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PROTEIN COMPOUNDS OF VITAMIN B_{12} AND ITS ANALOGS

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S u m m a r y

In order to get some more informations on the nature of the protein bound corrinoids, experiments were conducted, in which the properties of different preparations, hitherto isolated, from Propionibacterium shermanii cultures, were compared.

The analytical studis included mainly the amino acid composition, electrophoresis and measurements of the absorption spectra of the various compounds.

The isolated protein bound corrineids, when subjected to different purification procedures, showed marked varations in the amino acid condens. No cystine or cysteine could be evidenced chromatographically in the crude preparations. Electrophoretic studies, resulted in the seperation into fractions, may indicate the heterogenity of the investigated complexes. Thus, the isolated fractions were hydrolysed and subjected to amino acid analysis.

The absorption spectra of the protein baund corrineids differed markally from that of vitamin B_{12}.

Attempts were also made to chracterise the protein compounds of light sensitive corrinoids.

EXPERIMENTAL

Material and methods

Propionibacterium shermanii-1 was used as source of the corrinoid protein compounds. The isolation and purification procedures of these complexes were described in the previous annual report. Attempts were also made to isolate the protein compounds of the light sensitive corrinoids. Studies in this direction were performed according to Mulli and Schmidt, except that all the operations were carried out in dark or in the presence of red light.

Isolation of the light sensitive compounds: To 800 g of fresh cell paste 3 l of 75% ethyl alcohol were added. A fine suspension was obtained by mixing the material in a Warring blender for 10 min. The suspension was centrifuged, the supernatant removed, whereas the residual material was subjected to three similar treatments. 10 l of ethanol were used for all the extractions. The clear supernatants were combined and evaporated under reduced pressure at 30°C. To the residual aqueous solution, distilled water was added, to obtain a final volume of 4 litres. To this solution a portion of 1100g of solid ammonium sulphate was added. When a clear solution was obtained, a treefold extraction was performed, by means of 300 ml portions of n-butanol.

Subsequently, the combined butanol extracts were passed through a column of previously activated Al_2O_3. The column was washed first with 1000 ml of 90% acetone, and then with 1000 ml of 80% acetone.

This was followed by passing through a suitable volume of distilled water, resulting in the elution of a brick-coloured material.

The residual acetone was removed from the aqueous eluate by evaporation under reduced pressure. The clear solution, thus obtained, was subjected to several subsequent extractions, using small portions of phenol. The combined phenol extracts were carefully washed with distilled water, followed by treatment with a required amount of ethyl ether, to get a clear solution.

The coloured compounds were recvered quantitatively from this solution by water extraction. Traces of phenol were removed from the aqueous extract by washing it with appreciable volume of ethyl ether, the later being removed by evaporation.

The aqueous solution of the corrinoid compounds, thus obtained, was subjected to several phenol extractions. The combined phenol extracts were washed with distilled water and subsequently treated with nine volumes of acetone. This solution was allowed to stand for several hours. The precipitate of an amorphous brick-coloured material was seperated by centrifugation, washed with ethanol and ether, and finally dried in vacuum over H_2SO_4.

All the hitherto isolated compounds were subjected to further studies. Other methods applied in this research are given in appropriate sections of this text.

RESULTS AND DISCUSSION :

The following abbreviations are being used through out this report: M-1 and M-2/or B_{12}-M/for preparations, isolated by the method of Mulli and Schmidt,
B-1 and B-2 /or B_{12}-B/-for the compounds obtained by the modified Redborn procedure,
B_{12}-M-6 - for the light - sensitive corrinoid preparation.

Studies on the amino acid composition of B_{12}-M and B_{12}-B

The quantitative amino acid compositions of the B_{12}-M and B_{12}-B preparations, as well as of the major fractions of these compounds, obtained after electrophoretic seperation, are presented in Table 1.
Each of the isolated compound was hydrolyzed by means of 6N HCl in a way, described in the previous annual report. The amino acid analysis of the hydrollyzates has been performed using an automaticanalyzer /Phoenix-Precision Instr. Co., Model K-5000-A/.
The amino acid composition of M-1 and M-2 was found to be identical. No significant variations could be also stated between B-1 and B-2. Data presented in Table 1 are therefore mean values from the figures obtained for both preparations, respectively. The results are

expressed as percents of the amount of compound analyzed. The data illustrated in Table 1, however, indicate marked differences in the amounts of individual amino acid residues of the preparations, as far as the mode of purification is being concerned. Both compounds /B_{12}-M and B_{12}-B/ contained significant amounts of glutamic acid and proline, and did not show the presence of cystine or cysteine. All the protein bound forms of corrinoids, hitherto isolated, show a 1:1 ratio / by weight/, of the protein moiety to the corrinoid residue in the whole complex.

Studies on the amino acid composition of the protein compounds of corrinoids included also the analysis of fractions obtained after ionophoresis of both B_{12}-M and B_{12}-B preparations. The aim of this research was to find out which of the electrophoretically separated fraction show the highest protein contents. Qualitative amino acid analysis of the indicated individual fractions was performed, after acid hydrolysis /6 N HCl, 22 hrs, in sealed tubes at 110°C/, on Whatman No.1 filter paper, using n-butanol /acetic acid/ water 4:1:5 v/v 48 hrs/ and phenol saturated with pH 12 phosphate buffer /12 hrs/ as solvent system. No cystine, or cysteine, and tyrosine could be found in hydrolysates of the major fractions, obtained after electrophoresis of either M-2 or B-2. The absence of tyrosine differs markedly the protein moiety of the appropriate preparation, prior to, and

after the electrophoretic treatment. The remaining "small" fractions, however, were found to contain the tyrosine residue. The electrophoretically isolated fractions, which appeared to contain most of the protein were subjected to further quantitative studies on the amino acid composition. Results of this research are presented in Table 1.

Absorption spectra of M-1, M-2, B-1 and B-2 preparations

The absorption spectra of the isolated complexes were measured in aqueous solutions in the region of 200 - 700 mμ using the Unicam SP-700 spectrophotometer. Characteristic maxima were observed at the following wave lengths:

M - 1 - 357, 412, 467, 505 mμ

M - 2 - 356, 412, 467, 505 mμ

B - 2 - 357, 412, 467, 526 mμ

B - 2 - 356, 412, 476 and 515 mμ.

The lack of a peak at 278 mμ differs the absorption spectra of the investigated preparations from that, typical for the crystallibe vitamin B_{12}, showing characteristic maxima at 278, 361, 520 and 550 mμ, respectivel The remaining peaks were found also to be shifted somewhat into the region of ultraviolet light. The most striking differences appeared however, in the region between 370 - 470 mμ, which might be due to the heterogenity of the preparations studied.

Fig. No 1 illustrates the adsorption spectra of both the B_{12}-M and B_{12} -B preparations

Electrophoresis of the B_{12}-M and B_{12}-B compounds in the presence of CN-ions.

Fig.No 2 presents the location of different fractions obtained during ionophoresis of the B_{12}-M and B_{12}-B compounds in presence of KCN.

The electrophoretic studies were performed according to the Holdsworth's procedure. Elektrophoresis occurred in 1N CH_3COOH pH 2.7 to which 0.015 % KCN was added. Samples on Whatman No 3 filter paper were subjected to a potential gradient of 5 v per cm for 13 hrs.

The B_{12}-M complex separated into three main fractions, numbered as 1, 2 and 3, respectively. Three additional patterns were detected in traces.

Fraction No 1, containing most of the coloured material applied to the filter paper, appeared to be a neutral one. The mobility of this component, as well as its colour, was found to be similar to that of cristalline vitamin B_{12}. The yellow coloured fraction No 2 showed basic properties. The colour of this pattern would indicate the absence of the corrin moiety.

Fraction No 3 was reddish and more basic as compared with fraction No 2.

When B_{12}-B was subjected to electrophoretic studies, similar patterns were demonstrated except the fraction showing most basic properties, which was not evidenced.

As illustrated on Fig.No 2 both the position and colour of the major fractions were fairly close to these, obtained after electrophoretic treatment of B_{12}-M. Fraction No 1 showed a weakly acid character.

The qualitative amino acid analysis of different fractions, seperated electrophoretically in the presence of CN^- ions.

Fractions No 1,2 and 3, originating from both B_{12}-M and B_{12}-B preparations, respectively, were hydrolysed by means of 6N HCl and subjected to paper chromatography as described elsewhere.

Whereas fraction No 1 showed only traces of the ninhydrin positive compounds, the remaining major fractions, No 2 and 3, contained most of the amino acids present in the untreated samples of B_{12}-M and B_{12}-B.

On the base of these findings a conclusion could be drawn out, concerning the decomposition of these compounds within the course of electrophoresis in presence of CN^- ions, resulting in the separation of the corrinoid constituent /mainly vitamin B_{12} or its analog/ and the protein moiety.

Electrophoresis of the B_{12}-M and B_{12}-B compounds in the absence of CN^- ions.

The omission of CN^- within the course of electrophoresis led to a different separation of the compounds analyzed, as compared with the patterns obtained when KCl was added to the electrolyte./ Fig. No-3/.

The major fractions, originated from either B_{12}-M or B_{12}-B, numbered as 2 and 3, had distinct basic properties. Moreover, the B_{12}-M preparation separated into three trace fractions, one of which moved into the position of crystalline vitamin B_{12} and showed a similar colour. The remaining two fractions were found to be basic. On the other hand, the B_{12}-B compounds separated into fractions No 2 and 3, and a weakly acid component, fraction No 1 /in traces/.

The red coloured fractions No 3 obtained after electrophoretic treatment of B_{12}-M, and B_{12}-B as well as the fraction No 2 of the B_{12}-M were eluted, hydrolyzed and subjected to the quantitative amino acid analysis.

The OH^- ions, usully applied in the electrophoresis of corrinoids, were found to affect the stability of the investigated complexes.

The absorption spectra of the main fractions obtained within the electrophoresis of B_{12}-M preparation in the presence of OH^- ions

These measurements were conducted in order to confirm the data from the amino acid analysis, which allowed to assume that degradation of the complexes takes place, due to the action of OH^- ions.

Because of the identity of the main fractions originated either from B_{12}-M or B_{12}-B preparation, the measurements

of absorbancy were limited to one of the indicated compounds, namely B_{12}-M.
Decomposition of the preparation was clearly evidenced /fig. No 4/.
The absorption spectrum of the most intensive fraction No 1 was found to be identical with that of crystalline vitamin B_{12}, whereas the absorption of fraction No 2 could not be ascribed to any of the corrinoids.
It seems very likely that fraction No 3 was the only one which contained the unaltered protein complex of corrinoid. The absorbtion spectrum of this fraction differed from that of B_{12}-M preparation, particularly in the region 370 to 450 mu.

The absorption spectra of the main fractions of B_{12}-compound, separated within the course of electrophoresis in the absence of OH^- ions.

As Fig. No 5 illustrates the absorbtion bands of both No 2 and 3 show much lower maxima, somewhat shifted toward the region of short waves. This phenomenon could be also observed in the case of crude B_{12}- preparation. The most striking difference between the crude compound and the indicated above components appeared in the region of 370 to 450 mu, in which the spectra of the later compounds are fairly similar to that of vitamin B_{12}. These findings indicate that the compounds were liberated from the interferring substance. A difference between fraction No 2 and No 3 could be noted in the region between 450 and 550

Table 1

Amino acid contents of B_{12}-M and B_{12}-B preparations /mean values from M-1 and M-2, B-1 and B-2, respectively /and of the major fractions, obtained during electrophoresis in the absence of KCN

Amino acid	B_{12}-M % of the whole compound	B_{12}-B % of the whole compound	B_{12}-M Fraction No 3	B_{12}-M Fraction No 2	B_{12}-B Fraction No 3
			% of the total of amino acids		
Lysine	1.31	3.37	1.89	-	4.29
Histidine ..	2.65	2.46	2.92	-	2.63
Arginine ...	1.29	2.43	3.70	-	2.29
			8.51	9%	9.21
Aspartic acid	1.65	4.12	2.84	4.23	12.9
Threonine ...	2.24	1.72	4.61	5.00	4.49
Serine	0.91	2.62	2.91	2.75	7.79
Glutamic acid	8.90	9.12	21.2	19.2	20.7
Proline	13.3	7.34	25.7	23.7	16.9
Glycine	1.34	1.62	3.82	3.86	3.71
Alanine	0.71	1.83	2.91	1.70	5.43
1/2 cystine..	-	-	-	1.4	-
Valine	2.63	1.98	9.15	10.0	4.01
Methionine ..	1.34	1.13	2.13	1.16	2.19
Isoleucine...	1.23	2.85	2.66	2.86	5.75
Leucine	4.60	4.11	8.92	9.25	4.77
Tyrosine	0.92	0.99	-	1.5	-
Phenylalanine	2.30	1.26	4.65	5.25	4.05

The quantitative amino acid composition of the electrophoretically isolated fractions of B_{12}-M and B_{12}-B preparations in the absence of CN^- ions.

17 aminoacid residues were determined in fraction No 3 of either the B_{12}-M /Fig.No 6/ or B_{12}-B complexes /Table No 1/.

The presence of 14 amino acid residues was evidenced in fraction No 2 of the B_{12}-M preparation ; the basic amino acids; lysine, arginine and histidine were not detected. This fraction has been previously described as "small".

Because of the lack of an appreciable amount of the experimental material, the electrophoretically seperated fractions, after elution, were not evaporated to dryness and weight, but hydrolyzed directly. Therefore the concentration of individual amino acid is being calculated as percent of the total amino acid contens determined, and assumed as 100.

The same reason made the determination of the basic amino acids in fraction No 2 of the B_{12}-M preparation impossible. To compare fraction No 2 and No 3, both originated from the same compound, it has been assumed, that the contents of basic amino acids in fraction No 2 would not exceed the figure of 9%. This assumption was made according to the experimentall data obtained for the fraction No 3 of B_{12}-M /8,5%/ and for fraction No 3 of B_{12}- compound /9,2%/ as shown in Table 1.

In order to compare the amino acid composition of the crude preparation with that of isolated fractions, all the figures corresponding to the preparation should be multiplied by a factor of 2. This seems to be reasonable because of the 1:1 ratio between the total of amino acids and the weight of the corrinoidresidue.
In contrast to the crude complexes fraction No 3 of both the B_{12}-M and B_{12}-H preparations did not show the contents of tyrosine. Moreover no cystaine orcystine could be determined, either in crude preparations, as well as in the indicated fractions. Tyrosine and probably cystine, was found, however, to be present in the hydrolysate of fraction No 2 of the B_{12}-M preparation. More arginine, serine, glycine and valine and less histidine was found in fraction No 3 of the B_{12}-M compound as compared with the crude preparation, from which this fraction originated. The percentage of the remaining amino acid residues appeared to be rather equal in both substances. The amino acid compositions of fraction No 2 and No 3 are similar, with the exception of cystine and tyrosine, only found in fraction No 2. For example, both fraction contain 20% of glutamic acid and 2% of proline. The same gures were obtained for the crude B_{12}-M preparation. Fractions No 3 of the B_{12}-H compound contained, however, a higher concentration of aspartic acid, threonine, serine alanine and phenylalanine, and lesser amounts of lysine, histidine, arginine and leucine.

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From the hitherto obtained data a general conclusion could be drawn out, concerning the heterogenity of the crude B_{12}-M preparation. This preparation seems to be a mixture of at least two components differing in the protein residue.

It seems very likely this property could be also ascribed to the B_{12}-S compund.

Studies on the light sensitive complexes :

The light sensitive compounds B_{12}-M-S isolated by the procedure described in the experimental, showed an characteristic orangereddish colour.

1. Electrophoresis:

This was performed, according to Holdsworth, except in the absence of light. In the presence of CN^- ions the preparation separated into two fractions. The neutral one showed a characteristic colour, specific to vitamin B_{12} /1/ whereas the other contained most of the orange-reddish coloured material /2/. Electrophoresis of the B_{12}-M-S compound in the absence of CN^- ions, resulted in a single fraction, which has not been subjected to further studies, within the course of research covered by this report.

2. Spectrophotometry:

Fig.7 illustrates the absorption spectra which correspond to the B_{12}-M-S preparation as well as to the preparations obtained after electrophoresis. The spectra of B_{12}-M-S preparation and of its orange-reddish feaction, isolated electrophoretically, are similar to

that of 5,6-dimethylbenzimidazole B_{12} coenzyme reported by Barker /Fed.Proc.20 956 /1961/ . The absorption spectrum of fraction No 1 was similar to that of crystalline vitamin B_{12}.

Formation of this fraction within the course of electrophoresis was probably due to the action of CN^- ions the B_{12} coenzyme. Another possibility, which might lead to the appearance of this fraction, would be the unsufficiant light protection during the isolation of the light sensitive form of corrinoids.

3. Qualitative amino acid analysis of the B_{12}M-S preparation an its fractions.

This was performed by means of paper chromatography. Butanol /acetic acid/ water system was used as solvent. The procedure for hydrolysis and chromatography was identical with that described elsewhere. Glycine and glutamic acid were the only amino acids found in appreciable amounts, whilest other amino acids could be detected only in traces.

In fraction No 1 which seems to be a vitamin B_{12}-like compound only glutamic acid was evidenced. On the other hand, glycine was the only one amino acid residue found in the hydrolyzate of fraction No 2 /B_{12}-coenzyme-like compund/.

The light-sensitive forms of corrinoids will be subjected to further studies, as indicated in the plan of future research.

Chromatography on Sephadex A-25

An appropriate volume of the M-2 water solution was passed trought a column of Sephadex A-25 /medium, 16 x 1.4 cm/.

This was followed by water elution of the coloured fraction. No resolution could be observed. The recovered compound was hydrolyzed by 6N HCl /22 hrs, in sealed glass tube, at 110°C/ and after the acid has been removed, analyzed by means of paper chromatography as mentioned elsewhere.

All the amino acids, commonly present in a protein hydrolyzate were detected, except cystine /or cysteine/ which could not be evidenced. This research will be continued.

Microbiological assays:

Assays of the growht factor activity of the isolated complexes for E.coli were performed according to the Burkholder's tube procedure, slightly modified in this laboratory. E.coli 113-3 served as test organism. The activity of the isolated complexes /expressed as percent of that of the crystalline vitamin B_{12}/ for the E.coli was as follows :

Sample	Activity in %
M - 1	34
M - 2	40
B - 1	20
B - 2	68

Following compounds, isolated within the course of study, were also tested:

- the light sensitive corrinoid preparation after electrophoretic treatment / no CN^- ions added/,

- two fractions of the light sensitive corrinoid preparation separated isoelectrophoretically in the presence of CN^- ions ;

- the position of the electroneutral fraction corresponds to that of crystalline vitamin B_{12} ; whilest the basic fraction moved into the position of factor B.

The absorption spectra of these components may suggest the conversion of the protein compound of light sensitive corrinoid, resulting in the formation of two di - CN^- forms of vitamin B_{12}.

Plan of future research :

1. Continuation of the studies on light sensitive corrinoids bound to protein /isolation, purification, analytical studies, including electrophoresis and dialysis at various pH values, amino acid analysis and chromatography.

2. Elaboration of suitable conditions for separation of the protein moiety from the investigated compounds.

3. Isolation of the protein bound corrinoids by adopting the procedures hitherto applied in research on the "vitamin B_{12}-enzyme".

4. Determination of the growth factor activities of the various fractions, isolated after electrophoretic treatment.

5. Preparation of tetrahydrofolic acid; this reagent is required for studies on the enzymic activity of the isolated "vitamin B_{12}-enzyme" - like preparation