconostoc citrovorum* and *Streptococcus fæcalis*.

4. The standardisation of liver preparations is discussed on the basis of these results.

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