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On Vitamins in Sewage Sludge

IV. Isolation of New Vitamin B_{12}-like Factors

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Of eleven vitamin B_{12}-like factors isolated from digested sewage sludge three behave as nucleotide-containing compounds and show considerable activity towards *Ochromonas malhamensis*. One of them (the most abundant) has been identified as cyanocobalamin, another may be identical with Factor III (Bernhauer) but the third has not, as yet, been identified with any known factor.

Eight of the isolated factors show behaviour typical of the factors lacking a nucleotide component. Three of them have considerable activity towards *E. coli* and one of these factors has been identified as factor B; the other two, or at least one of them, are probably identical with factor C. The microbiological activity of the remaining five factors is practically negligible.

Some properties of the isolated factors are given, and their behaviour during the isolation process discussed.

The abundance and variety of known vitamin B_{12}-like factors has increased considerably during the last two years. In particular, faecal sources — raw faeces of different animals and treated sewage sludges — have been found to contain many new factors suggesting that hitherto unknown forms of this vitamin may occur in nature.

At the same time there arose the problem of a rational method of nomenclature. At present several authors use individual systems of their own invention when describing factors isolated by them — a fact which complicates the naming of new factors when isolated by other workers. In previous work on vitamin B_{12}-activity in sewage sludge [1-3] the present author adapted the terminology of the Reading-[4-7] and Glaxo-[8,9] groups, and of Pfiffner *et al.*[10] who isolated a series of vitamin B_{12}-like factors from the faeces and rumen contents of certain ruminants. The factors found in sewage sludge (in addition to cyanocobalamin) were designated A, B, C, (C_1 and C_2) and pseudovitamin B_{12} after their identification by bioautography and paper electrophoresis with samples of the respective factors kindly provided by the above mentioned authors. Such identification was in most cases performed on crude sludge extracts or concentrates.

Armitage and co-workers [11] have described two series of vitamin B_{12}-like factors obtained by the hydrolytic cleavage of cyanocobalamin and have shown that those factors lacking a nucleotide component form stable *violet* * dicyanocomplexes at pH 6.5, while those containing a nucleotide component factors yield *red* monocyanocomplexes at this pH. Bernhauer and co-workers [12,13] have used this property and have characterized factors isolated from sewage sludge as "complete" (containing nucleotide) or "incomplete" (lacking nucleotide). Among several vitamin B_{12}-like factors isolated by them from sewage sludge was the previously unknown Factor III ("complete") which was later shown by two independent groups [14,15] to differ from cyanocobalamin in containing 5(6)-hydroxybenzimidazole instead of 5,6-dimethyl-benzimidazole. Bernhauer and co-workers [12] have also isolated from sewage sludge a substance, originally called "Factor V", which on electrophoresis was resolved into five different factors ** corresponding to the five nucleotide-lacking carboxylic acids described by Armitage *et al.*[11] (*cf.* Bernhauer [12]).

From portions of digested sewage sludge totalling 100 l (containing 10 % dry solids) 11 vitamin B_{12}-like factors of which three are red and eight violet in the presence of cyanide at pH 6.5, have now been isolated together with an orange substance of similar R_F-value to the slow moving vitamin B_{12}-like factors. Thus repeated chromatographic and electrophoretic separations have shown "factor A + pseudovitamin B_{12}"[1-3] to be a mixture of six factors with R_F-values close to those of factor A and pseudovitamin B_{12} but differing in their microbiological activities and spectrophotometric characteristics. Since most of these substances have not, as yet, been identified with known factors they may be conveniently described as: factor B, cyanocobalamin, factors V and W, factors X1, X2, X3 and X4, factors Z1, Z2, Z3, and factor Y which is not, however, a vitamin B_{12} factor. The investigation of the nature of these factors is continued with a view to their identification with similar substances isolated from other sources. Factor Z3 appears to be identical with factor C [6,7]. In the earlier stages of purification by chromatography, it behaved as factor C_2[1,2] (or B_{12s} [18,19]), while after numerous chromatographic and electrophoretic separations its behaviour changed to that of factor C_1[1,2].

EXPERIMENTAL

Isolation of factors

2—3 l portions of digested sludge (10 % total solids) were diluted (4:1) with water boiled at pH 6 for 15 min. in the presence of KCN (100 mg/l) and centrifuged. The combined supernatants (1 % total solids) were subjected to absorption on activated charcoal (Norit. 2 % w/v) at pH 6 and 3° C for 2 hours in 10 l portions. The charcoal suspension (together with 2 % w/v filteraid (Hyflo-Super Cel)) was removed by filtration through

* The term "violet" as used in this paper corresponds to the term "purple" used by the above mentioned authors.

** The electrophoretic separation of these factors was performed by the author in Professor Bernhauers laboratory, using the two zone electrophoresis apparatus described by Flodin [16] and Carlsson [17].

filteraid layers of 2—3 cm thickness in large Büchner funnels and then washed with a 5 % (w/v) solution of phenol in water. Considerable amounts of brown impurities, but negligible amounts of vitamin B_{12}-active material, were removed by the washing. Every filtercake was washed with 2—3 l of the phenolic solution. The washed filtercakes were boiled for 5 minutes under reflux with a volume of 70 % ethyl alcohol equal to about four times that of the filtercake and quickly filtered. This operation was repeated four times. The filtrates were cooled rapidly and the alcohol distilled *in vacuo*. The remaining liquid was concentrated by further evaporation *in vacuo* and the precipitated material was removed by filtration at 80° C with some filteraid. The red-brown liquid was then extracted with an equal volume of *iso*propanol containing 30 % $(NH_4)_2SO_4$ and the emulsions and precipitates formed were reextracted until the solution was only very slightly coloured and showed negligible vitamin B_{12} activity. The combined purple extracts were concentrated *in vacuo* in order to remove *iso*propanol and about half the water and the concentrate was subjected to a series of extractions with *p*-chlorophenol solutions the vitamin B_{12} activity being finally precipitated on kieselguhr as described by Bernhauer [12]. Each extraction was repeated until the red material passed completely into the extracting phase and until microbiological tests (mainly cup plate assay with *E. coli* 113—3) showed the almost total absence of vitamin B_{12} activity in the extracted phase. In some cases as many as 10—15 extractions were necessary.

For the final stages of purification and separation of the different factors the vitamin B_{12} preparation was subjected to partition chromatography on cellulose powder columns and on Whatman 3MM filterpaper using, as solvents, water saturated *sec.* and *n*-butanols containing 0.01 % KCN and saturated with $KClO_4$[20]. The height of the columns varied between 8—12 cm; the filter paper sheets were 50 cm long and 20 cm broad.

Although in earlier experiments the vitamin B_{12} material was dissolved in few ml of the eluting solvent and applied directly to the top of the column it was later found more convenient to apply the kieselguhr concentrate itself as described by Friedrich and Bernhauer [20].

The first stage of chromatography was performed with *sec.* butanol on cellulose columns when the vitamin B_{12} material resolved into four bands. The fastest, a violet band (I), was followed by a red band (II) which in turn was followed by a very slow violet band (III) and another, almost immobile, violet band (IV). The degree of separation was poor and considerable amounts of yellow and brown impurities could be seen in the column moving both faster than (I) and as slow as or slower than (IV). The kieselguhr layer on the top of the column retained a large amount of brown immobile material. Although (I) and (II) could easily be eluted and collected in two distinctly separated fractions the bands (III) and (IV) could not be eluted even after an extended period of time. The cellulose column was then extruded and the two violet bands cut off, extracted with water, evaporated to small volumes in *vacuo* and the vitamin B_{12} activity precipitated on kieselguhr as before. The remainder of the column including the kieselguhr layer was cut in sections and analyzed for vitamin B_{12} activity. Only that extract corresponding to the top of the column, including the kieselguhr layer, contained noteworthy amounts of vitamin B_{12}-activity. This extract was evaporated to dryness and rechromatographed several times on thick Whatman paper using, respectively, *sec.* and *n*-butanol but the separation of the vitamin B_{12} activity from the yellow and brown impurities was not successful (*cf.* p. 926). The four kieselguhr preparations were then rechromatographed on cellulose columns with *n*-butanol. All four preparations contained besides the main band smaller amounts of the three other bands and of brown or yellow impurities which could be separated from the vitamin B_{12}-active substances; (III) divided into two poorly separated violet bands (IIIa) and (IIIb). Again (I) and (II) could be easily eluted, while (IIIa), (IIIb) and (IV) were cut from the columns and treated as before. After repeated precipitation on kieselguhr the five fractions were rechromatographed on cellulose columns using *n*-butanol for (I), (II) and (IIIa) and *sec.* butanol for (IIIb) and (IV). After this third fractionation (I) appeared to have divided into two bands, one of which was a weak violet, faster-moving band (Ia) poorly separated from the main violet band (I). Fraction Ia was collected but could not be thoroughly investigated due to an accident. Its R_F value (relative to that of cyanocobalamin) was about 1.5 while the corresponding R_F-value of (I) was 1.35 and on mixed bioautograms the two fractions appeared as distinct zones. In the following, fraction Ia will be referred to as factor Bl. When fraction (I) was again chromatographed on thick Whatman paper with *n*-butanol it appeared as a

single violet band free of yellow impurities. Ionophoretic test confirmed the homogeneity of the fraction but an attempt to crystallize it from aqueous acetone gave only a precipitate. It could be identified by bioautography and paper electrophoresis as factor B, its other properties can be seen in Tables 1 and 2 referred to as factor B.

Fraction II appeared as a distint single red band on the column but to ensure its purity it was rechromatographed on thick Whatman paper with *n*-butanol. It was then crystallized from aqueous acetone and identified as cyanocobalamin.

Fraction (IIIa) appeared as a broad violet band which could be removed from the column only by protracted elution. Spectrophotometric measurements of the eluate fractions indicated the presence of three different factors in this band but repeated chromatography on thick Whatman paper did not lead to a satisfactory separation. On paper electrophoresis at pH 3.0 (0.5 N HAc containing 0.01 % KCN) the mixture separated into three zones: one basic (X2), one neutral (W) and one strongly acidic (X1). The degree of separation of zones X2 and W was not quite satisfactory but could be improved by electrophoresis at pH 6.5 (phosphate, $\mu = 0.1$). The three factors thus obtained were rechromatographed on thick Whatman paper with *n*-butanol. Factor W appeared as a single distinct red band, while factors X1 and X2 moved as violet zones. Considerable amounts of factor B separated from each of these two latter factors.

Fraction IIIb appeared on the column as a broad violet band. Spectrophotometric measurements of the eluate fractions gave no indication of its heterogeneity but to ensure its purity it was rechromatographed on thick Whatman paper with *n*-butanol. After a considerable time it appeared to separate into three very close zones but a satisfactory separation of the factors could not be obtained. Paper electrophoresis of the mixture at pH 3.0 led to separation into one neutral (V) and two basic zones (X3 and X4) and the three factors were rechromatographed on thick Whatman paper with *n*-butanol. Factor V gave a single, rather wide pink zone while factors X3 and X4 appeared as distinct violet bands. As in the case of the factors isolated from (IIIa) considerable amounts of factor B could be separated from factors X3 and X4 during this final purification.

Fraction (IV) appeared on the column as a very broad violet band and the presence of several factors was confirmed by spectrophotometric measurements of the eluate fractions. The degree of separation was very poor and the band appeared to contain considerable amounts of yellow substances. Paper-electrophoresis at pH 6.5 followed three times by chromatography on thick Whatman paper with *n*-butanol resulted in the isolation of three violet factors (Z1, Z2 and Z3) and one orange factor (Y). Again, considerable amounts of factor B could be separated at the same time.

All chromatographically pure factors were tested for their homogeneity by paper electrophoresis and further purified in that way if necessary. Attempts to crystallize the isolated factors were unsuccessful except in the case of cyanocobalamin. The amounts isolated were in most cases too small to be weighed and they are listed in Table 2 on the basis of the optical density of their solutions. The factors were desalted and kept in 70 % EtOH at $-20°$ C.

Characterization of factors

Absorption spectra. For determination of spectra appropriate amounts of the alcoholic solutions were evaporated to dryness and dissolved in 5 ml water. 1 drop of 0.1 % KCN solution was added and the pH adjusted to 6.5 with HAc or NH_4OH; in most cases these additions were negligible. The pH of the solution was also measured after each determination and in no case did the pH change exceed 0.2 pH units. The measurements were performed on a Beckman Spectrophotometer Model DU. Although the spectrophotometric data on the non-crystalline factors listed in Table 2 must be taken with reserve, it is hoped that they can help to elucidate the relationships between these factors and those previously identified.

Partition coefficients were determined in the solvent pair 20 % *p*-chlorophenol in trichloroethylene-water. The amount of vitamin B_{12}-like factor in each phase was measured spectrophotometrically.

Paper electrophoresis was performed with two buffer systems; at pH 3 with HAc and at pH 6.5 with $K_2HPO_4-KH_2PO_4$, both containing 0.01 % KCN and having an ionic strenght of 0.1. Voltage gradients of 10 V/cm at $+4°$ C during 18 hours were standard experimental conditions using LKB Paper Electrophoresis Apparatus Type 3276 B.

Both the bioautographic technique using *E. coli* 113—3 and the technique with visible spots were used in detection.

Microbiological tests were performed with *E. coli* 113—3 in both cup plate and tube assays and with *Ochromonas malhamensis*. Using Ford's technique most tests were repeated on 3—5 different days and the values listed in Table 2 are averages of all determinations. The microbiological potency of the factors is calculated on the basis of the optical density of their solutions at $\lambda = 361$ mμ for the red and at $\lambda = 367$ mμ for the violet factors.

Bioautography was used to determine the R_F-values of the factors and in certain cases visible spots were chromatographed for comparison. Chromatograms (descending technique) were run for 48 h at 21° C in water-saturated *sec.* butanol containing 3 % HAc and 0.01 KCN, and developed on *E. coli* 113—3 plates; authentic specimens were run as standards. The R_F-values listed in Table 1 are calculated in relation to that of cyanocobalamin and will be referred to as R_c-values. Mixtures of known and unknown factors as well as of groups of unknown factors were chromatographed but usually little information was obtained regarding the relationships between different factors (Table 1). The R_c-values appeared to be influenced by the impurities present in the samples and usually increased with the degree of purity.

RESULTS AND DISCUSSION

The factors isolated are listed in Table 1 and are grouped vertically according to their ability to form violet (di)cyano complexes at neutral pH, and horizontally according to their R_c-values. The horizontal arrangement yields four main groups (left column in Table 1), with R_c-values 1.6—1.3 (I), 1.0 (II), 0.7—0.6 (III) and 0.5—0.4 (IV), respectively. The R_c-values of the factors within each group have been determined at least six times in each case giving variations of up to ±0.05. Although the values listed in Table 1 agree

Table 1. Vitamin B_{12} factors isolated from sewage sludge. (Behaviour at pH 6.5 in the presence of CN^-.)

Group of factors	R_c * interval	Red factors	R_c *	Violet factors	R_c *	Other factors not yet defined	R_c *	Known (unisolated) factors	R_c *
I	1.6—1.3	—	—	factor B	1.35	factor B1 ** red or violet?	ca 1.5	factor B	1.4
II	1.0	cyanocobalamin	1.00	—	—	—	—	cyanocobalamin	1.0
III a	(0.70—0.65)	W	0.69	X1	0.67	—	—	"factor A +pseudo-vitamin B_{12}"	0.7
				X2	0.66	—	—		
III	0.7—0.6								
				X3	0.62	—	—		
III b	(0.65—0.60)	V	0.61	X4	0.60	—	—		
IV	0.5—0.4	—	—	Z1	0.46	Y(orange)	0.46	factor $C_{(1)}$	0.4
				Z2	0.43				
				Z3	0.40			factor $C_{(2)}$	0.0

$$* \; R_c = \frac{R_F \text{ of factor}}{R_F \text{ of cyanocobalamin}},$$ solvent system: water-saturated *sec.* butanol + 3 % HAc + 0.01 % KCN, 48 h, descending technique.

** This factor could be found in many sludge preparations. No further investigation has, however, been made.

Table 2. Some properties of Vita-

Factor *	Physical properties			
	Absorption maxima	Points of inflexion	Electrophoretic behaviour	
	H_2O, pH 6.5, in presence of CN^-		pH 3.0	pH 6.5
cyanocobalamin	278 (306 323) 361 (523) 550. —	—	neutral	slightly acidic
W	278 — — 361 — 550 —	288 410 526	neutral	slightly basic ($>$factor A)
V	275 — — 361 — 550 —	—	neutral	slightly basic ($<$W)
B	278 (306) — 368 — 540 580	—	basic	neutral
X1	? — — 366 — 540 580	350 440	strongly acidic	slightly acidic
X2	277 — — 366 — 540 —	300	basic	slightly acidic ($<$X1)
X3	— — — 367 — 540 580	270 300	basic($<$B)	slightly acidic ($>$X1)
X4	277 (305) — 367 — 540 580	350 415 510	basic $\approx$B)	acidic($\approx$X3)
Z1	— — — 368 — 540 580	512	basic($\approx$X4)	acidic($\approx$X4)
Z2	276 (304) — 368 — 540 580	350 415	basic($<$Z1)	acidic($>$Z1)
Z3	280 — — 367 — 540 580	512	neutral	acidic($<$Z1)
Y	264 — — — — — —	530		

* for the corresponding R_c values see Table 1.

** $C_W/C = \frac{\text{concentration of the factor in water phase}}{\text{concentration of the factor in solvent phase}}$
solvent: 20 % *p*-chlorophenol in trichloroethylene.

fairly well with the order in which the factors moved on chromatograms during the isolation procedure (*cf.* p. 920) it is evident that R_c-values are of limited use in the characterization of these factors unless they are well differentiated. It is possible that group III can be divided into two subgroups with R_c-values 0.70—0.65 (IIIb), respectively.

Some properties of these factors are listed in Table 2. The four main groups (left column in Table 1) are in full agreement with earlier findings [1] (*cf.* right column in Table 1) except in the case of "factor C_2".

Factors "Z". The presence in sewage sludge of a virtually immobile B_{12}-like factor ($R_c = 0.0$) has been noted in this laboratory before [1-3]. In the present isolation experiment such a factor was detected by bioautography in the original sludge and after repeated purification by chromatography it appeared as a very distinct, immobile, violet band at the top of the column (*cf.* p. 919). Its behaviour was similar to that of "factor C_2" or B_{12s} [18,19] and was unchanged after being twice chromatographed on

min B_{12} factors from sewage sludge.

Partition coefficients C_w/C_s **		Microbiological activities *** $\frac{\mu g}{D}$ *Escherichia coli* 113—3		*Ochromonas malhamensis*	Identified as	Amount isolated from 100 l sludge, ml of a soln. of optic dens.	
CN⁻	no CN⁻	plate assay	tube assay	tube assay			
		49	49	49	cyanocobalamin	40 mg	
1.5	1.0	300	50	25		10	1.0
		50	45	20	Factor III?	10	0.9
		200	25	1	factor B ††	10 mg	
		10—50†	1—3†	2		3	0.4
1.5	1.0	10—50†	5—50†	4		3	0.8
2.5	0.5	10—80†	5—50†	1		3	0.8
3.0	0.5	15	5	0		25	7.0
4.5	2.0	5—15†	0.2—2†	0		25	0.9
1.5	1.0	100—200†	15—45†	0	factor C?	25	0.5
		100—500†	10—300†	0			
		traces	traces				

*** Calculated as cyanocobalamin on the basis of optical densities of the solutions
$\frac{\mu g}{D}$ {at 361 mμ for red factors, at 367 mμ for violet factors} {Beckman spectrophotometer Modell DU}

† non linear response

†† by chromatography, electrophoresis and spectra

cellulose powder columns although there was then some indication that this factor was, in fact, not homogeneous. After further chromatography (on thick Whatman paper) and electrophoresis this factor could be separated into three violet factors (Z1, Z2, Z3) and one orange factor (Y), all of which had R_c values within the range 0.5—0.4. This R_c range corresponds to "factor C_1" (*cf.* right column in Table 1). The explanation of this fact is not yet known. It appears that the immobile violet factor has been transformed to other violet forms during the extended time of isolation.

Similarly it is difficult to decide whether the orange factor (Y) was initially present in the sludge or merely formed during the transformation of the original immobile violet material into the violet factors of R_c 0.5—0.4. The slight microbiological activity of this factor may be due to traces of impurities; its absorption spectrum indicates that it is not a vitamin B_{12}-like factor. The "intermediate" character of "factors C_2" and "C_1" which are present only in

the initial stages of bacterial fermentation, "factor C_2" being the first one to disappear, has already been noted [2]. Assuming that "Fraction IV" (p. 920), the source of the three factors "Z", corresponds to the "factor C_2" of bioautograms then the present investigation offers further confirmation of the labile nature of this factor. Similarly the labile nature of "factor C_1" may be confirmed by the separation of considerable amounts of factor B at each of the numerous chromatographings of "Fraction IV" (*cf.* p. 920).

It is difficult to decide which, if any, of the factors "Z" most closely resembles factor C but on the basis of microbiological activity it cannot be Z1 whose activity for *E. coli* is insignificant and probably due only to traces of the other two factors "Z" still present.

Earlier information on the electrophoretic behaviour of factor C (C_1 and C_2) or B_{12s} concerns mostly crude extracts or concentrates. Holdsworth [21] reports the mobility of factor C in 0.5 N acetic acid containing KCN; as -1.4×10^{-5}. According to Ericson [19] vitamin B_{12s} should move to the anode under these conditions as should factors C_1 and C_2. Brown and coworkers have determined the acidic character of factor C by electrophoresis in neutral and alkaline buffers [9].

As can be seen in Table 2 all three factors "Z" show acidic electrophoretic behaviour at pH 6.5 but at pH 3.0 factor Z3 is neutral while factors Z1 and Z2 behave as bases. This corresponds to the findings of Brown and coworkers [9] on factor C but is contrary to the earlier information [19] on the electrophoretic behaviour of this factor at pH 3.0.

However, it should be remembered that some determinations were carried out on crude extracts whose content of salts and other impurities may have influenced the electrophoretic behaviour of the B_{12}-like factors. Again if "factor C" has "changed" during the isolation procedure, this would explain its changed electrophoretic behaviour. (*cf.* p. 923).

It is, therefore, probably factor Z3 which most closely resembles the earlier description of factor C (C_1), both in its microbiological activity and in its chromatographic and electrophoretic behaviour.

Factors X. The four violet factors of R_c values 0.7—0.6 occurred together with two red factors V and W on the bioautograms (see Table 2). On mixed chromatograms (bioautograms) with standard substances — factor A, ψ B_{12}, and factor III — they gave in most cases single spots. In a few cases the spots were oval suggesting the presence of at least two components. It is evident, therefore, that this method of identifying B_{12} factors must be used only with very great caution. The "incomplete" character of these factors is shown by their ability to form violet (di)cyano complexes at neutral pH. Not only is their R_c range distinct from that of "factors C" but their microbiological activity for *E. coli* is also extremely low — this slight activity, together with the low activity for *O. malhamensis*, may be explained by the presence of traces of factors V and W (*cf.* Table 2). Although the electrophoretic behaviour of factors X2, X3 and X4 resembles that of factors Z, insofar as they are acidic at pH 6.5 and basic at pH 3.0; factor XI differs markedly from the rest of the group in being strongly acidic at both pH's although much less so at pH 6.5. (*cf.* Table 2).

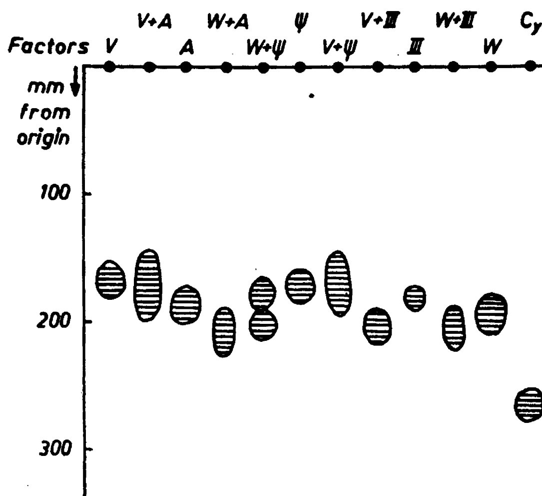

Fig. 1. Mixed bioautograms of factors W and V with known factors. Solvent system: water satur. sec. BuOH containing 3 % HAc and 0.01 % KCN; 21° C, 48 h. run. Developed with E. coli 113—3.

ψ = pseudovitamin B_{12}
C_Y = cyanocobalamin

Factors V and W. These two red factors with R_c-values 0.7—0.6 have absorption spectra closely resembling that of cyanocobalamin. They are neutral at pH 3.0 and slightly basic at pH 6.5 (*cf.* Table 2). In order to establish their identity with known factors, bioautography, electrophoresis and, in the case of Factor III, spectrophotometric measurements were carried out several times on mixed solutions in order to compare them with known standards.

Since a mixture of factor W and ψ B_{12} is resolved by paper chromatography (Fig. 1) it is obvious that the two substances are not identical. Although other mixed solutions gave single spots these have a markedly oval shape, except in the case of V+III, while the single spots of pure solutions are more circular. It seems likely therefore that factor W is not identical with any of the three factors A, ψ B_{12}, and III available for comparison, while factor V is possibly identical with Factor III.

It was not possible to identify the factors V and W with the above mentioned known standards by electrophoresis or by spectrophotometric measurements. The spectrophotometric data for ψ B_{12} were unavailable. The band at 295 mμ characteristic for the monocyano complex of Factor III could not be detected in either factor W or V but the monocyano complexes of these factors showed, instead, bands at 275 mμ and 278 mμ, respectively. The absence of a band in this region may not be incompatible with the postulate of a relationship to Factor III, since the measurements were performed on a noncrystalline product. Moreover factor V resembles Factor III in its microbiological activity towards *E. coli* by both the cup plate and tube methods. The activity towards *O. malhamensis* is about 40 % of that of cyanocobalamin.

Factor W shows a strikingly high microbiological activity towards *E. coli* in plate assay. Its activity towards *E. coli* in tube assay is of the same order as that of cyanocobalamin. Although Factor H, isolated by Brown and co-workers [9] also has a very high activity towards *E. coli* in plate assay its activity in tube assay is lower than that of factor W and the activity towards *O. malhamensis* is very small. Further differences between these two factors are found in their absorption spectra [9] (*cf.* Table 2).

GENERAL CONSIDERATIONS

Violet factors. Of the 8 violet factors only three (B, Z2 and Z3) have a pronounced microbiological activity (Table 2) while the remaining five factors (X1, X2, X3, X4 and Z1) can be considered as inactive, (*cf.* p. 924). They form violet (di)cyanocomplexes at pH 6.5 which show the characteristic maxima at 367—368, 540 and 580 mμ. They exhibit acidic properties upon electrophoresis at the same pH. When acidified to pH 4.0 the colour of the solutions changes rapidly to orange, presumably due to the formation of hydroxo (or aquo) compounds.

The microbiologically active factors Z2 and Z3 (as well as factor B) were identical with the inactive factors in the formation of (di)cyano and hydroxy-forms, respectively, and in their electrophoretic behaviour (*cf.* Table 2 and p. 924). In cup-plate assay with *E. coli* these factors showed characteristically *diffuse* growth zones which may account for their high microbiological potencies when calculated by this method. The phenomenon was much more pronounced in the case of factors Z2 and Z3 than in the case of factor B.

Behaviour of the different factors during some isolation steps. It is possible that the inactive factors, like the violet factors (Z2 and Z3) of high activity, tended to remain in precipitates, emulsions *etc.* during extraction. For this reason the solutions were exhaustively extracted beyond the point judged necessary by the behaviour of the red-brown substances (*cf.* p. 919) but constant microbiological testing is essential if the yellow hydroxo (or aquo) compounds formed by the active violet factors at pH 4.0—5.0 are not to be mistaken for "impurities" (most of which are also yellowish) and lost during the isolation procedure. Although these factors can be easily detected in solution by the violet colour formed immediately upon addition of CN^- this may not be the case in emulsions and precipitates. However, such extended extractions may give impure preparations necessitating repeated chromatography in later purification. It is not clear, whether the microbiological activity contained in the "kieselguhr layer" (p. 919) was due to still another factor or to the "tail" of the immobile violet factor which later resolved into factors Z1, Z2 and Z3 (*cf.* p. 919). Furthermore, the factors apparently differ in their ability to form the phenolic complexes described by Bernhauer since they behaved in different ways during extractions with phenolic solutions; some preliminary values for partition coefficients in one solvent system are given (Table 2). Unfortunately in many cases the amount of material available was insufficient to allow a thorough investigation of the problem.

It has been pointed out above (*cf.* p. 920) that considerable amounts of factor B could be separated from the violet factors upon repeated chromato-

graphy. This phenomenon suggests the possibility that the violet factors (or some of them) correspond merely to some different cyanide complexes of factor B. However, the relatively stable character of these factors during the process of determination of their spectrophotometric and electrophoretic properties rather points against this possibility. The problem was not further investigated.

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REFERENCES

1. Sjöström, A. G. M., Neujahr, H. Y. and Lundin, H. *Acta Chem. Scand.* **7** (1953) 1036.
2. Neujahr, H. Y. *Acta Chem. Scand.* **9** (1955) 622.
3. Neujahr, H. Y. *Acta Chem. Scand.* **9** (1955) 803.
4. Ford, J. E. *Brit. J. Nutrition* **6** (1952) 324.
5. Ford, J. E. *Nature* **171** (1953) 148, 149.
6. Ford, J. E., Kon, S. K. and Porter, J. W. G. *Chemistry & Industry* **1952** 495.
7. Ford, J. E. and Porter, J. W. G. *Brit. J. Nutrition* **7** (1953) 326.
8. Gant, D. E., Smith, E. L. and Parker, L. F. J. *Biochem. J. London* **56** (1954) xxxiv.
9. Brown, I. C. Cain, Gant, D. E., Parker, L. F. J. and Smith, E. L. *Biochem. J. London* **59** (1955) 82.
10. Pfiffner, J. J., Dion, H. W. and Calkins, D. G. *Federation Proc.* **11** (1952) 269.
11. Armitage, J. B., Cannon, J. R., Johnson, A. W., Parker, L. F. J., Smith, E. L., Stafford, W. H. and Todd, A. R. *J. Chem. Soc.* **1953** 3849.
12. Bernhauer, K. und Friedrich, W. *Angew. Chem.* **66** (1954) 776.
13. Friedrich, W. und Bernhauer, K. *Angew. Chem.* **65** (1953) 627.
14. Friedrich, W. und Bernhauer, K. *Angew. Chem.* **67** (1955) 619.
15. Robinson, F. M., Miller, J. M., Mc Pherson, J. F. and Folkers, K. *J. Am. Chem. Soc.* **77** (1955) 5192.
16. Flodin, P. and Porath, J. *Biochim. et Biophys. Acta* **13** (1954) 175.
17. Carlson, L. A. *Acta Chem. Scand.* **8** (1954) 510.
18. Ericson, L.-E. and Lewis, L. *Arkiv Kemi* **6** (1953) 427.
19. Ericson, L.-E. *Acta Chem. Scand.* **7** (1953) 703.
20. Friedrich, W. und Bernhauer, K. *Z. Naturforsch.* 106 (1955) 755.
21. Holdsworth, E. S. *Nature* **171** (1953) 148.

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