3. The interrelationships of the peptides in the acid hydrolysate were determined by subjecting them to further partial acid hydrolysis and to the Edman degradation procedure.

4. The individual residues in these peptides were identified from the ionophoretic and chromatographic mobilities of the peptides, by specific reactions and from the amino acid composition of highly purified peptides.

5. It was concluded that the amino acid sequence around the serine phosphate (SerP) residue was Thr. Ala. SerP. His. Asp (or Asp-NH₂).

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A Native Cobalamin-Polypeptide Complex from Liver: Amino Acid Composition and Terminal Amino Acid Analyses of the Peptide Part

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The isolation of a cobalamin-polypeptide from bovine liver was described by Hedbom (1955, 1960). In view of the current interest in protein or peptide conjugates of corrinoids for basic metabolic studies (Wagle, Mehta & Johnson, 1958*a, b*; Helleiner & Woods, 1956; Barker, Weissbach & Smyth, 1958) and for the oral treatment of pernicious anaemia (cf. Heathcote & Mooney, 1958)

it has seemed desirable to investigate this complex in more detail. Knowledge of its composition and structure might form a basis for general conclusions regarding relationships between structure and biological activity of the corrinoids. The present paper deals with the quantitative amino acid analysis and the determination of the terminal amino acids of the peptide part of the complex.

All results obtained indicate the following composition [for abbreviations see *Biochem. J.* (1957), **66**, 6]: Gly₂₂; Ala₉; Thr₈; Val₄; Leu₁₀; Ileu₁; Asp₄; Glu₅; Tyr₄; His₆; (CySH)₂; Pro₃; Lys₂, with histidine and glycine as the *N*- and *C*-terminal amino acids respectively.

EXPERIMENTAL

Materials

The samples of cobalamin-peptide used for this work (lots no. 3, 4, 6, 7 and 10) were prepared from ox liver by the method of isolation previously published (Hedbom, 1960). The purified material appeared as a monodisperse substance in the ultracentrifuge. On paper electrophoresis (in citrate, acetate, phosphate and veronal-acetate buffers), within the pH range 2.0–8.6 over which the complex is stable, it migrated as a single entity. Elementary analysis of lot no. 3 after drying gave: C, 52.9; H, 6.7; N, 16.6; S, 0.71; P, 0.342; Co, 0.646%. The moisture content of this was 2.46% as determined by drying *in vacuo* for 24 hr. over P₂O₅ at 100°. Authentic reference samples of dinitrophenyl amino acids were prepared synthetically by Dr H. Kiessling and were kindly supplied by him.

Methods

Isolation of the peptide part. In order to split the cobalamin complex into a corrinoid and a polypeptide part, pure samples of 5–10 mg. were dissolved in 5 ml. of water containing 1 drop of a 10% KCN solution. This process is known to cause a change in the light-absorption so as to give a spectrum resembling that of cyanocobalamin. The shift may be explained by the assumption that a cyanide group has replaced the peptide ligand of the original complex and thus formed a cyanocobalamin and a free peptide.

Preliminary examination by paper electrophoresis at pH 4.5 showed, however, that the cyanocobalamin was still attached to the peptide to a considerable degree by some weak forces of attraction.

Upon slow addition of 1 ml. of 10% trichloroacetic acid the polypeptide precipitated, while the cyanocobalamin remained in solution. After centrifuging and washing of the sediment with diethyl ether the polypeptide was isolated from the last traces of cyanocobalamin by column electrophoresis according to Porath (1956). A volatile buffer of 0.5M-pyridine and 0.1M-acetic acid, pH 4.5, was used. Under these conditions the peptide migrated towards the cathode as a single substance leaving the neutral cyanocobalamin at the starting position. After freeze-drying, the polypeptide was obtained as a white homogeneous powder.

Hydrolysis of the polypeptide. Accurately weighed samples of 5–6 mg. were hydrolysed for 20 and 70 hr. in sealed Pyrex tubes at 105–107°, each with 1 ml. of constant-boiling (5.7N) HCl, which had been redistilled three times in glass. The hydrolysates, which were quite colourless, were processed in the manner described by Smith & Stockell (1954), which involved repeated concentration *in vacuo* at 40–50° followed by dilution with water. The N contents were determined by duplicate micro-Kjeldahl analyses on portions of the hydrolysates.

Amino acid determinations. Initially the dinitrophenylation method developed by Levy (1954) was employed for the amino acid analysis. This method, which is based on paper-electrophoretic separation of dinitrophenyl (DNP) amino acids, gave poor results in our hands. For this reason the column-chromatographic technique of Moore & Stein (1951) had to be used for the accurate determination. It was later possible to confirm the reproducibility of this determination by use of the Spinco automatic amino acid analyser, when this apparatus became available.

Dinitrophenylation method. The quantitative dinitrophenylation was carried out both on 20 and 70 hr. hydrolysates with a slight excess of 1-fluoro-2,4-dinitrobenzene (FDNB) at pH 9.0 and 40°. Excess of reagent was removed by sublimation according to the method described by Mills (1952). The separation of the DNP amino acids was effected by two-dimensional chromatography on Whatman no. 4 paper with toluene-chloroethanol-pyridine-aq. 0.8N-NH₃ soln. (Biserte & Osteux, 1951) or with 2-methylbutan-2-ol (Blackburn & Lowter, 1951) in the first dimension and with aq. 1.5M-phosphate buffer in the second. The DNP derivatives were identified by comparison with known DNP amino acids. For quantitative estimation, the coloured spots were cut out together with appropriate blanks, and eluted by shaking for 15 min. at 60° in 4 ml. portions of aq. 1% (w/v) Na₂CO₃. The extinctions of the extracts were read against the blank extract at the maxima near 360 mμ (385 mμ for DNP-proline) in a Beckman DU spectrophotometer. These readings were converted into μmoles of DNP amino acids as described by Levy (1954) with the same conversion factors. Duplicate analyses were performed for each hydrolysate and each analysis was carried out with three different chromatograms.

Column chromatography. For the more accurate amino acid analyses the method described in detail by Moore, Spackman & Stein (1958) and Spackman, Stein & Moore (1958) was used. The separation and determination of the amino acids were performed in a Beckman/Spinco model MS amino acid analyser. The columns were packed with a sulphonated styrene, 8%, divinylbenzene co-polymer, labelled 'Aminex-MS cation-exchange resin', obtained from Bio. Rad Laboratories, Richmond, Calif., U.S.A. The resin was graded according to the method of Hamilton (1958). A particle size of 31–45 μ was used for the 150 cm. column and 19–25 μ for the 15 cm. column.

Oxidation with performic acid. To cleave possible disulphide bridges in the molecule, a polypeptide sample of 5 mg. dissolved in 1 ml. of 85% formic acid was oxidized by the addition of 0.1 ml. of 30% H₂O₂. The reaction was allowed to proceed for 30 min. at room temperature (22°) after which the solution was diluted with 10 ml. of ice-water. The excess of reagent was removed by freeze-drying. The residue was redissolved in 2 ml. of water and evaporated to dryness *in vacuo*.

N-Terminal analysis. The polypeptide was dinitrophenylated by shaking amounts of 5–6 mg. with excess of FDNB in 67% ethanol containing NaHCO₃ or trimethylamine; essentially the method of Sanger (1945) was used. Excess of reagent was removed by extraction with ether, after acidifying the product with HCl. The precipitated yellow DNP peptide was then hydrolysed with 5.7N-HCl for 8 or 20 hr. After removal *in vacuo* of the excess of HCl, the yellow residue was dissolved in 0.5N-HCl and extracted four times with diethyl ether. The ether extracts and the

water phase were evaporated to dryness, and the residues dissolved in water and used for paper-chromatographic analysis of the DNP amino acids, as described above.

C-Terminal analysis. Some preliminary attempts were made to identify the C-terminal amino acids by reduction of the free carboxylic groups with LiAlH₄. Hydrolysis of the reaction product gave a mixture of amino acids and amino alcohols which was investigated by paper chromatography.

In a typical experiment 8 mg. of polypeptide was dried *in vacuo* for 24 hr. at 100° over P₂O₅. The sample was suspended in 10 ml. of dry ether and an ether solution of 100 mg. of LiAlH₄ was slowly added. The reaction was allowed to proceed for 10 hr. during which time the mixture was stirred continuously. The reaction mixture was then neutralized with HCl and evaporated to dryness. The residue, containing the reduced polypeptide, was hydrolysed for 20 hr. in 5·7N-HCl and analysed for amino acids and amino alcohols by paper chromatography as described by Hedbom (1960).

RESULTS

Amino acid composition. The dinitrophenylation method furnished preliminary data regarding the amino acid composition. DNP-leucine and DNP-isoleucine were not separated, and sometimes DNP-glutamic acid and DNP-aspartic acid were poorly resolved, but otherwise the separation was satisfactory. The results of the amino acid analysis by the Levy method is reported in Table 1. The amounts of glycine, leucine, glutamic acid, tyrosine and proline are low compared with the values obtained by the Moore & Stein procedure (see below). As mentioned in the Discussion, a possible reason for this may be that the factors for the conversion of the extinctions into molar ratios, given by Levy for insulin, are not completely applicable to other polypeptides. It would appear that the

following factors, which are on average about 6% higher than those given by Levy, are more nearly correct for the cobalamin-polypeptide: Asp 1·04; Thr 1·12; Glu 1·15; Pro 1·04; Gly 1·11; Ala 1·08; Val 0·94; Leu 1·15; Tyr 1·96; Lys 0·69; His 1·88; CySH 1·12.

Control experiments with a commercial amino acid calibration mixture (Spinco 120-220 lot 191131) also indicated that the Levy factors were 5-8% too low. Here also, the chromatographic resolution of the coloured spots was satisfactory, and the blank readings were very low (less than 0·001/cm.²). For these reasons it would seem that the discrepancy is significant.

Ion-exchange chromatography gave satisfactory results for the amino acid analysis. Some features of the composition are illustrated qualitatively in the elution curve of Fig. 1, obtained with a sample hydrolysed for 20 hr. From these data it is apparent that the polypeptide has a relatively high content of glycine and that serine, arginine, phenylalanine and methionine are not present. The absence of tryptophan has previously been shown by spectroscopic examination of an unhydrolysed sample of the polypeptide. No evidence was found suggesting incompleteness of the hydrolysis. The base-line showed no significant variations, and the peaks were entirely symmetrical, and were apparently not contaminated with residual peptides. Tables 2 and 3 summarize the data from the experiments with 20 and 70 hr. hydrolysates of two samples of the polypeptide. In instances where there was evidence of destruction, as with aspartic acid, threonine, cystine and tyrosine, corrections for the losses were calculated by extrapolating the analytical data to zero time, assuming first-order kinetics for the decomposition. The value which

Table 1. *Amino acid analysis of the cobalamin-polypeptide by the dinitrophenylation method*

Samples were from preps. 3 and 4. Average quantity found is the average of duplicate analyses of 20 and 70 hr. hydrolysates. The variation is expressed as the average deviation. The calculated no. of amino acid residues are on a basis of a mol.wt. of 7900 from the cobalt content (Hedbom, 1960).

Amino acid	Average quantity found (μmoles)	Calculated no. of amino acid residues	Residues taken to nearest integer
Asp	0·482 ± 0·014	3·81	4
Thr	1·03 ± 0·035	8·11	8
Glu	0·530 ± 0·012	4·19	4
Pro	0·224 ± 0·011	1·77	2
Gly	2·58 ± 0·070	20·4	20
Ala	1·29 ± 0·034	9·2	9
CySH	0·366 ± 0·019	2·89	3
Val	0·535 ± 0·014	4·23	4
Leu + Ileu	1·32 ± 0·040	10·4	10
Tyr	0·406 ± 0·027	3·21	3
Lys	0·232 ± 0·013	1·85	2
His	0·645 ± 0·024	5·11	5
		Total	74

may be subject to the greatest error is that for cystine because of its instability to hydrolysis. The calculated number of two cysteine residues per molecule is in good agreement with the sulphur content. Experiments with performic acid-oxidized samples also support this value. Paper-electrophoretic examination of such samples at pH values of 3.0, 4.5 and 8.0 showed that the peptide still migrated as one homogeneous substance. No evidence for the degradation of the peptide to smaller fragments was found. At pH 4.5 the untreated peptide migrated towards the cathode, whereas the oxidized peptide under the same conditions slowly moved in the opposite direction, with a mobility that could be explained by the

presence of about two additional negatively charged groups in the molecule.

The foregoing results account for the entire composition of the polypeptide. The recovery of amino acids was in accordance with the total weight, and the nitrogen content was within the limits of the experimental method used.

End groups. No ether-soluble DNP amino acids were obtained from the 20 hr. hydrolysate of the polypeptide nor from a sample that had been hydrolysed for 8 hr. Coloured material in the ether phase proved to be dinitrophenol, dinitroaniline and small amounts of the water-soluble DNP derivatives. Increased time for the reaction with FDNB failed to produce any ether-soluble DNP amino acids.

Fig. 1. Elution curves from the column-chromatographic analysis of a 20 hr. hydrolysate of the cobalamin polypeptide on a sulphonated styrene resin. A, 15 cm. column; 0.35N-sodium citrate buffer, pH 5.25. B, 150 cm. column; 0.2N-sodium citrate buffer, pH 3.25 and 4.25. Buffer change at the arrow.

The water phase contained ϵ -DNP-lysine, bis-DNP-histidine, imino-DNP-histidine and minute amounts of α -DNP-histidine. The dinitrophenylation seemed to be complete since no free lysine could be detected in the hydrolysate.

Calculations from the ultraviolet spectra of the eluted bis-DNP-histidine spots gave an average recovery of 0.15 mg. of histidine/8 mg. of polypeptide, i.e. 0.92 mole of terminal histidine/mole of polypeptide. These data show that histidine is the *N*-terminal amino acid.

After reduction with LiAlH₄ and hydrolysis, the

sample, when subjected to paper chromatography, gave two additional ninhydrin-positive spots compared with the hydrolysate of the untreated peptide. Only one of these could be identified with reasonable confidence as an amino alcohol. This substance had the same *R_F* as ethanolamine obtained from a control sample of leucylglycine reduced and hydrolysed in the same way. Although no reliable quantitative estimation could be made from the chromatograms it seems likely from the data obtained that glycine constitutes the *C*-terminal group.

Table 2. *Amino acid analysis of the cobalamin-polypeptide by column chromatography*

The values given for the 20 and 70 hr. hydrolysates represent duplicate analyses of samples from two different preparations.

		Amino acid residue (g./100 g. of polypeptide)									Av. or extra- polated value
Amino acid	Time of hydrolysis, 20 hr.					Time of hydrolysis, 70 hr.					
	Sample from prepn. 7		Sample from prepn. 10		Av.	Sample from prepn. 7		Sample from prepn. 10		Av.	
Asp	5.73	5.67	5.81	5.79	5.75	5.33	5.28	5.37	5.40	5.35	5.92*
Thr	10.16	10.26	10.14	10.16	10.18	9.63	9.57	9.69	9.67	9.64	10.42*
Glu	8.54	8.56	8.62	8.60	8.58	8.46	8.42	8.54	8.50	8.48	8.53
Pro	3.76	3.70	3.76	3.74	3.74	3.68	3.74	3.72	3.76	3.71	3.73
Gly	16.18	15.85	16.51	16.29	16.21	15.86	15.98	15.95	15.83	15.91	16.06
Ala	8.30	8.24	8.25	8.17	8.24	8.64	8.74	8.46	8.64	8.62	8.43
CySH†	2.36	2.43	2.52	2.60	2.48	2.08	2.16	2.07	2.09	2.10	2.64*
Val	5.16	5.30	5.14	5.16	5.19	5.16	5.10	5.10	5.24	5.15	5.17
Ileu	1.54	1.54	1.48	1.44	1.50	1.42	1.38	1.45	1.42	1.42	1.46
Leu	15.26	15.24	15.21	15.09	15.20	14.48	14.60	14.61	14.39	14.52	14.86
Tyr	8.40	8.49	8.55	8.57	8.46	8.16	8.19	8.20	8.23	8.20	8.57*
Lys	3.52	3.47	3.47	3.33	3.45	3.24	3.15	3.34	3.18	3.23	3.34
His	10.54	10.46	10.40	10.46	10.43	10.71	10.69	10.68	10.56	10.66	10.55
Amino (NH ₂)	0.35	0.41	0.44	0.36	0.39	0.39	0.49	0.47	0.45	0.45	0.42

* Extrapolated value.

† Measured as cystine but calculated as cysteine.

Table 3. *Amino acid composition of the cobalamin polypeptide*

The result is in agreement with the following structure: Gly₂₂; Ala₉; Thr₈; Val₄; Leu₁₀; Ileu₁; Asp₄; Glu₅; Tyr₄; His₈; (CySH)₂; Pro₃; Lys₂.

Amino acid	Amino acid residue (g./100 g. of polypeptide)	Min. mol.wt.	Mol.wt.	Amino acid (g./100 g. of polypeptide)	N (% of total N)	Calculated no. of residues for mol.wt. 7712
Asp	5.92	1944	7776	6.84	4.21	3.97
Thr	10.42	970.2	7762	12.27	8.44	7.95
Glu	8.53	1513	7565	9.72	5.41	5.10
Pro	3.73	2604	7812	4.42	3.15	2.96
Gly	16.06	355.2	7814	21.13	23.06	21.70
Ala	8.43	843.2	7589	10.56	9.71	9.15
CySH	2.64	3905	7810	3.10	2.11	1.97
Val	5.17	1917	7668	6.11	4.27	4.02
Ileu	1.46	7753	7753	1.69	1.05	0.99
Leu	14.86	761.8	7618	17.22	10.75	10.10
Tyr	8.57	1904	7616	9.51	4.30	4.05
Lys	3.34	3838	7676	3.81	4.27	2.01
His	10.55	1300	7800	11.93	18.89	5.93
Amino (NH ₂)	0.42	4448	(8896)*	—	2.02	(1.73)*
Total	100.10	—	—	118.31	101.64	79.90
Mean	—	—	7712	—	—	—

* Omitted value.

DISCUSSION

Evidence for the homogeneity of the isolated cobalamin-polypeptide complex has been put forward earlier (Hedbom, 1960). The results reported in the present paper substantiate these findings. The general amino acid composition and the terminal amino acids are shown to be the same within the limits of experimental error, in four or five samples of peptide from different preparations. This was determined by dinitrophenylation in three cases, and by column chromatography in two. Concerning the dinitrophenylation method for the determination of the amino acid composition, Li & Chung (1956) reported excellent results from analyses of a hypophyseal growth hormone with a molecular weight of 45 000 and of bovine plasma albumin of 65 000. As the cobalamin-polypeptide has a molecular weight of less than 10 000, we had hoped that the method would give the exact and correct number of residues. Direct calculations from the extinction values of the eluted DNP amino acids gave molar ratios corresponding to only 75 residues. It is possible, however, to deduce the number of residues from the alkali uptake during the dinitrophenylation reaction. This was not done, but if the correct value of 80 residues (from Table 3) is used for the calculation the following composition is obtained: Asp 4.07; Thr 8.60; Glu 4.47; Pro 1.90; Gly 22.20; Ala 9.80; CySH 3.10; Val 4.50; Leu + Ileu 11.1; Tyr 3.44; Lys 1.95; His 5.47.

If these numbers are taken to the nearest integer the result is (the correct number, if different, is given in parentheses): Asp 4; Thr 9 (8); Glu 4 (5); Pro 2 (3); Gly 22; Ala 10 (9); CySH 3 (2); Val 4 or 5 (4); Leu + Ileu 11; Tyr 3 (4); Lys 2; His 5 (6).

The accuracy of the determination of the molar fractions was within $\pm 3\%$, and we must therefore conclude that the Levy method was not sufficiently precise to give the correct amino acid composition of the polypeptide. A simple, possible explanation would be that the dinitrophenylation reaction was not quantitative. The method has, however, some advantages of speed and sensitivity; for the present work it was of value for determining the approximate amino acid composition of the polypeptide, and later for analysing smaller degradation peptides of the cobalamin complex.

The accurate amino acid analysis, as reported in Tables 2 and 3, showed that the peptide part of the complex contained 80 residues: Gly₂₂; Ala₉; Thr₈; Val₄; Leu₁₀; Ileu₁; Asp₄; Glu₅; Tyr₄; His₆; (CySH)₂; Pro₃; Lys₂; with a calculated molecular weight of 7698. In the analysis 100.10% of the total weight and 101.64% of the nitrogen content was accounted for. The calculated molecular weight corresponds to 9028 for the whole complex. This is

in good agreement with the earlier reported figure of 9129 from the cobalt content and 9053 from absorption spectroscopy. That this number represents the true molecular weight rather than a multiple of it is supported by results of centrifuging (Hedbom, 1960).

The polypeptide has a single *N*-terminal amino acid, as judged from the finding of 0.92 mole of histidine as DNP derivative. The identification of ethanolamine in the reduced and hydrolysed peptide sample indicated glycine to be one *C*-terminal group. Degradation with carboxypeptidase gave glycine as the only *C*-terminal (A. Hedbom & R. Mosbach, unpublished observations). Treatment with performic acid to break possible S-S bonds did not result in the rupture of the peptide chain into smaller fragments. This evidence suggests a straight-chain gross structure of the polypeptide, possibly with a ring formed by a cross-linked cystine residue.

A close examination of the amino acid distribution reveals the presence of four aspartic acid, five glutamic acid and eight basic residues, i.e. six histidine and two lysine. It is thus likely that the isoelectric point of the peptide should be near a pH where the imidazole groups of histidine are in the cationic form. The measured isoelectric point of 6.25 is in good agreement with the analytical findings.

The glycine content is remarkably high; 22 residues of the total of 80 represents 16.27% of the total weight.

SUMMARY

1. The cobalamin-polypeptide from ox liver has been analysed for amino acids by the dinitrophenylation method and by ion-exchange chromatography. The terminal amino acids have been determined.

2. The dinitrophenylation method was inadequate for amino acid analysis of the peptide. The reasons for this are discussed.

3. The polypeptide has been shown to consist of a single chain of 80 amino acid residues, possibly with a ring formed by a cross-linked cystine residue.

4. The composition of the polypeptide is consistent with the following formula: Gly₂₂; Ala₉; Thr₈; Val₄; Leu₁₀; Ileu₁; Asp₄; Glu₅; Tyr₄; His₆; (CySH)₂; Pro₃; Lys₂. Glycine and histidine are the *C*- and *N*-terminal residues respectively.

5. The reported composition is in good agreement with the molecular weight and the nitrogen content of the polypeptide part as well as of the whole cobalamin complex.

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The Mechanism of the Glyoxalase I Reaction, and the Effect of Ophthalmic Acid as an Inhibitor

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The glyoxalase system catalyses the two reactions:

and each reaction is catalysed by a separate enzyme, glyoxalases I and II respectively (Racker, 1951; Crook & Law, 1952). The work described is concerned with glyoxalase I only, which, for brevity, will be referred to simply as glyoxalase. The thiol group of glutathione plays a part in the reaction, and ophthalmic acid, a tripeptide, isolated from calf lens, which is structurally similar to glutathione but lacks the thiol group (Waley, 1958), might be expected to inhibit the reaction. This has been demonstrated. Synthetic analogues of glutathione are known to act as inhibitors in the glyoxalase reaction (Kermack & Matheson, 1957).

In an enzymic reaction in which there are two substrates, it is possible that the two substrates might react with each other before reacting with the enzyme. In general, this does not happen. The evidence presented here, however, suggests that this

occurs with the glyoxalase reaction. In that case, the intermediates in the complete glyoxalase system will be as set out in Scheme I.

A preliminary account of this work has already appeared (Cliffe & Waley, 1959).

EXPERIMENTAL

Materials

Methylglyoxal was purified and standardized as described by Kermack & Matheson (1957), and the glutathione (GSH) was obtained from The Distillers Co. Ltd. (Biochemicals). Ophthalmic acid and norophthalmic acid were synthetic samples (Waley, 1957, 1958). We are indebted to Dr K. W. Daisley for the preparation of glyoxalase I, which had been purified to step 4 in the procedure of Racker (1951), and was free from glyoxalase II.

Methods

The formation of *S*-lactoylglutathione was followed by the increase in $E_{240\text{ m}\mu}$ in a Beckman DU spectrophotometer (Racker, 1951); optical cells with 1 cm. light-path were used. The reactions were carried out in 33 mM-potassium