New members of the vitamin B_{12} group isolated from sewage sludge

In sewage sludge[1,2,3,4], vitamin B_{12} is accompanied by factors *A* (vitamin B_{12m}[5]), *B* (vitamin B_{12p}[6]), *C* ($C_1 + C_2$, probably identical with vitamin B_{12s}[7,8]), ψ-vitamin B_{12}[9], two acidic factors[1], clinically active "B_{12}-factor III"[3] (factor *I*[10]) and "B_{12}-factors *V*"[3] which belong, according to BERNHAUER, to the group of the so-called etiocobalamins. In addition to these factors there have been isolated from other sources: vitamin B_{12f}[11] and factor *WR*[5] (both mixtures of known B_{12}-factors[12]), factors *D*, *E*, *F*, *G*, *H*[10], ψ-vitamin B_{12b}[9], ψ-vitamin B_{12a}[13] and ψ-vitamin B_{12f}[14].

The present paper reports the isolation, by means of the chromatographic and electrophoretic techniques, of three new factors from a concentrate prepared from fermented sludge of the Prague sewage works. They are provisionally designated as vitamin B_{12}-factors *J*, *K* and *L*. The existence of factor *M* as a new compound remains unsolved.

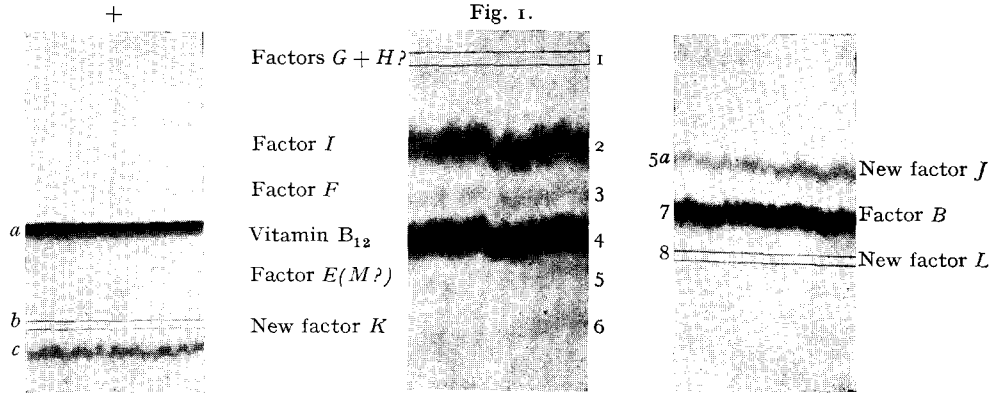
The preparation of vitamin B_{12} concentrates from sewage sludge

200 l of liquid sludge were autoclaved for 30 min at pH 3 and 120° C. The supernatant was adsorbed on 0.25 % (w/v) charcoal at pH 6 and the adsorbate was eluted for 10 min with two portions of 50 l 65 % hot ethanol. The eluate was concentrated to 500 ml by evaporation at reduced pressure and was precipitated with acetone which was added to obtain a concentration of 50 %. The precipitate was discarded and the concentration of acetone was increased to 85 %; the resulting brown sticky paste was extracted three times with 2,500 ml cold absolute methanol. 100 ml of the brownish-red concentrate containing vitamin B_{12} factors was chromatographed on a column of neutral alumina; pink fractions from 75 % and 50 % aqueous acetone were united, and the remaining yellow pigments were extracted with *n*-butanol. The active fraction was extracted with a mixture of phenol and benzene (1 : 2), precipitated with ether and reprecipitated from a methanolic solution. Microbiological activity of the precipitate tested on a *E. coli* mutant 200 was found to correspond to 85 μg vit. B_{12} per mg dry wt.

Fractionation of vitamin B_{12} factors

Paper electrophoresis (in 2 *N* acetic acid containing 0.01 % KCN, Whatman 3 MM, 400 V, 15 h) effects separation of an electroneutral fraction (zone *a*, Fig. 1) from factor *A* (zone *b*) and a cathodic fraction (zone *c*). Fractions *a* and *c* were cut out, eluted with water and subjected to paper chromatography (*sec.* butanol–acetic acid–water–saturated aqueous soln. of KCN in the ratio 100 : 1 : 50 : 0.25; 60–72 h).

Fig. 1.



Electrophoretic pattern of the factors

Chromatographic patterns (*a*) neutral fraction (red bands) (*c*) basic fraction (violet bands)

In simultaneous chromatography of zone *a* + *c*, band 5 overlies band 5*a*, and band 6 closely adheres to band 7.

The absence of growth-active contaminants in repeatedly purified fractions was tested by bioautography using *E. coli* mutant M 200. Factors *A*, *B*, *I* and vitamin B_{12} were identified by comparison with standard samples, and factor *F* was compared with data found in the literature[10]. Position of band 1 corresponds to that of the group of factors *A*, *G*, *H* and ψ-vitamin B_{12}; since factor *A* had already been separated by previous electrophoresis which did not show ψ-vitamin B_{12}, the possibility remains that band 1 represents factor *G* or *H*; identity with factors *C* appears excluded by the electroneutral character of this fraction and by the close similarity of the R_F of this band with that of factor *A* which is reported to be twice the R_F of factor *C*. Material

from band 5 corresponds by its relative position on chromatograms and by its electrophoretic mobility in acetic acid and sodium phosphate to the factor (or factors) E^{10}; it differs from the microbiologically active factor E by the absence of growth-promoting activity. This difference can be attributed either (a) to the contamination of factor E^{10} by traces of adjacent factors, or (b) to different growth requirements of our *E. coli* mutant (M 200) or (c) to the non-identity of both factors; in the latter case we suggest a provisory designation as factor M.

TABLE I

Band factor	Colour of aqueous soln.		R_F rel. to B_{12}		Paper electrophoresis		Bioautography
	$pH < 7$	pH 7	I*	II**	III	IV***	*E. coli* M 200
$5a = J$	orange	violet	1.25	1.08	basic	acidic	inactive
$6 = K$	red	red	1.49	1.21	neutr.	acidic	inactive
$8 = L$	orange	violet	1.76	1.34	basic	acidic	inactive
$5 = M$?	red	red	1.25	1.08	neutr.	acidic	inactive

I. Sec-BuOH, AcOH, H_2O, satd. soln. KCN (100:1:50:0.25).
II. Sec-BuOH, AcOH, H_2O (100:1:50), atmosphere satd. with HCN.
III. 2 N AcOH with 0.01 % KCN (according to HOLDSWORTH).
IV. 0.05 M sodium phosphate with 0.01 % KCN, pH 6.5.
* R_F of factor B = 1.59; ** R_F of factor B = 1.25.
*** Whereas mobility of factor J and L is the same, mobility of factor K and M is slightly less than that of J and L.

Absorption spectra show a shape typical for members of the vitamin B_{12} group (main maximum at 361 mμ for factor K and M, 353 mμ for factor J); detailed results will be published separately.

Whereas vitamin B_{12}-factor K is most probably a further member of the series of nucleotide-containing compounds, factors J and L (orange in acid medium, violet at pH 7) appear to belong to nucleotide-free etiocobalamins. A comparison between the electrophoretic behaviour in acetic acid and in phosphate suggest that factors J, K and L (and factor from band 5) contain a similar number of carboxylic groups. They differ substantially from BERNHAUER's "vitamin B_{12}-factors V" by their position on paper chromatograms.

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