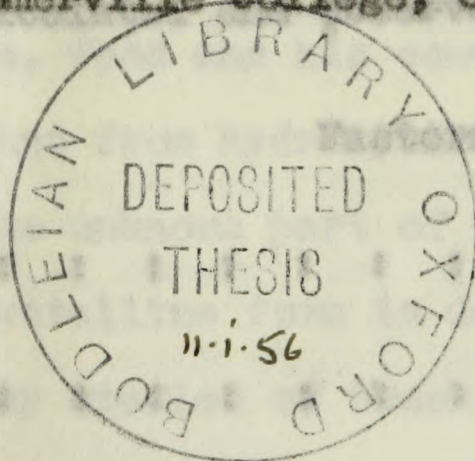


CRYSTALLOGRAPHIC STUDIES ON THE STRUCTURE	
OF VITAMIN B ₁₂ AND RELATED COMPOUNDS	
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by	
Jenny Pickworth	126

Somerville College, Oxford.



June, 1955

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The methods used were empirical, but studies by other workers, on the vitamin, had indicated a

possible mode of approach to the problem. As methods for the interpretation of X-ray diffraction patterns obtained from single crystals develop, it becomes increasingly possible to apply them to the determination of structures of compounds of unknown chemical formulae. This may be invaluable, particularly when chemical degradative methods have failed to indicate the formula. The determination of the structure of vitamin B₁₂ gives an example of the power of this method, for, while the structure of part of the molecule has been found chemically from hydrolysis, there remains a part which has so far defied chemical attempts at a complete identification by degradation.

At Cambridge, Todd and his co-workers isolated a degradation product from hydrolysis mixtures, and this contained only the unknown part of the molecule. It was obtained in a crystalline form in October 1953, and, in this thesis, X-ray studies of these crystals, to give a complete determination of the structure of this compound, will be described.

The methods used were empirical, but studies, by other workers, on the vitamin, had indicated a possible mode of approach to the calculations.

Fortunately the method used was successful and has since been applied to X-ray data from the vitamin. This investigation has raised many points of crystallographic interest during its course.

The chemical formula suggested is a little unexpected, because it bears a very close resemblance to that of a metal porphyrin, and yet there are some very significant differences which will be discussed.

I should like to thank Mrs. D.M. Hodgkin who supervised all this work. She has provided help, encouragement and inspiration throughout all these studies. Dr. J.H. Robertson taught me and assisted with all the procedures in the preliminary experimental work.

The crystals which were investigated were kindly provided by Dr. J.R. Cannon, Dr. A.W. Johnson and Professor A.R. Todd, with whom I have had many interesting and helpful discussions.

Two of the three-dimensional electron density

calculations which will be described were done in Oxford. Such large problems can only be tackled if speedy and efficient calculating equipment is available. All the other three-dimensional calculations were made by Dr. K.N. Trueblood and Dr. R.J. Prosen on an electronic machine in California. Without this assistance the work would have taken many more years.

My thanks extend to all colleagues for their help, and especially to Mrs. U.K. Ledwith who drew out most of the three-dimensional series, and to Mrs. H.M. Oliver, who assisted with computing.

I wish to acknowledge gratefully a maintenance grant from the Department of Scientific and Industrial Research.

Castle² suggested that there was a preliminary reaction between an antitoxic factor present in food (for example liver) and an antitoxic factor in the stomach to give a factor which is active in the body. Pernicious anemia is then an unusual deficiency disease for it results from an inability to absorb a factor present in food.

Folkers and his co-workers³ at Harvard Laboratories

Introduction

Vitamin B₁₂

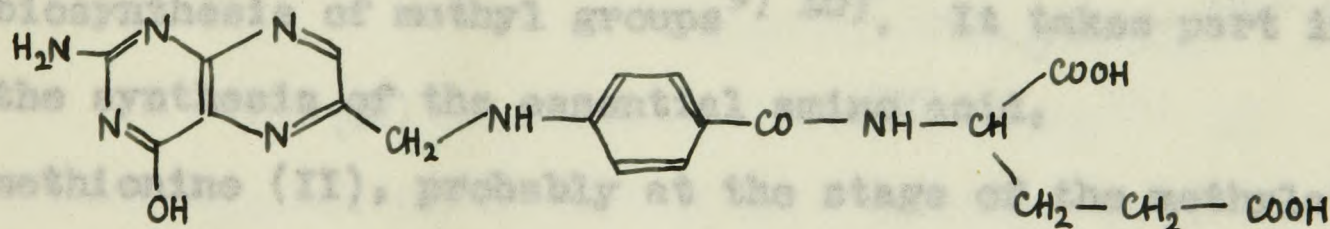
The effectiveness of liver in the dietary treatment of pernicious anaemia was reported by Minot¹⁾ and Murphy in 1926. In this disease the red blood cells divide but do not mature. They are reduced in number and are larger than usual. The stomach lining atrophies and no hydrochloric acid is secreted. Degeneration of the spinal cord results in the death of an untreated patient. At first, whole liver was given as treatment, but there must be a particular component present in this which prevents the disease. For many years workers attempted unsuccessfully to isolate it. Castle²⁾ suggested that there was a preliminary reaction between an extrinsic factor present in food (for example liver) and an intrinsic factor in the stomach to give a factor which is active in the body. Pernicious anaemia is then an unusual deficiency disease for it results from an inability to absorb a factor present in food.

Folkers and his co-workers³⁾ at Merck Laboratories

in America isolated the anti-pernicious anaemia factor from liver in 1948, using chromatography and microbiological assays. They named it vitamin B₁₂ because it was found to be an essential growth factor for certain organisms. Lester Smith⁴⁾ at Glaxo Laboratories isolated it independently in the same year. He had used only clinical methods of assay. The vitamin is present only to the extent of one part per million in liver but it is extremely potent, the effective dose being about one microgram daily. It is now generally prepared from culture broths of streptomyces griseus used for the preparation of streptomycin⁵⁾.

This vitamin is Castle's extrinsic factor.

Folic acid (I)———

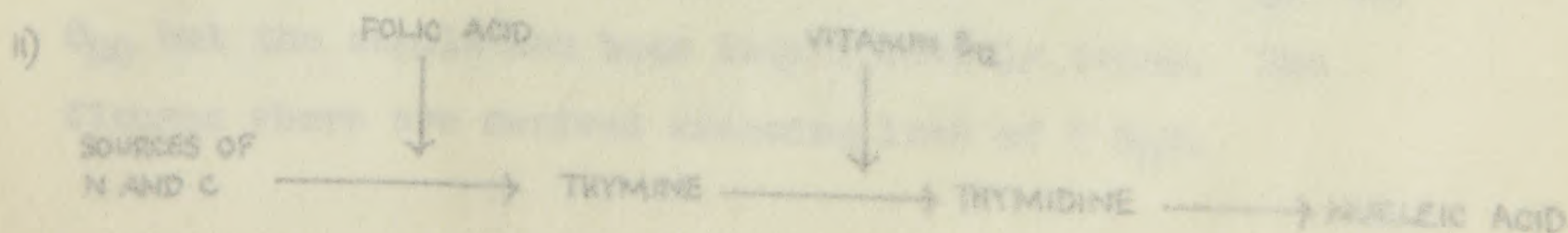


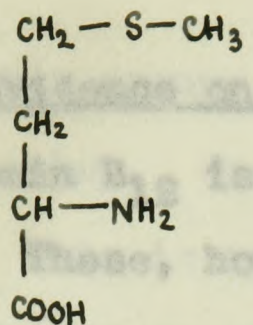
(1)

once given in the treatment of pernicious anaemia_____

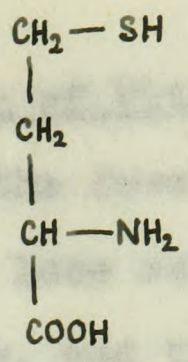
will restore the red cell count to normal but will not relieve the accompanying spinal cord degeneration⁶⁾. Latner⁷⁾ has recently described the isolation of intrinsic factor from hog gastric mucosa. It is probably mucoprotein in nature. Further researches by many workers on intrinsic factor are now in progress. It has been suggested⁸⁾ that vitamin B₁₂ is bound by one protein in the liver, where it is stored, and carried by another — which binds it more firmly — in the serum to the tissues. The importance of a binding factor is being stressed increasingly, for it seems that without intrinsic factor in the stomach nearly all vitamin B₁₂ taken orally is excreted.

It is difficult to find all the functions of a vitamin in the body. Vitamin B₁₂ is involved in the biosynthesis of methyl groups^{9) 10)}. It takes part in the synthesis of the essential amino acid, methionine (II), probably at the stage of the methylation of homocysteine (III). It may also be concerned in the



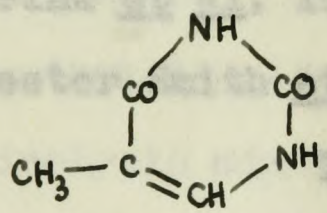


(II)

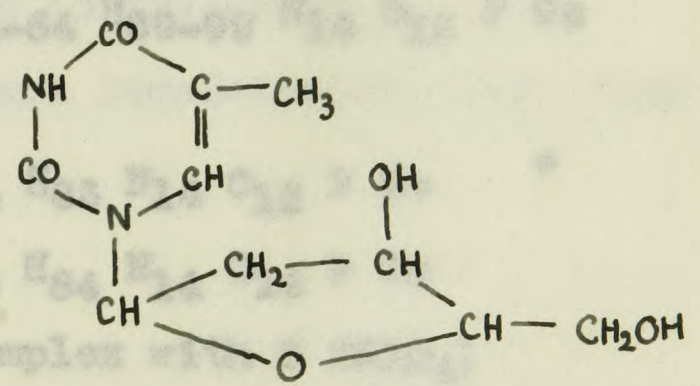


(III)

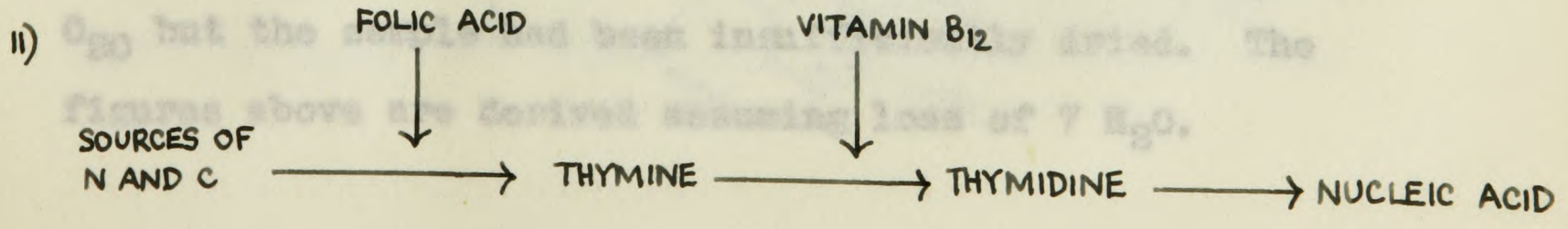
reduction of disulphide groups. The vitamin, like folic acid, probably also takes part in the synthesis of nucleic acids. Folic acid may be involved in the formation of thymine (IV) and vitamin B₁₂ with its conversion to thymidine (V).



(IV)



(V)



Chemical Evidence on the Structure of Vitamin B₁₂

Vitamin B₁₂ is isolated in the form of deep red crystals. These, however, slowly lose solvent of crystallisation on exposure to air, and this fact complicates chemical analyses and crystallographic molecular weight determinations. X-ray studies at Oxford¹²⁾ suggested that the molecular weight of the vitamin lies between 1360 and 1575. The molecule contains cobalt, phosphorus, carbon, hydrogen, nitrogen and oxygen. Various molecular formulae have been suggested:-

13) Brink et al, 1949: $C_{61-64} H_{89-92} N_{14} O_{13} P Co$

14) Lester Smith et al,

the hydrolysis of 1950: $C_{63} H_{83} N_{14} O_{13} P Co$ *

15) Alicino, 1951: $C_{63} H_{84} N_{14} O_{14} P Co$

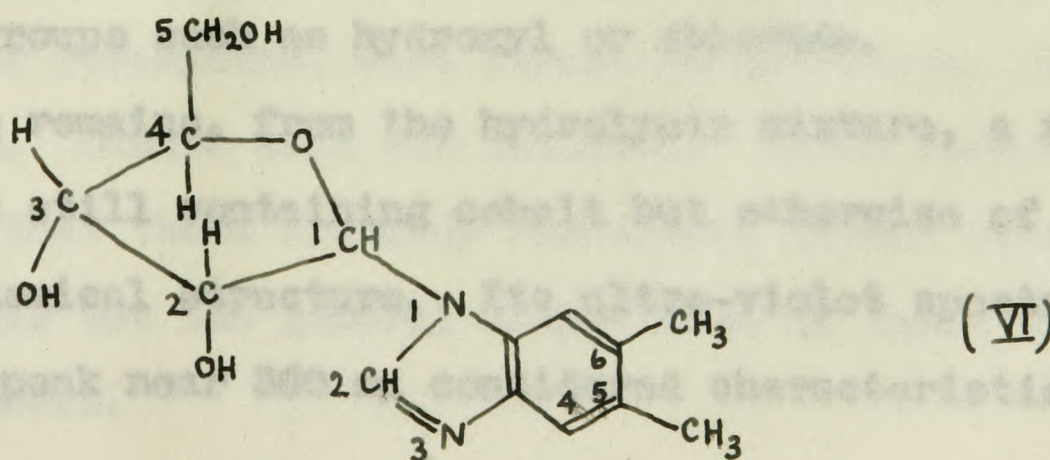
(complex with 6 $HClO_4$)

A study of the magnetic susceptibility¹⁶⁾ of vitamin B₁₂ has shown that it is a covalent cobaltic

* The first published formula of Lester Smith had H_{97} and O_{20} but the sample had been insufficiently dried. The figures above are derived assuming loss of 7 H_2O .

complex. Six groups are arranged octahedrally around the cobalt atom. One of these groups is a cyano group¹⁷⁾. This is non-ionic since experiments have shown¹⁸⁾ that excessively large doses of the vitamin are not toxic to mice.

The chemical nature of almost half of the molecule has been found by acid hydrolysis and chromatographic separation of the products. Their identification showed the presence of 5:6-dimethylbenziminazole¹⁹⁾, a phosphate group²⁰⁾, D-ribose²¹⁾, D-1-amino-2-propanol²²⁾ and five or six amide groups²³⁾ in the vitamin. Folkers and co-workers²¹⁾ announced the isolation of 5:6-dimethyl-1- α -D-ribofuranosyl benziminazole (VI) from the hydrolysis mixture,



confirming this by comparison with a synthetic specimen. The phosphate group must be linked to the ribose on C₂ or C₃ as the benziminazole nucleotide contains no free α -glycol grouping²⁴⁾. Ultra-violet spectroscopic studies suggest that the N₃ of the benziminazole is linked co-ordinately to the cobalt atom²⁵⁾.

Other forms of vitamin B₁₂ have been isolated. Certain organisms, for instances those in the gut, can synthesise not only vitamin B₁₂ but also vitamin B₁₂-like factors, given suitable precursors such as adenine or 2-methyladenine instead of 5:6-dimethylbenziminazole. These factors probably play the same part as the vitamin in the metabolism of the micro-organism. They are not absorbed by the animal, possibly because they do not form a complex with intrinsic factor. The cyanide group in vitamin B₁₂ may be replaced by other groups such as hydroxyl or chloride.

There remains, from the hydrolysis mixture, a red gummy mass still containing cobalt but otherwise of unknown chemical structure. Its ultra-violet spectrum²⁶⁾ shows the peak near 360 m μ considered characteristic of

the chromophoric group within the molecule. This must contain a system of conjugated double bonds. Pyrolysis of the vitamin with alkali is said to lead to the formation of vapours giving a test characteristic of pyrroles²⁶⁾. It was supposed that vitamin B₁₂ contained a porphyrin group, but as this suggestion was not confirmed by the ultra-violet spectrum such a group, if present, must be modified in some way.

All attempted oxidative degradations resulted in the loss of cobalt and the complete disruption of the molecule. This is not characteristic of porphyrins which may lose only the metal. Karrer²⁷⁾ isolated oxalic, succinic, methylsuccinic and dimethylmalonic acids and four unidentified acids from the potassium permanganate oxidation of the vitamin. At Cambridge, carefully controlled experiments were carried out to see if any derivatives of maleimide, degradation products of porphyrins, were formed on oxidation. None were found. Oxamic acid was isolated. These researches indicated that there are many functional groups within the molecule but the general disposition of these could not be deduced.

Crystallographic Studies on Vitamin B₁₂

A crystallographic investigation of vitamin

B₁₂ was started in Oxford in 1949⁽¹⁾ under the

supervision of Dr. R. B. Martin. The molecule contains

a fairly heavy atom, cobalt. The space group for this

compound is still unknown. The molecule is non-symmetric.

It was found that the cobalt atom position

was not given the place of each other

to a small extent of the molecule.

Other work is being done.

Three related compounds were studied.

Brookhead collected three-dimensional intensity

for crystals of the vitamin which had been dried in air.

Similar data were collected by Dr. J. R. Hiltner at

Princeton. These crystals, however, deteriorated in time

owing to loss of solvent of crystallization. In these

Figure I. Formula of Vitamin B₁₂ from Chemical

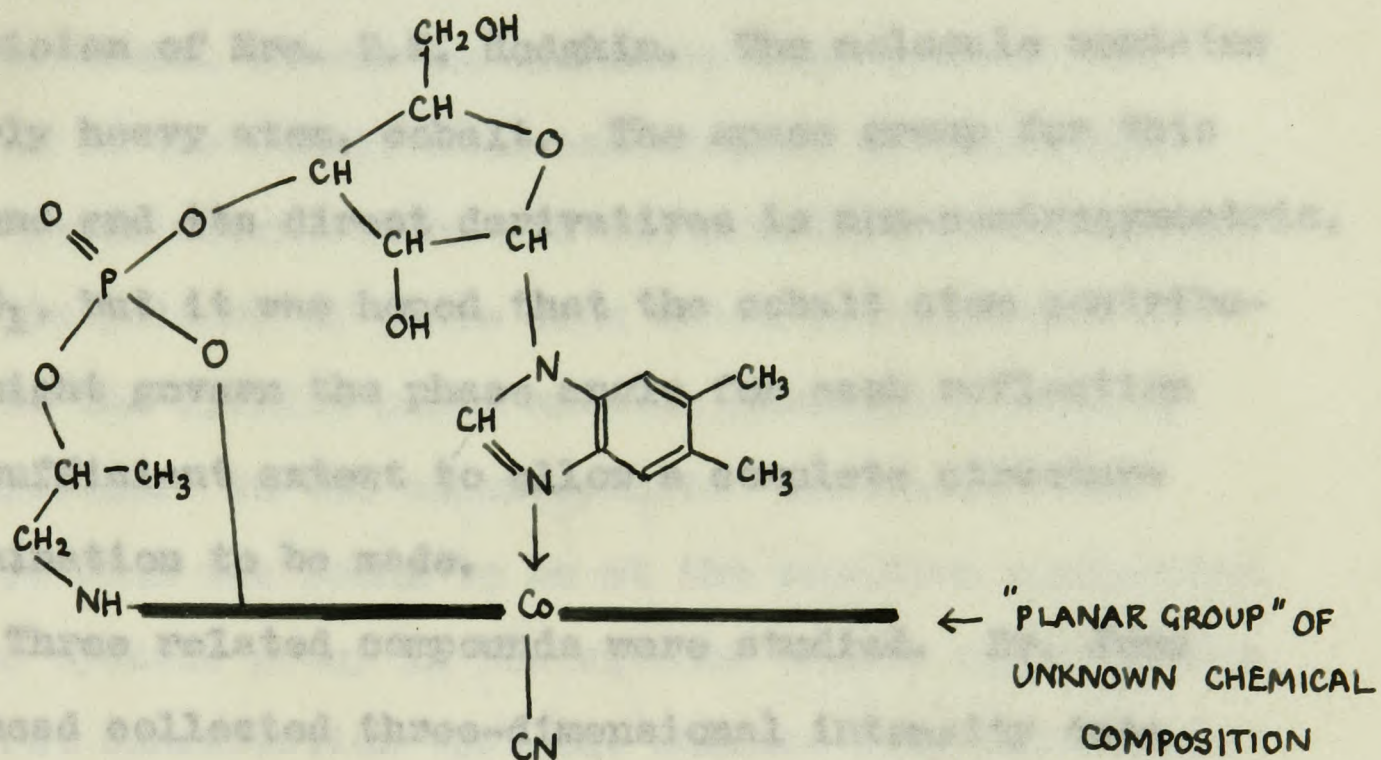
degradation by Degradation by Hydrolysis.

worked on a selenocyanate derivative of the vitamin.

It was hoped that uncertainties of phase determination

from the cobalt atom position alone could be overcome

by the introduction of a second and heavier atom into



(VII)

Crystallographic Studies on Vitamin B₁₂

A crystallographic investigation of vitamin B₁₂ was started in Oxford in 1948¹²⁾ under the supervision of Mrs. D.M. Hodgkin. The molecule contains a fairly heavy atom, cobalt. The space group for this compound and its direct derivatives is non-centrosymmetric, $P2_12_12_1$, but it was hoped that the cobalt atom contribution might govern the phase angle for each reflection to a sufficient extent to allow a complete structure determination to be made.

Three related compounds were studied. Dr. June Broomhead collected three-dimensional intensity data for crystals of the vitamin which had been dried in air. Similar data were collected by Dr. J.G. White at Princeton. These crystals, however, deteriorate in time owing to loss of solvent of crystallisation. Dr. Clara Brink²⁸⁾ therefore collected data for the crystals surrounded by their mother liquor. Dr. J.H. Robertson worked on a selenocyanato derivative of the vitamin. It was hoped that uncertainties of phase determination from the cobalt atom position alone would be overcome by the introduction of a second and heavier atom into

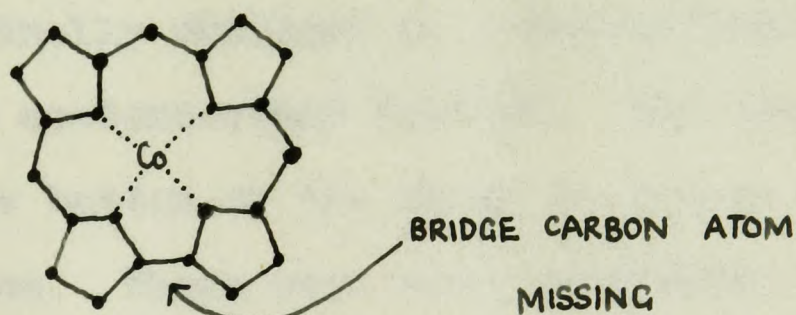
the molecule.

All of the three-dimensional electron density calculations, with phases determined by the heavy atom positions, showed an extremely high peak in the position of the cobalt atom and similar arrangements of low peaks around this. It was possible to recognise from this pattern of peaks some which corresponded to atoms in the cyanide group and the nucleotide part of the molecule. In the selenocyanato derivative the selenium atom was found to be at the position postulated for the cyanide group in the vitamin itself and this was the first confirmation that the choices made for atomic positions were reliable. Dr. J. Callomon²⁹⁾ studied oriented crystals with polarised infra-red radiation and confirmed the approximate direction of the cyanide group in the crystal. More recently Miss M.J. Harrison³⁰⁾ has worked on a vitamin B₁₂-like factor synthesised by micro-organisms in a culture medium containing 5:6-di-chlorobenziminazole instead of 5:6-dimethylbenziminazole. The positions of the chlorine atoms were those expected for such a substitution in isomorphous crystals.

The innermost part of the molecule appeared to be

porphyrin-like in dimensions and nearly planar.

Detailed sorting of peaks, especially from the electron density distribution for the selenocyanato derivative, suggested that this group around the cobalt atom contained four five-membered rings with bridge atoms, as in a porphyrin, but with one bridge atom missing (VIII).



(VIII)

Dr. White had attempted to refine a structure containing a true porphyrin-like arrangement of atoms. In his calculated distributions, however, there was always an anomaly in this region of the direct link which suggested that this porphyrin-like arrangement was incorrect.

When, in October 1953, the red cobalt-containing hydrolysis product was finally obtained in a crystalline

form at Cambridge, a crystallographic investigation was immediately started. The compound had a lower molecular weight than the vitamin and so the cobalt atom should be more phase determining. It was hoped to find the chemical formula of this degradation product and so, possibly, that of vitamin B₁₂ itself.

and hexabasic acids. (A hepta-acid was obtained on acid hydrolysis). The hexa-acid was present in the largest proportion and was finally obtained in a crystalline form from an aqueous acetone-ether mixture. The crystals were deposited on the bottom of the flask in masses with a rock-like appearance. These were sent to Oxford. The preparation of these "rocks" has not since been repeated, and, as the yield was only of the order of micrograms, no chemical analyses could be performed on them.

Fine red needles, which were too small to photograph singly, were obtained in bulk from the mother liquor. Attempts in Oxford to recrystallize some of these slowly in order to obtain larger crystals were unsuccessful and only a heavy mass was obtained. Later, on one occasion at Cambridge, the needles in contact with their mother liquor were converted into prisms.

SOLUTION OF HEXABASIC ACID

Experimental WorkPreliminary Investigation

A deep red cobalt-containing gum was prepared by Dr. J. R. Cannon³¹) at Cambridge by hydrolysis of the vitamin with sodium hydroxide solution (30%) at 150° C for one hour. It behaved as a mixture of tetra-, penta- and hexabasic acids. (A hepta-acid was obtained on acid hydrolysis). The hexa-acid was present in the largest proportion and was finally obtained in a crystalline form from an aqueous acetone-ether mixture. The crystals were deposited on the bottom of the flask in masses with a rock-like appearance. These were sent to Oxford. The preparation of these "rocks" has not since been repeated, and, as the yield was only of the order of micrograms, no chemical analyses could be performed on them.

Fine red needles, which were too small to photograph singly, were obtained in bulk from the mother liquor. Attempts in Oxford to recrystallise some of these slowly in order to obtain larger crystals were unsuccessful and only a gummy mass was obtained. Later, on one occasion at Cambridge, the needles in contact with their mother liquor were converted into prisms.

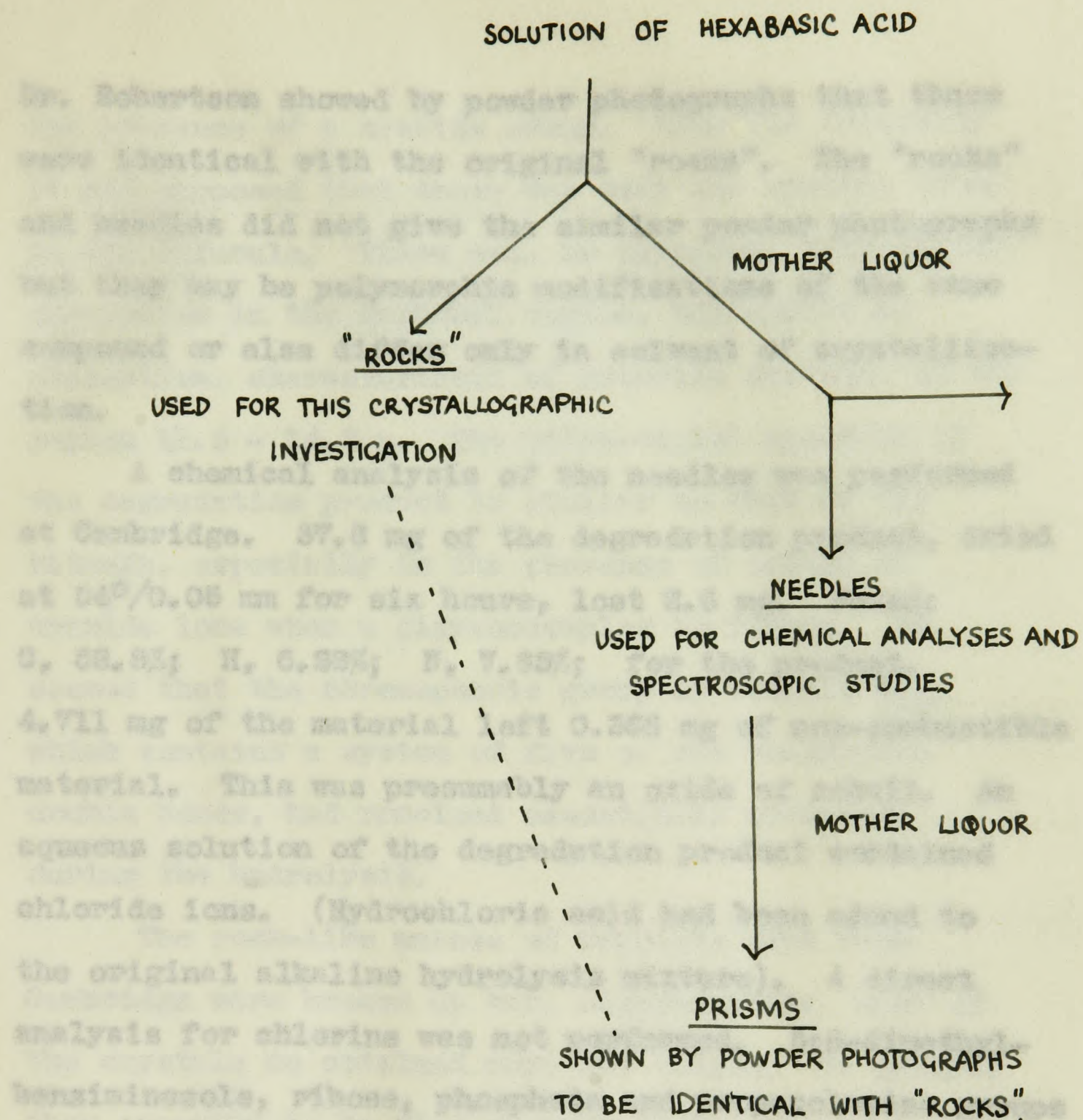


Figure 2. : Crystalline modifications of the
on a "Rufol Red Hexabasic Acid"

Dr. Robertson showed by powder photographs that these were identical with the original "rocks". The "rocks" and needles did not give the similar powder photographs but they may be polymorphic modifications of the same compound or else differ only in solvent of crystallisation.

A chemical analysis of the needles was performed at Cambridge. 37.8 mg of the degradation product, dried at 54°/0.05 mm for six hours, lost 2.6 mg. Found: C, 52.8%; H, 6.23%; N, 7.35%; for the product. 4.711 mg of the material left 0.366 mg of non-combustible material. This was presumably an oxide of cobalt. An aqueous solution of the degradation product contained chloride ions. (Hydrochloric acid had been added to the original alkaline hydrolysis mixture). A direct analysis for chlorine was not performed. 5:6-dimethylbenziminazole, ribose, phosphate and propanolamine groups had been removed during the hydrolysis and the amide groups of the vitamin had been converted to carboxylic acid groups giving a hexacarboxylic acid.

The infra-red spectrum of the needles, determined on a "Nujol" mull, showed a maximum at 4.67μ indicating

the presence of a cyanide group. From its intensity it was supposed that there was only one cyanide group in the molecule. There was, as expected, very strong absorption in the carbonyl region, but almost no absorption characteristic of aromatic systems in the region $11.5 - 14.5 \mu$. The ultra-violet spectrum of the degradation product is similar to that of the vitamin, especially in the presence of excess of cyanide ions when a dicyanocomplex is formed. It seemed that the chromophoric group in vitamin B₁₂, which contains a system of five or six conjugated double bonds, had remained essentially unchanged during the hydrolysis.

The rock-like masses of crystals sent from Cambridge were broken up but, unfortunately, most of the crystals so obtained were not single. In general they appeared to be flat diamond-shaped plates. The one selected for X-ray studies had the dimensions $0.29 \times 0.25 \times 0.043 \text{ mm}$ (figure 3). When a solution of the hexabasic acid was run through an ion-exchange column several bands were obtained. This was, at first, considered to show that the specimen was impure but the

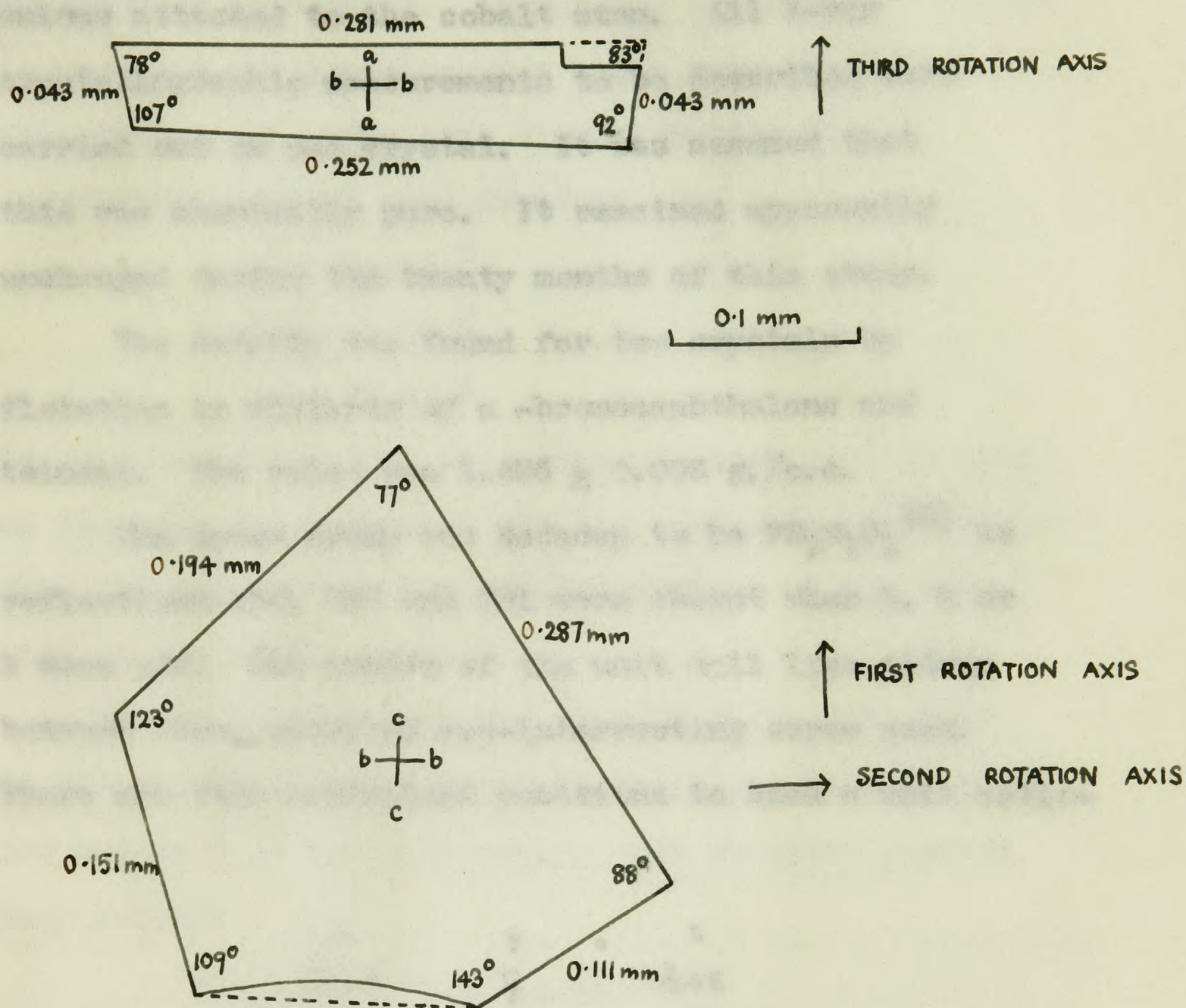


Figure 3. Diagram of Crystal Used for Intensity Measurements

effect is probably due to the ease of exchange of anions attached to the cobalt atom. All X-ray crystallographic measurements to be described were carried out on one crystal. It was assumed that this was chemically pure. It remained apparently unchanged during the twenty months of this study.

The density was found for two crystals by flotation in mixtures of α -bromonaphthalene and toluene. The value was 1.396 ± 0.005 g./c.c.

The space group was deduced to be $P2_12_12_1^{32}$ as reflections $h00$, $0k0$ and $00l$ were absent when h , k or l were odd. The origin of the unit cell lies midway between three pairs of non-intersecting screw axes. There are four equivalent positions in such a unit cell:-

the contents of the unit cell). The analysis of the data then suggest

x	y	z
$\frac{1}{2} - x$	$\bar{y}$	$\frac{1}{2} + z$
$\frac{1}{2} + x$	$\frac{1}{2} - y$	$\bar{z}$
$\bar{x}$	$\frac{1}{2} + y$	$\frac{1}{2} - z$

Symmetrical a-axis oscillation photographs were taken using chromium $K\alpha$ radiation ($\lambda = 2.29 \text{ \AA}$). Reflections with $\theta = 80-90^\circ$ were used to obtain b and

c. A value for "a" was found from the h0l layer line Weissenberg photograph. Copper K α radiation ($\lambda = 1.54$ Å) was used and copper powder lines were superposed on this photograph. The cell dimensions for the degradation product were found to be:

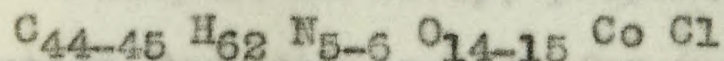
$$a = 24.58 \text{ Å}$$

$$b = 15.52 \text{ Å}$$

$$c = 13.32 \text{ Å}$$

It is interesting that the value of about 16 Å is found in the cell dimensions of all B₁₂ compounds measured so far. It may represent a similar packing of the same part of the planar group.

The cell dimensions and density value give 1072 as the weight of the asymmetric unit (one quarter of the contents of the unit cell). The analysis figures then suggest



as an approximate formula for the dried compound.

Intensity Measurements

There are about 290 atoms, excluding hydrogen atoms, in the unit cell, so that it was most unlikely that the molecular structure could be determined from

work on projections. Three-dimensional intensity data were therefore collected. Multiple-film equi-inclination Weissenberg photographs were taken with filtered copper $K\alpha$ radiation. Exposure times were from 48 to 90 hours for the three more intense photographs. c-axis photographs with l 0 to 8, b-axis photographs with k 0 to 9 and the zero layer line photograph $0kl$ were taken.

c-axis Weissenberg photographs showed reflections to the limit of $\sin \theta$ for the copper $K\alpha$ radiation used. An example is shown in figure 4. b-axis photographs did not show reflections to such high $\sin \theta$ values. Very few extra reflections were obtained from the zero layer a-axis photograph.

Intensities of reflections were estimated visually. A comparison strip, prepared by photographing one reflection from the crystal for varying lengths of time, was used. Dr. Robertson and Mrs. Hodgkin assisted with the tedious task of intensity measurement. The intensities of a few reflections with very low $\sin \theta$ values were measured from a-axis oscillation photographs taken by Dr. Robertson. Of 6360 reflections which were

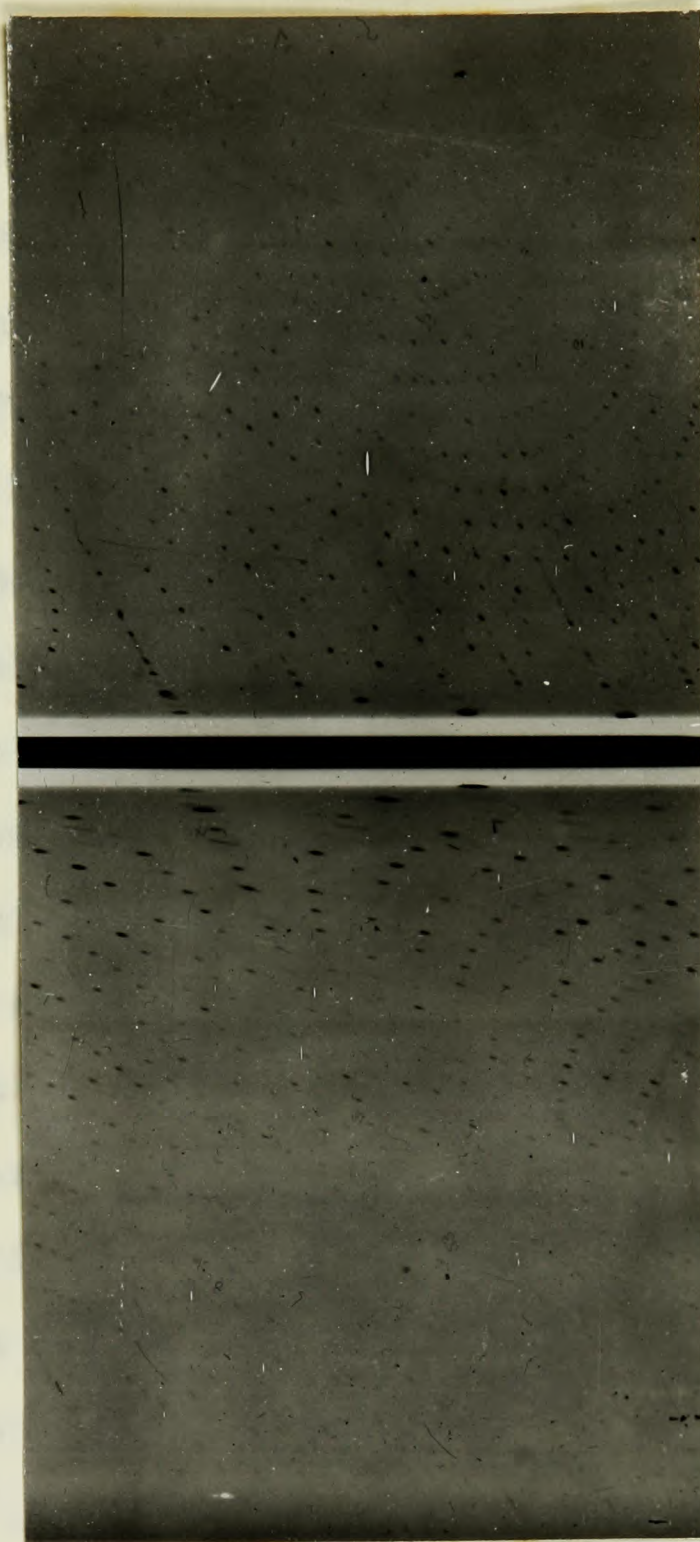


Figure 4 Typical Weissenberg Photograph—hk7

possible when copper $K\alpha$ radiation was used, 3350 were measured. 2550 were too faint on the photographs to be measured. 460 reflections were not photographed but most of these were probably also too faint to be observed. These last were reflections with k greater than 9, l greater than 8 and h not zero.

There was an unexplained intensity anomaly. Some reflections with the same h , k and l indices did not have the same intensities on different sides of the symmetry axis on a Weissenberg photograph. The systematic absences of reflections required by the space group were rigidly obeyed. The effect was probably partly an absorption effect as the crystal was plate-like in shape. Also the absorption edge of cobalt is near the wavelength of the copper radiation used and effects due to the asymmetry of the structure might be expected³³). A third explanation could be that the crystal symmetry is actually lower than orthorhombic. Perhaps, for example, there is a variation in the position of a side chain in the unit cell. The effect was, however, so small that it was neglected in the following analysis. No absorption corrections were made.

All intensities measured were divided by the Lorentz factor and the polarisation factor for the particular $\sin \theta$ value. The function suggested by Cochran³⁴⁾ was used. These values were derived by Mrs. U.K. Ledwith from a reciprocal lattice net. Thus relative values of F^2 were found for each layer line.

Initially corrected intensities for the same reflection from b- and c-axis Weissenberg photographs were compared, but the fall-off in intensity, as $\sin \theta$ increases, is greater for the b- than c-axis photographs. This is equivalent to different effective "temperature factors" for the two sets of photographs. Therefore a statistical method was used to find "temperature factors" and scaling factors in order to convert corrected intensities on each photograph to an absolute scale. The data for each layer line were plotted separately using a method suggested by Wilson³⁵⁾ for two and three dimensional data. Plots for c-axis Weissenberg photographs are shown in figure 5. Reflections which were not observed on a photograph were given a mean intensity of half the thresh-hold value. The "temperature factors", represented by the slopes of the lines in the

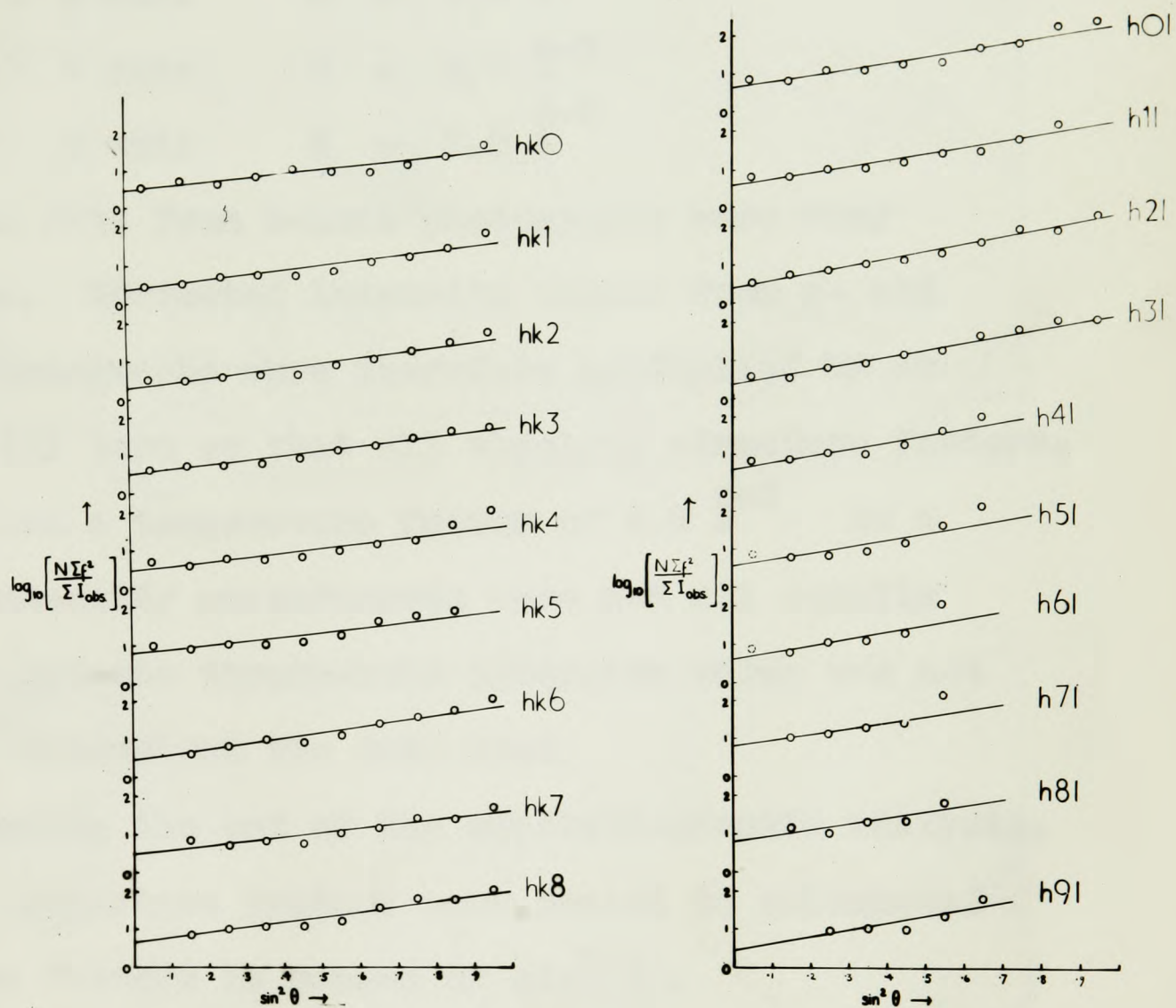


Figure 5.

Temperature and Scaling Factors — Wilson's Method

graphs, were taken to be the same for all layer lines of photographs taken about a given axis. The average "temperature factors", B , were:-

c axis	$B = 3.0 \text{ \AA}^2$
b axis	$B = 4.6 \text{ \AA}^2$
a axis	$B = 5.0 \text{ \AA}^2$

The data from c-axis photographs were very extensive. Corrected intensity values from a- and b-axis photographs were therefore multiplied by an exponential term so that all absolute structure factors, $|F|$, had a temperature factor of $3.0 \text{ \AA}^2$. As a result intensity measurements were not all equally accurate and the threshold intensity value was not constant throughout the data used.

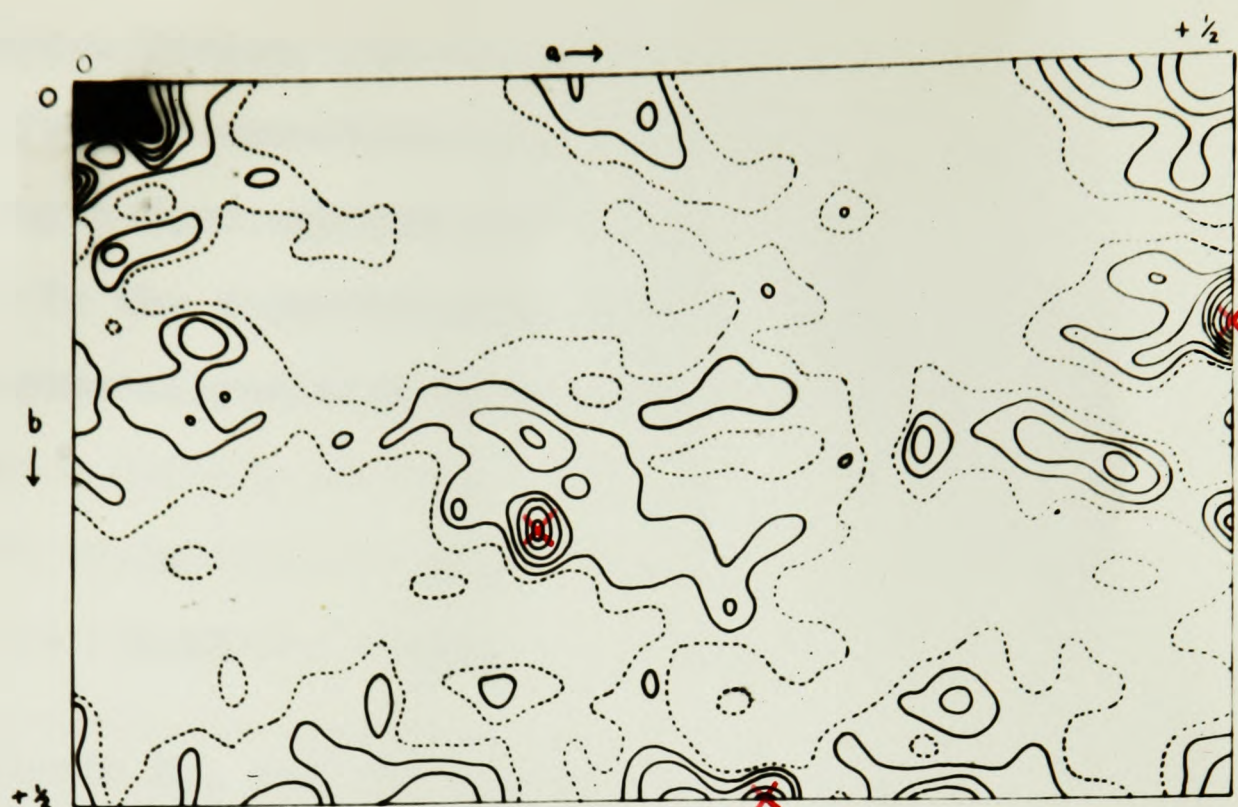
Towards the end of the crystallographic analysis, observed structure factors were scaled to calculated structure factors in ranges of $\sin^2 \theta$.

The Position of the Cobalt Atom in the Unit Cell

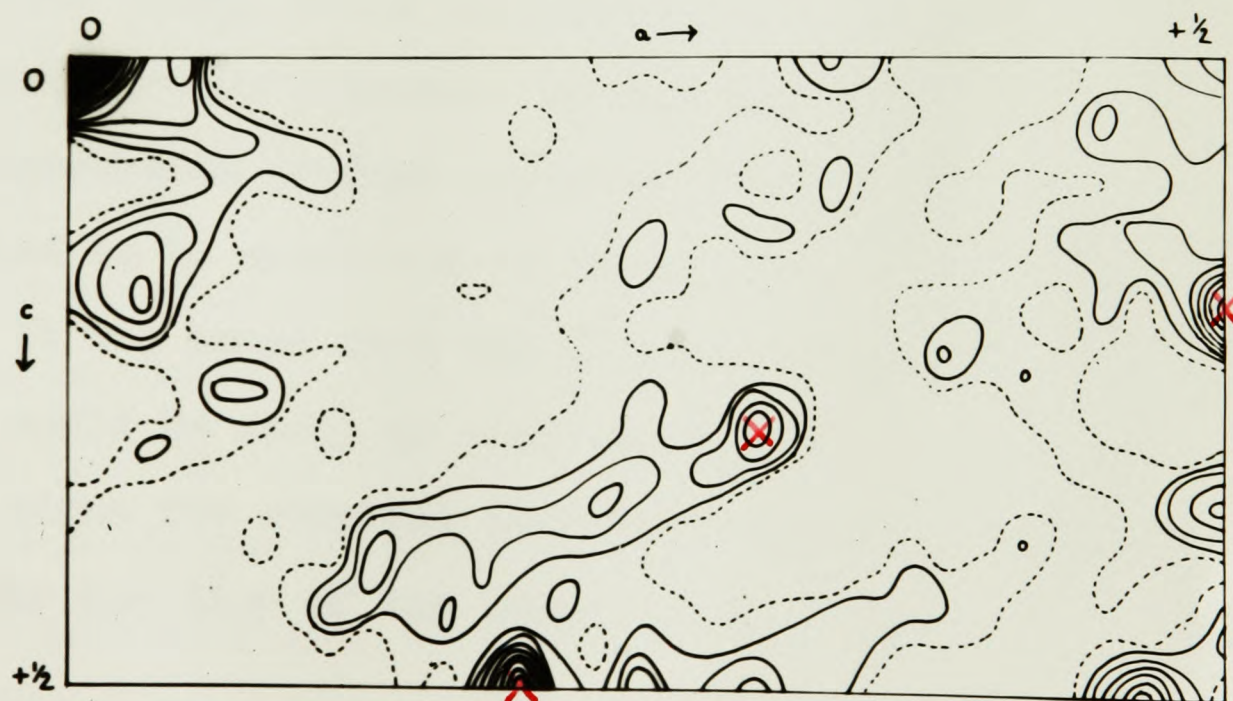
The general position (x, y, z) of the cobalt atom in the unit cell was found by Patterson $hk0$ and $h0l$ projections. The result was later confirmed by Dr. Robertson who calculated a three-dimensional

Figure 6. Patterson Projections.

Co-Co vector peaks



$hk0$ Sharpened Patterson Projection.



$h0l$ Sharpened Patterson Projection.

Figure 6. Patterson Projections.

X

Co-Co vector peaks

Patterson series. In all cases the F^2 values used for the summation were sharpened by multiplying them by a term $\exp(2B \sin^2 \theta / \lambda^2)$.

In the degradation product the cobalt atom is in a general position in the unit cell. Its parameters are:-

$x = 0.15$, $y = 0.16$, $z = 0.10$

In vitamin B_{12} and its direct derivatives the x and z parameters of the cobalt atom are nearly zero.

Therefore three-dimensional electron density distributions for these, based on cobalt atom phases, showed similar spurious effects. In the same calculation for the degradation product spurious effects would be expected to be unrelated to those found for the vitamin.

It was hoped that the orientation of the planar group would be shown by electron density projections using signs for observed structure factors calculated from the position of the cobalt atom. This was not the case.

It has been thought that, for a successful

Three-dimensional electron Density Calculations

The space group of the degradation product is non-centrosymmetric and so a phase angle, α , which may have any value from 0-360°, must be determined for each reflection before a three-dimensional electron density distribution can be calculated. A direct evaluation of α is not practicable but there are two procedures which may be considered in endeavouring to overcome this difficulty. So far the only method which has been used in an attempt to determine α values directly is the study of a pair of isomorphous compounds, as described by Bijvoet et al³⁶⁾. This will limit α to two alternative values. In the present structure analysis too little of the material was available to allow the preparation of isomorphous compounds. Therefore phases calculated from the positions of the cobalt atom were used, as for the vitamin (see page 13), with the hope that at least some of the molecular structure would be indicated in the resulting electron density map. It has been thought³⁷⁾ that, for a successful

Figure 7 indicates the electron density calculations

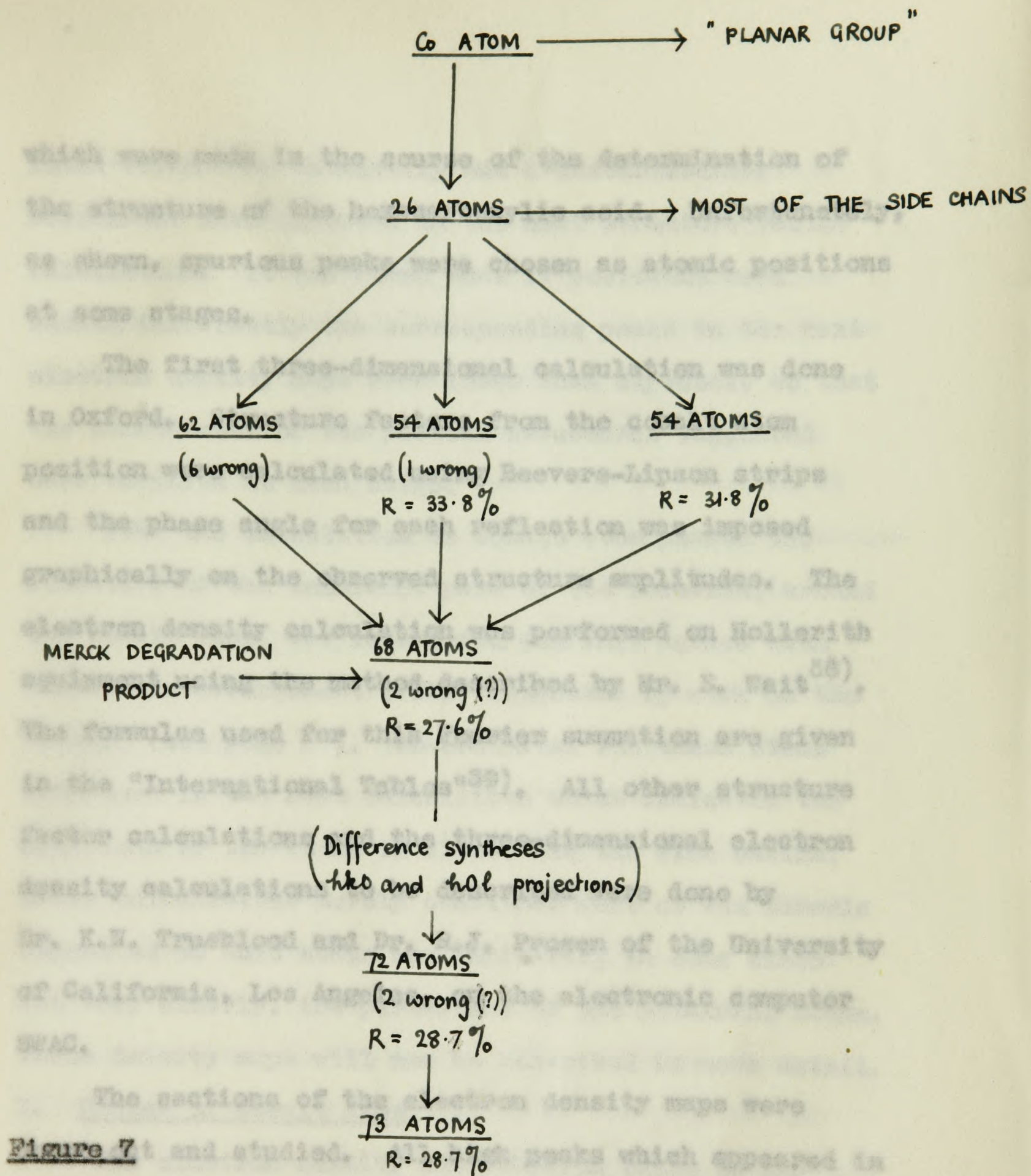
structure determination, even in a centrosymmetric case, the average contributions of the heavy atoms to the intensity of a reflection should be approximately equal to the average contribution of the rest of the molecule. This is certainly not so for vitamin B₁₂ or even the degradation product. For this reason it is surprising that it has been possible to determine the structures of these compounds crystallographically.

Table I

Values of $\sum f^2$

	Degradation Product	Vitamin B ₁₂	B ₁₂ seleno-cyanide
Heavy atoms (Co and Se)	729	729	1885
Rest of structure (one asymmetric unit)	3505	5469	5469

As the chemical structure of the compound was unknown the idea of introducing only a partial structure into the phase angle calculation was, of necessity, continued and, at each stage in the refinement, only positions of peaks which were chemically and stereochemically reasonable were included in the calculation. Figure 7 indicates the electron density calculations



Atoms included in the Phase Angle Calculation at Each Stage in the Three-dimensional Fourier Refinement.

which were made in the course of the determination of the structure of the hexacarboxylic acid. Unfortunately, as shown, spurious peaks were chosen as atomic positions at some stages.

The first three-dimensional calculation was done in Oxford. Structure factors from the cobalt atom position were calculated using Beevers-Lipson strips and the phase angle for each reflection was imposed graphically on the observed structure amplitudes. The electron density calculation was performed on Hollerith equipment using the method described by Mr. E. Wait³⁸). The formulae used for this Fourier summation are given in the "International Tables"³⁹). All other structure factor calculations and the three-dimensional electron density calculations to be described were done by Dr. K.N. Trueblood and Dr. R.J. Prosen of the University of California, Los Angeles, on the electronic computer SWAC.

The sections of the electron density maps were drawn out and studied. All high peaks which appeared in addition to those included in the calculation were considered to indicate possible positions for atoms, and

those which were chemically and stereochemically reasonable were included in the next structure factor calculation. It was found that if positions were chosen incorrectly the corresponding peaks in the next electron density maps were lower than expected, so that the correctness of the partial structures suggested were verified at each stage.

From the calculation on cobalt atom phases the structure of the innermost part of the molecule, around the cobalt atom, was determined and this agreed with the atomic arrangement (VIII) suggested by work on the vitamin (see page 15.). Co-ordinates for these atoms were used in the next calculation which indicates the positions of the atoms in almost all the side chains. Later calculations merely confirmed most of the formula suggested at this stage and indicated, in some cases not very clearly, the parameters of the remaining atoms. These density maps will now be described in more detail.

1. Phases of Cobalt Atom

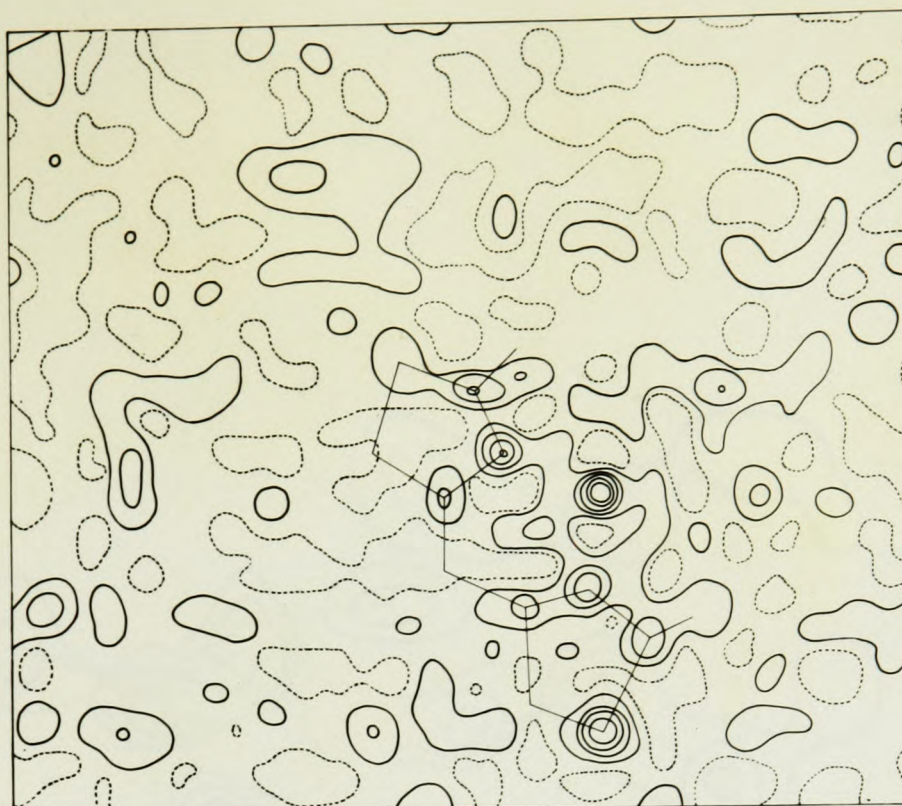
The electron density distribution based on the phases of the cobalt atom is dominated, like similar in calculations for vitamin B₁₂, by a high peak in the

position of the cobalt atom with strong diffraction maxima and minima around it. There were many low peaks around this, some representing atomic positions and others which were spurious. The appearances of two sections of the calculation are shown in figure 8.

While, in general, throughout this density map, there were too many spurious peaks for a selection of correct positions to be made, in the immediate neighbourhood of the cobalt atom the choice was more limited. There were four peaks, approximately $1.9 \text{ \AA}$ from the cobalt atom and, leading out from these, the positions of other atoms in four five-membered rings could be found using solely the condition that successively linked atoms should be about $1.5 \text{ \AA}$ apart and have a reasonable stereochemical relationship to one another. Two of these five-membered rings were directly linked together. Peaks representing atoms bridging two rings were quite clear in two cases. The position of the third bridge atom was doubtful since the peak at the required geometrical site was only a protuberance from the diffraction maximum. This "planar group", shown in figure 9, was therefore similar in form to that

Figure 8. Two sections of electron density calculation based on cobalt atom position.

Figure 8. Two sections of electron density calculation based on cobalt atom position.



B₁₂ Fragment. Section at $x=7$, 1954.



B₁₂ Fragment. Section at $x=13$, 1954.

Figure 8. Two sections of electron density calculation
based on cobalt atom phases.
(Interval $1e/A^3$. Zero level dotted)

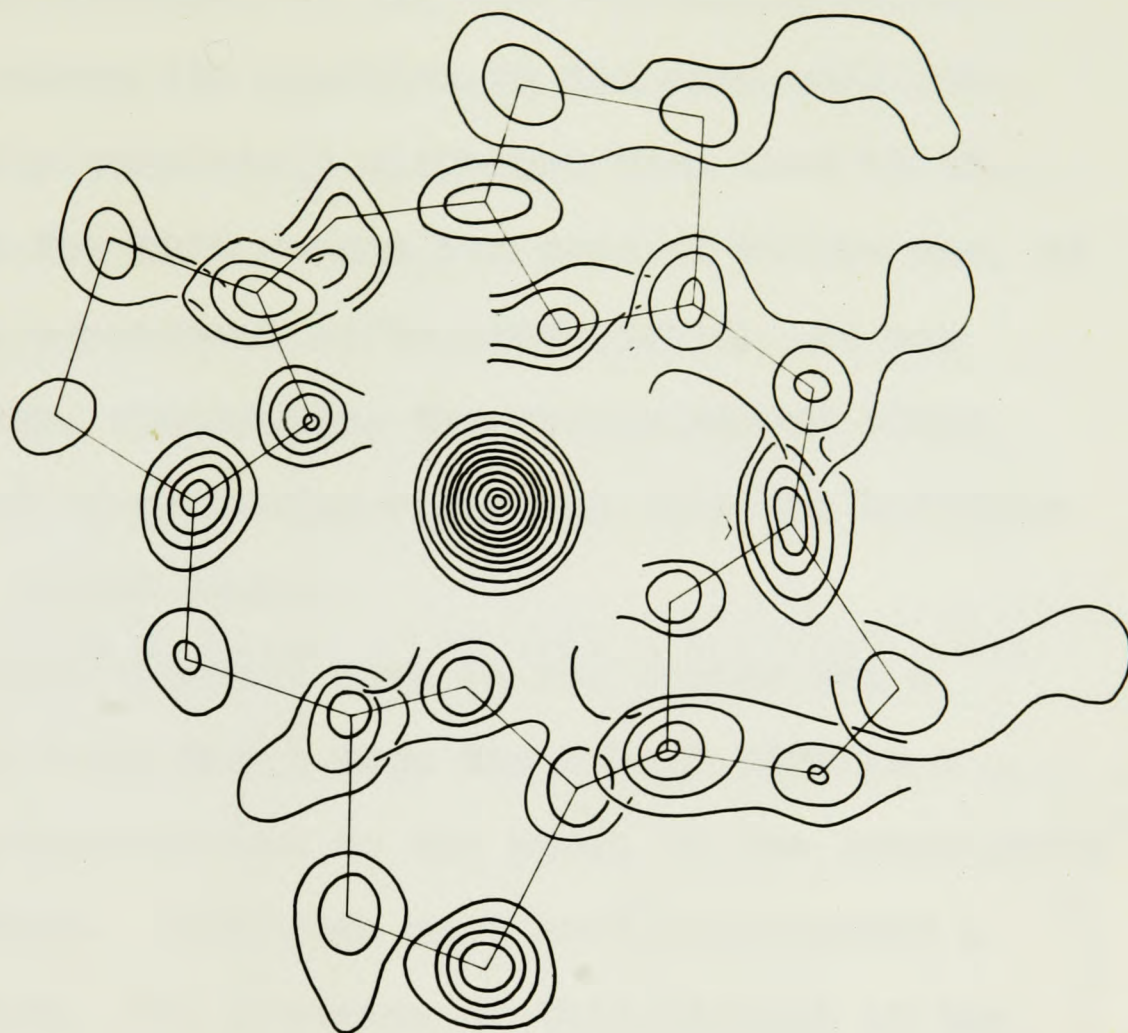


Figure 9.

B₁₂ Fragment. Planar Group June 1954

(Calculation on phases of cobalt atom)

Contours at $1e/A^3$ interval except at cobalt atom.

suggested by work on the vitamin, with some doubt only about the existence of the last bridging atom.

The group as a whole was not quite planar. It did not lie parallel to any of the sides of the unit cell but lay inclined to all the crystallographic axes. Therefore its position in the unit cell was geometrically completely different from that in the vitamin and for this reason its general nature was, at this stage, considered to be established. It was supposed that, chemically, the five-membered rings were pyrrole or pyrroline rings and that the bridging atoms were carbon atoms.

Table 2 Peaks in the calculation on Cobalt Atom

The second highest peak in the series was a distance of about $2.3 \text{ \AA}$ from the cobalt atom in a direction perpendicular to the plane of the large group just described. This peak must have represented a chlorine atom. The presence of this element in the degradation product was chemically very uncertain. The atoms of the cyanide group, possibly present from the spectroscopic evidence, were not very clearly resolved. The position of the nitrogen atom was best shown by the projection on (001) based on signs from the cobalt atom. The next structure factor and electron density calculation which was performed in California. The general

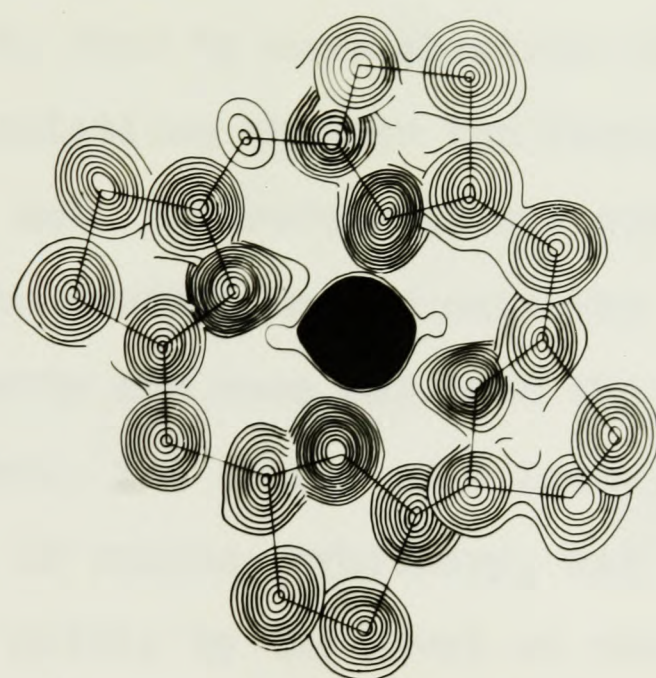
appearance of the second three-dimensional electron density distribution was very different from that of the first. Atoms put into the phase angle calculation appeared with approximate peak heights:-

Co	61.7 e/A ³	
Cl	31.2 e/A ³	
N	11.6 e/A ³	
C	8.6 e/A ³	average values

The appearance of the "planar group" is shown in figure 10. The atoms are all well-defined and atoms put into the calculation with the same atomic number appear as peaks with about the same heights. Some atoms moved slightly from the positions in which they had been put but these movements were less than 0.2 Å (see table 10, page 74). The diffraction rings round the cobalt atom had greatly diminished in height and a peak of height 2.8 e/A³ appeared in the geometrical position expected for the third bridge atom. Its existence was therefore considered to be established.

There remained several round, well-defined peaks with heights up to 4.4 e/A³. These generally appeared in positions which corresponded to a possible stereo-

based on phases of 2b atoms.



26 atoms.

Contours of atoms in the planar group. Each atom drawn in the section nearest its centre. Interval $1e/\text{\AA}^3$. Zero contour omitted.

Figure 10. "Planar group" in electron density calculation based on phases of 26 atoms.

chemical and chemical formula. The appearances of some of these peaks are shown in figure 11. At this stage almost the entire molecular formula (IX), shown in figure 12, was deduced from the positions of these peaks.

Although the chemical nature of most of the molecule had, at this stage, been guessed, it was necessary to find by more electron density and structure factor calculations whether the formula suggested was correct or not. Therefore it was considered best to proceed with caution and so only the highest peaks found in the density map were put into the next round of calculations. The choice of a lower limit of peak height is, of course, arbitrary, and here it was determined mainly by the level at which peaks did not always seem to be chemically significant. All peaks over $2.5 \text{ e}/\text{\AA}^3$ in height, 28 in all, were included in the next phase angle calculation and these atoms were given a mean atomic number of seven.

The Appearance of Some Side Chains in the
2b Atom Fourier

(Interval $1\text{\AA}/\text{\AA}^2$)

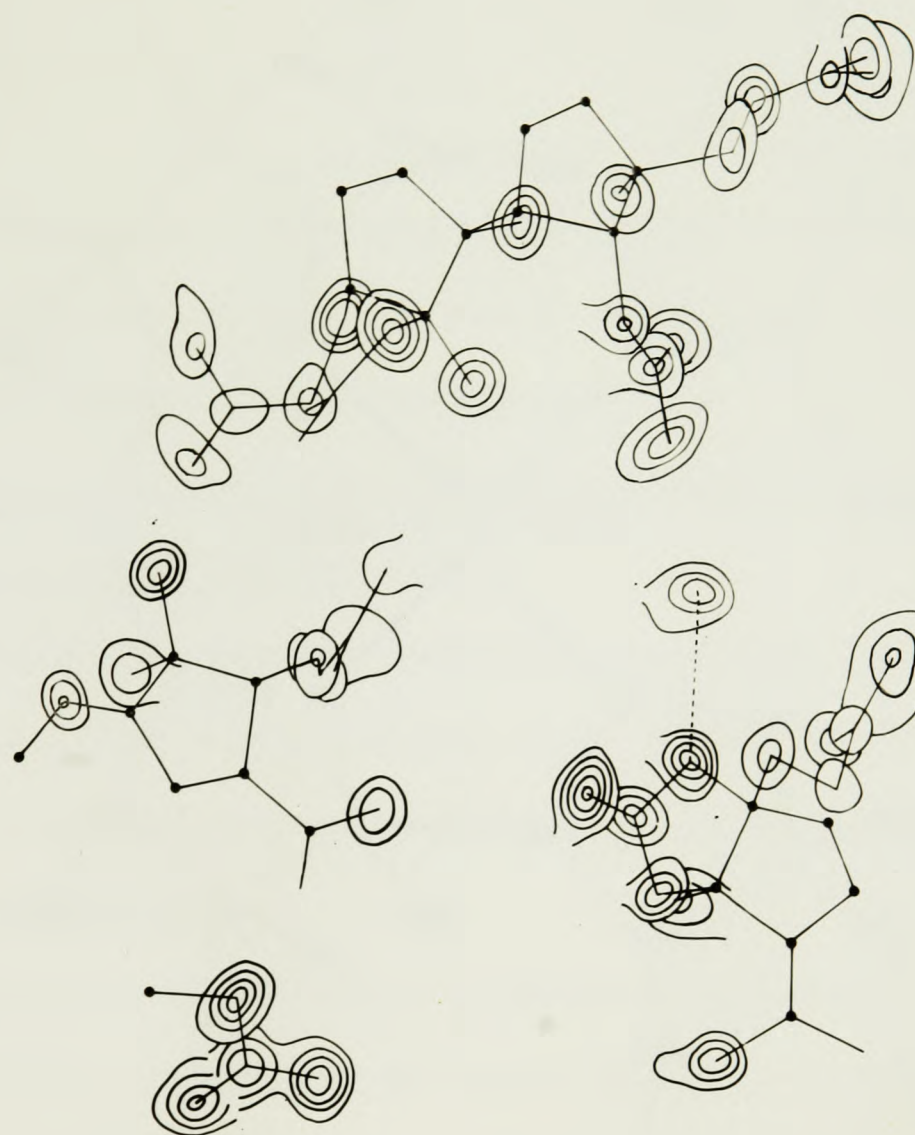


Figure II.

The Appearance of Some Side Chains in the
26 Atom Fourier.

(Interval $1e/A^3$)

Table 3 Peaks in the Calculation on Phases of
28 Atoms

Height range e/A^3	Number found	Number in final total structure
4.5 - 5.5	28	28
2.5 - 3.5	15	15
1.5 - 2.5	55	10
	91	47

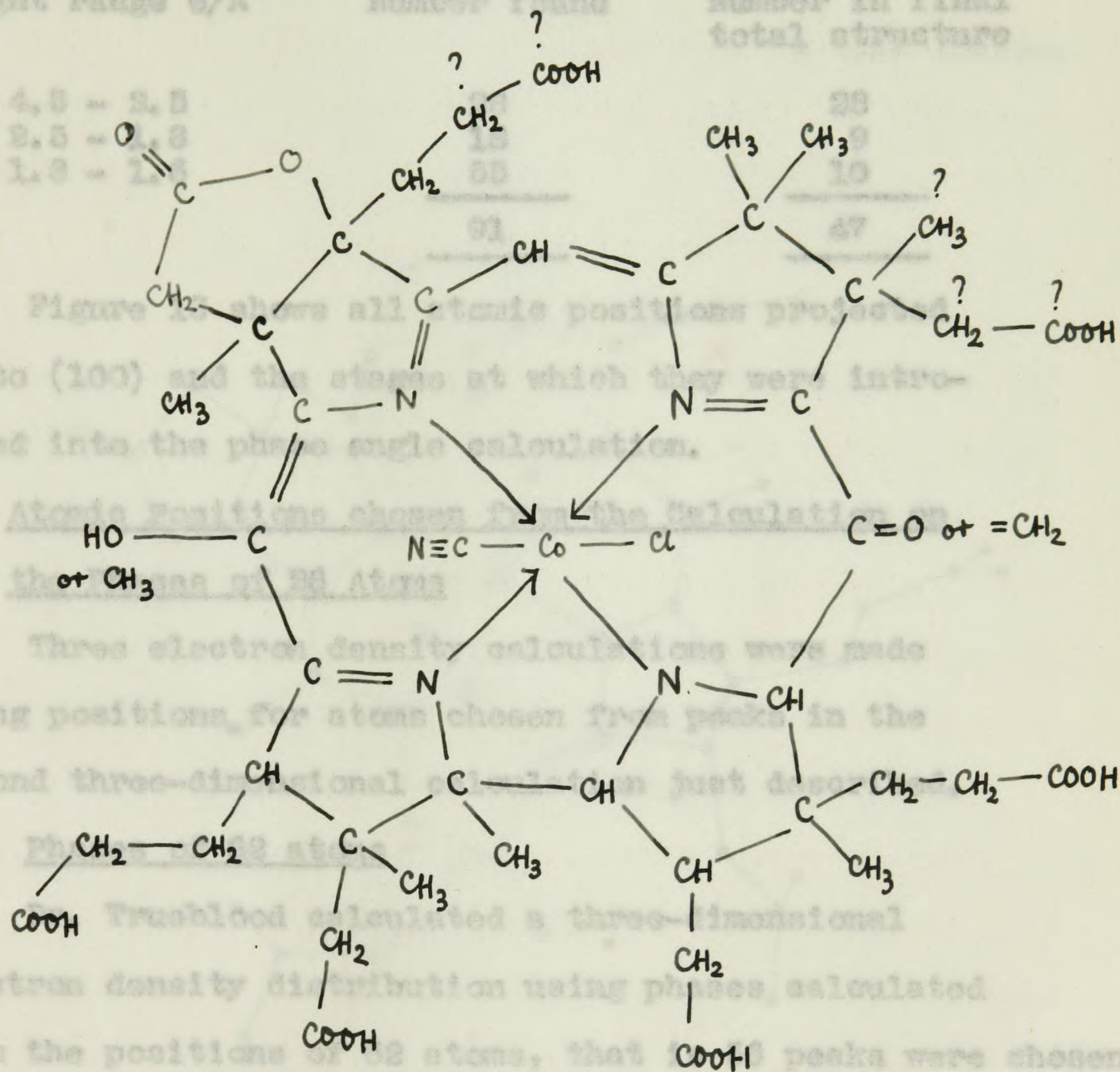
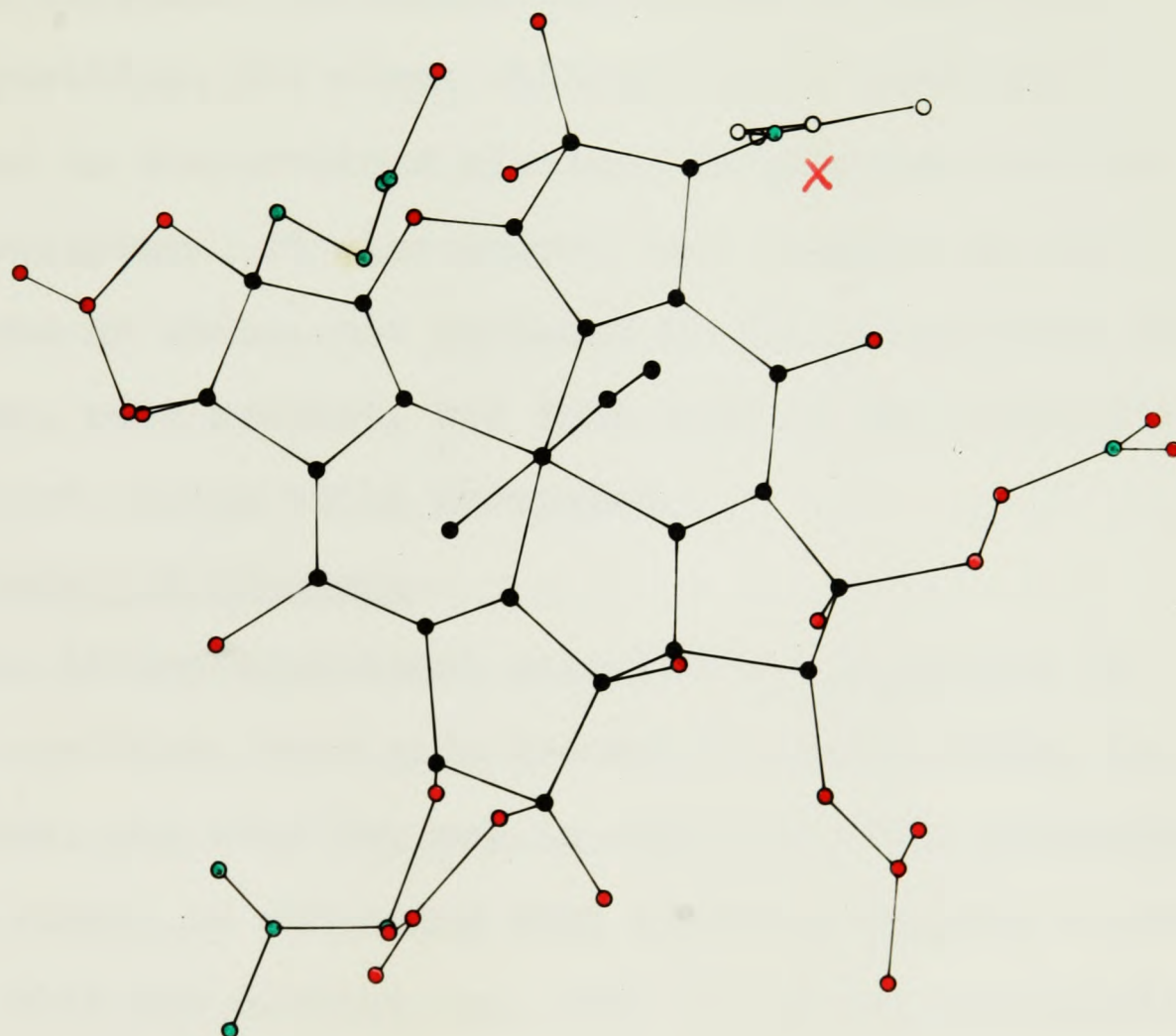


Figure 12

Chemical Formula Tentatively Suggested for the
Degradation Product. September 1954

KEY:

NEW ATOMS	IN THE	PHASE	ANGLE	CALCULATION
●	26			
●	54			
●	68			
○	LATER	CALCULATIONS		



Vitamin B₁₂ Fragment : February 1955.

Figure 13. Atomic positions projected on to (100)
and the calculations in which they were used.

chose (below the $2.5 \text{ e}/\text{\AA}^3$ level, of course) were incorrect. For instance, one atom was put in the middle of a five-membered ring on a diffraction peak there. This calculation was very useful, however, for several reasons. It showed the result of choosing wrong positions for atoms although small peaks had appeared in the previous electron density map in those very positions. It also showed that several of the positions he chose, not included in the calculation for 54 atoms, were correct, and that some of the peaks that he did not choose still persisted.

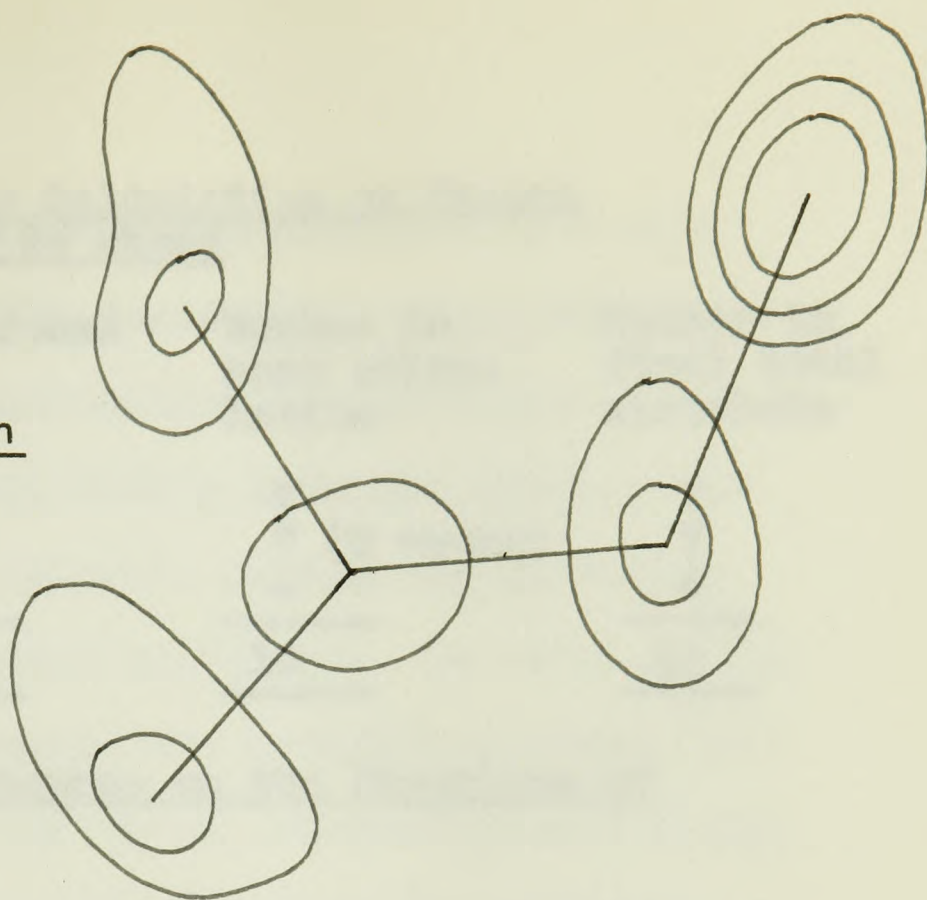
b) Phases of 54 atoms

Two three-dimensional calculations involving 54 atomic positions were made because, unfortunately, in the first, one atom was put in with one wrong parameter. It was found, on comparing this electron density distribution with the correct one, that there was very little difference in the appearances of the two density maps except in the regions directly affected, namely, the region into which the atom had been wrongly placed and the region in which it should have been put. These two areas are shown in figure 22 (see page 70).

It had been hoped that, as more atoms were included in the phase angle calculation, the peaks in the positions of the remaining atoms would become progressively higher in the stages of refinement. Even from the 62 and 54 atom calculations it became evident that this would not be the case. Figure 14 shows that, even when one atom in a side chain is included in the calculation the heights of peaks due to the other atoms are scarcely affected. On the other hand all atoms, once put correctly into the calculation, appeared with about the same peak height. The atoms left out of the calculation may have contributed mainly to certain reflections and their contributions to these were ignored by incorrect phasing. There may also have been a certain amount of disorder in the positions of these atoms and it may be significant that, as shown by figure 13, the positions of atoms in the longer side chains, were the last to be found. It was disappointing that the final complete structure was not obvious from the (correct) calculation on phases of 54 atoms.

Figure 14. A side chain in two calculations

26 atom calculation



54 atom calculation

(X included in the phase angle calculation)

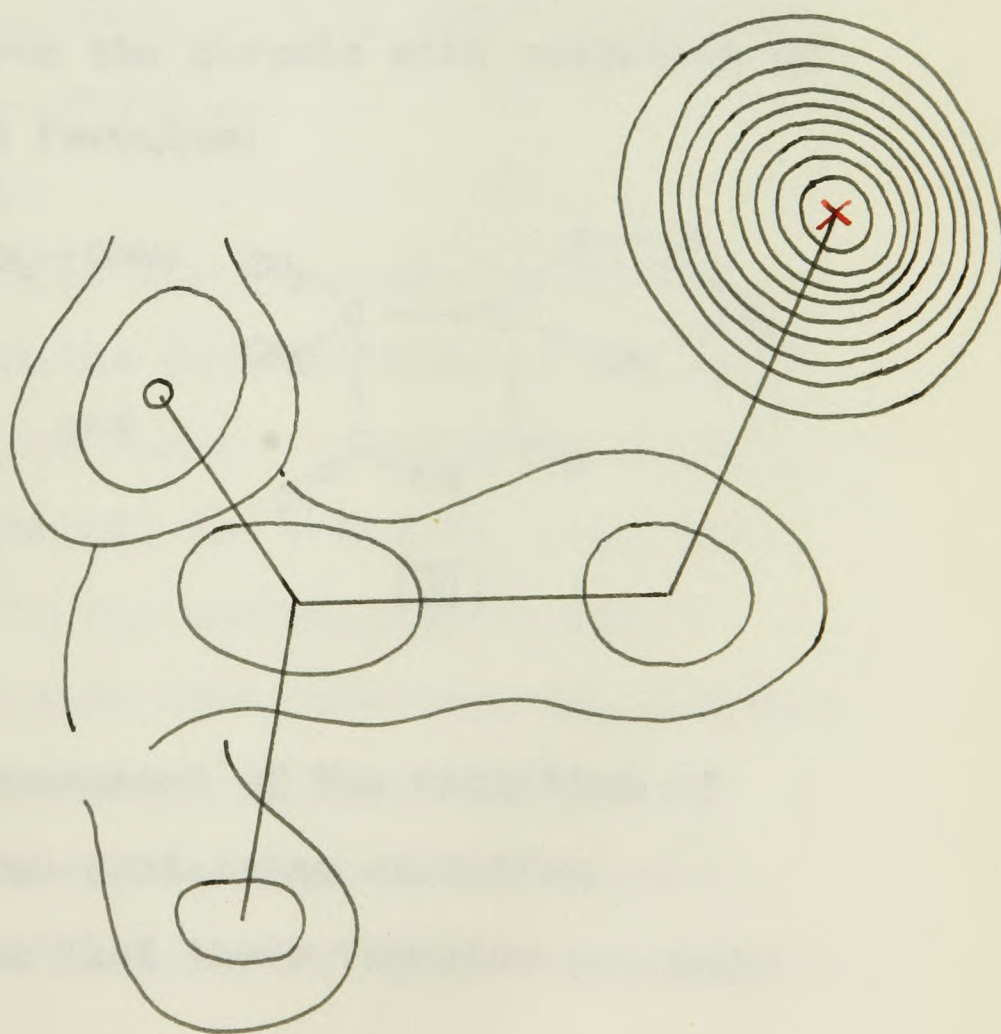


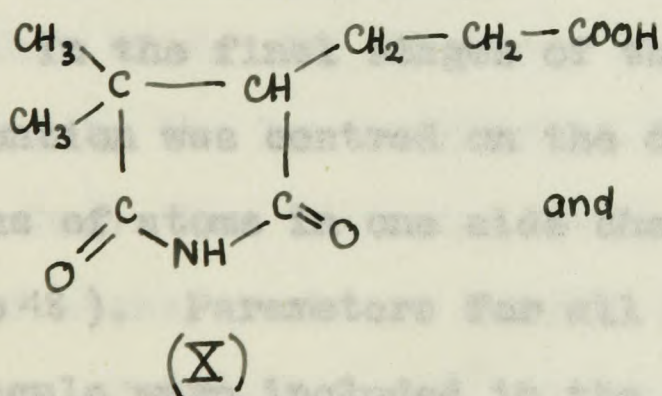
Figure 14. A side chain in two calculations.

Table 4. Peaks in the Calculation on Phases of 54 atoms

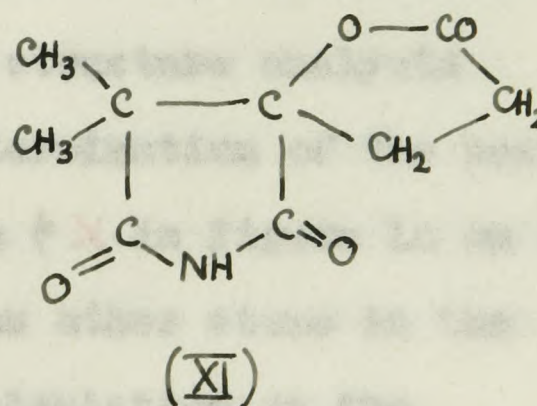
Height range $e/\text{\AA}^3$	Number found	Number in next calcu- lation	Number in final total structure
3.8-2.0	7	7	7
2.0-1.7	13	7 (2 wrong)	7
1.7-1.3	54	-	5
	<u>74</u>	<u>14</u>	<u>19</u>

4. Further Chemical Evidence on the Structure of Vitamin B₁₂

In January, 1955, Folkers and his co-workers at Merck Laboratories⁴⁰⁾ announced the isolation of two degradation products from the chromic acid oxidation of vitamin B₁₂. These had formulae:



and



This was the first announcement of the isolation of large identified nitrogen-containing oxidation products and it was seen that their formulae are con-

sistent with possible degradation products of a compound with the formula (IX) tentatively suggested for the cobalt-containing degradation product (see page 46). It was assumed that a hydroxyl group could be introduced into the molecule during the oxidation and so a lactone ring could be formed. It was a little ironic that these compounds were probably derived from the five-membered ring for which, crystallographically, it was most difficult to find the long side chain. The announcement of the isolation of these compounds at this stage showed the validity of this crystallographic method of structure determination.

5. Phases of 68 atoms

In the final stages of the structure analysis attention was centred on the determination of the positions of atoms in one side chain (X in figure 13 on page 48). Parameters for all the other atoms in the molecule were included in the calculation on the phases of 68 atoms. By this time, however, the chemical nature of this last side chain — a propionic acid group —, was known from the formulae of the succinimide derivatives (X and XI, see page 52). Very recently,

Fourier refinements for vitamin B₁₂ have confirmed this and have also indicated its stereochemical relationship to the planar group. All this had been guessed previously from the nature of the rest of the molecule.

There are several peaks in this area, as shown in one three-dimensional section in figure 15 (A and B), and it is difficult to choose the correct ones. It is, of course, not impossible for a disordered state to exist here. Three three-dimensional calculations involving phases of 62 atoms, 68 atoms and 72 atoms have been made in which atoms, corresponding to a possible side chain, have been put in various positions in this area. In all these cases the positions were shown, by the low heights of the resulting peaks, to be wrong. For instance, in the calculation with phases of 68 atoms, peaks of heights $5.6 \text{ e}/\text{\AA}^3$, compared with values of $6.8\text{--}10.9 \text{ e}/\text{\AA}^3$ for all other such peaks, appeared in the positions chosen for two atoms. One of these peaks is shown in figure 15 (D) and the positions which were finally suggested, are indicated. For a structure determination of this kind such three-dimen-

(contour interval $1 \text{ e}/\text{\AA}^3$)

x Atom included in phase angle calculation.

x Position suggested for 72 atom calculation.

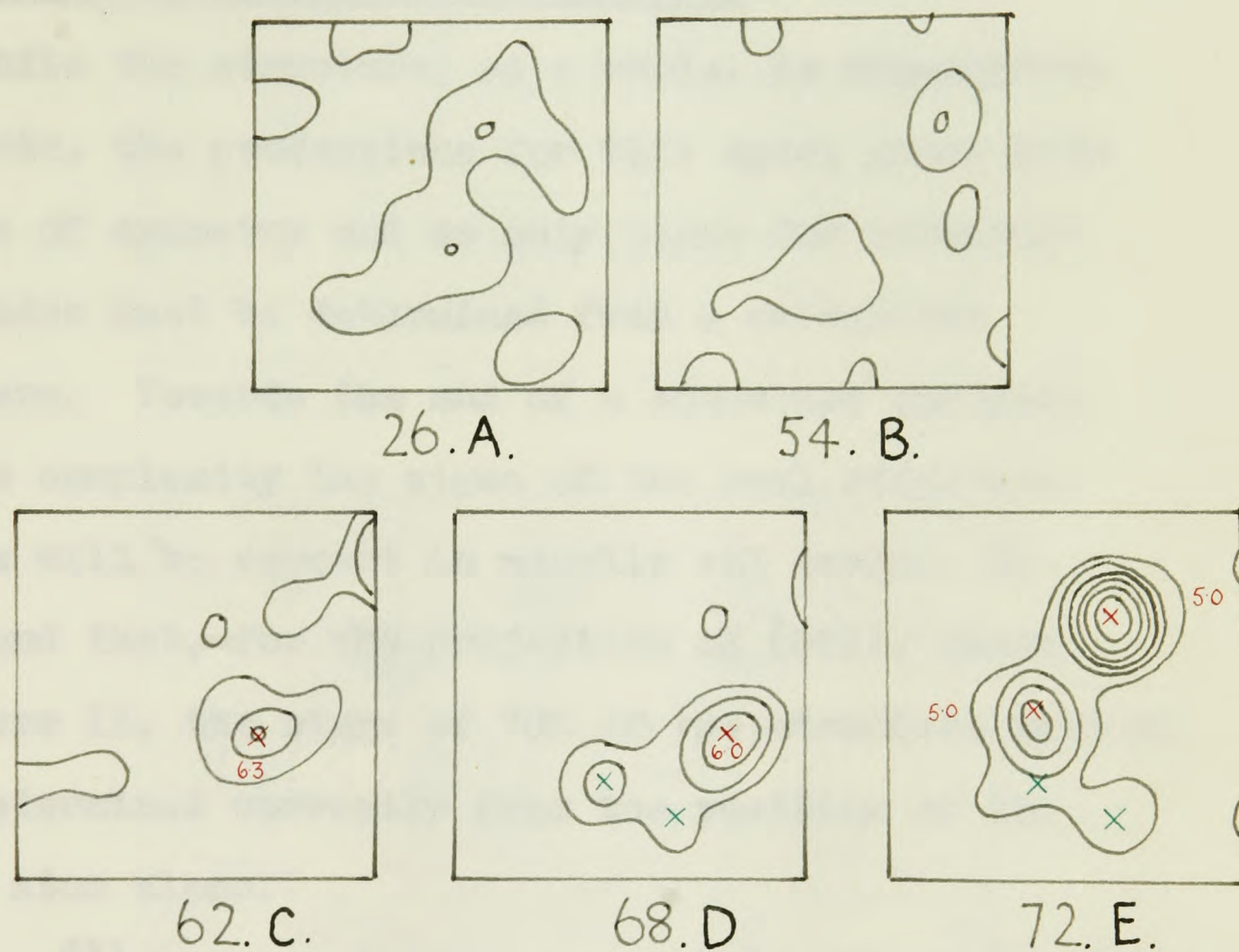


Figure 15. Sections at $x=5$ in region of

last side chain.

(contour interval $1 e/A^3$)

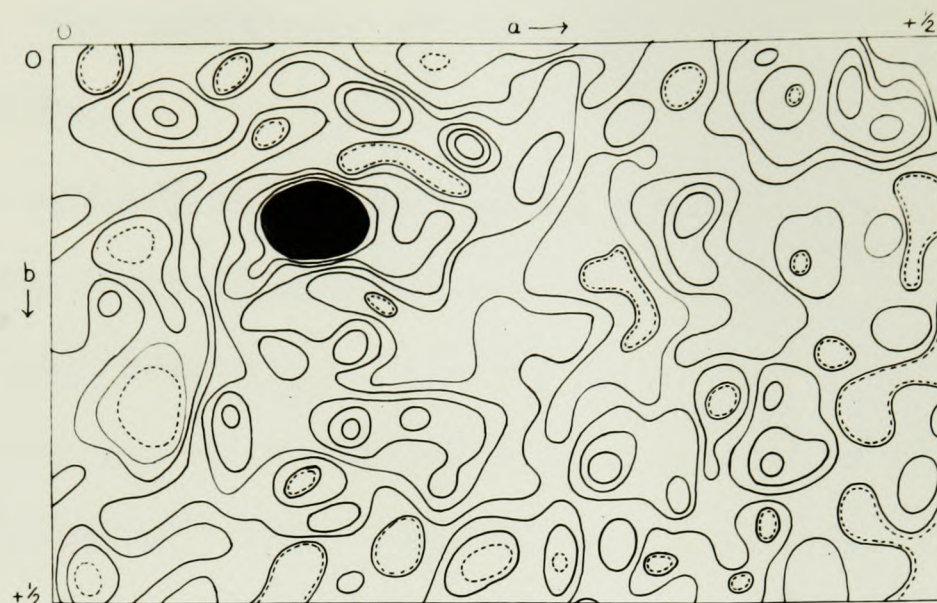
- × Atom included in phase angle calculation.
- × Position suggested for 73 atom calculation.

sional trial-and-error methods would not be generally practicable as the power of the result does not warrant such lengthy methods of calculation.

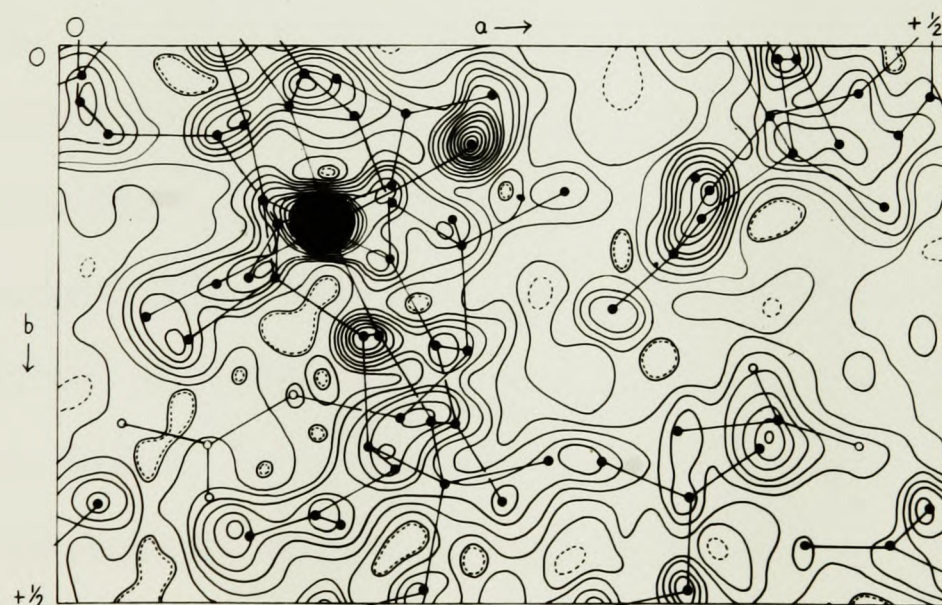
6. The Use of Difference Syntheses in the Final Stages of the Structure Analysis

While the structure, as a whole, is non-centrosymmetric, the projections for this space group have centres of symmetry and so only signs for structure amplitudes must be determined from a calculated structure. Towards the end of a structure analysis of this complexity the signs of the real structure factors will be correct in nearly all cases. It was found that, for the projection on (001), shown in figure 16, the signs of 70% of the structure factors were determined correctly from the position of the cobalt atom alone.

Bunn⁴¹) used an "error synthesis" with coefficients $(F_{\text{obs}} - F_{\text{calc}})$ to indicate discrepancies between the real and postulated structure of sodium benzylpenicillin. He used terms for which F_{obs} is small and F_{calc} large. In the later stages of a Fourier refinement of a structure all terms may be used. Cochran⁴²) called this



$hk0$ projection. Signs from cobalt atom position.



$hk0$ projection. Signs from positions of 68 atoms.

Interval of contours: $2e/\text{\AA}^2$

Figure 16. Projections on (001)

Scale for 54 atoms.

a "difference synthesis" and used it to show, very elegantly, the positions of hydrogen atoms in adenine hydrochloride.

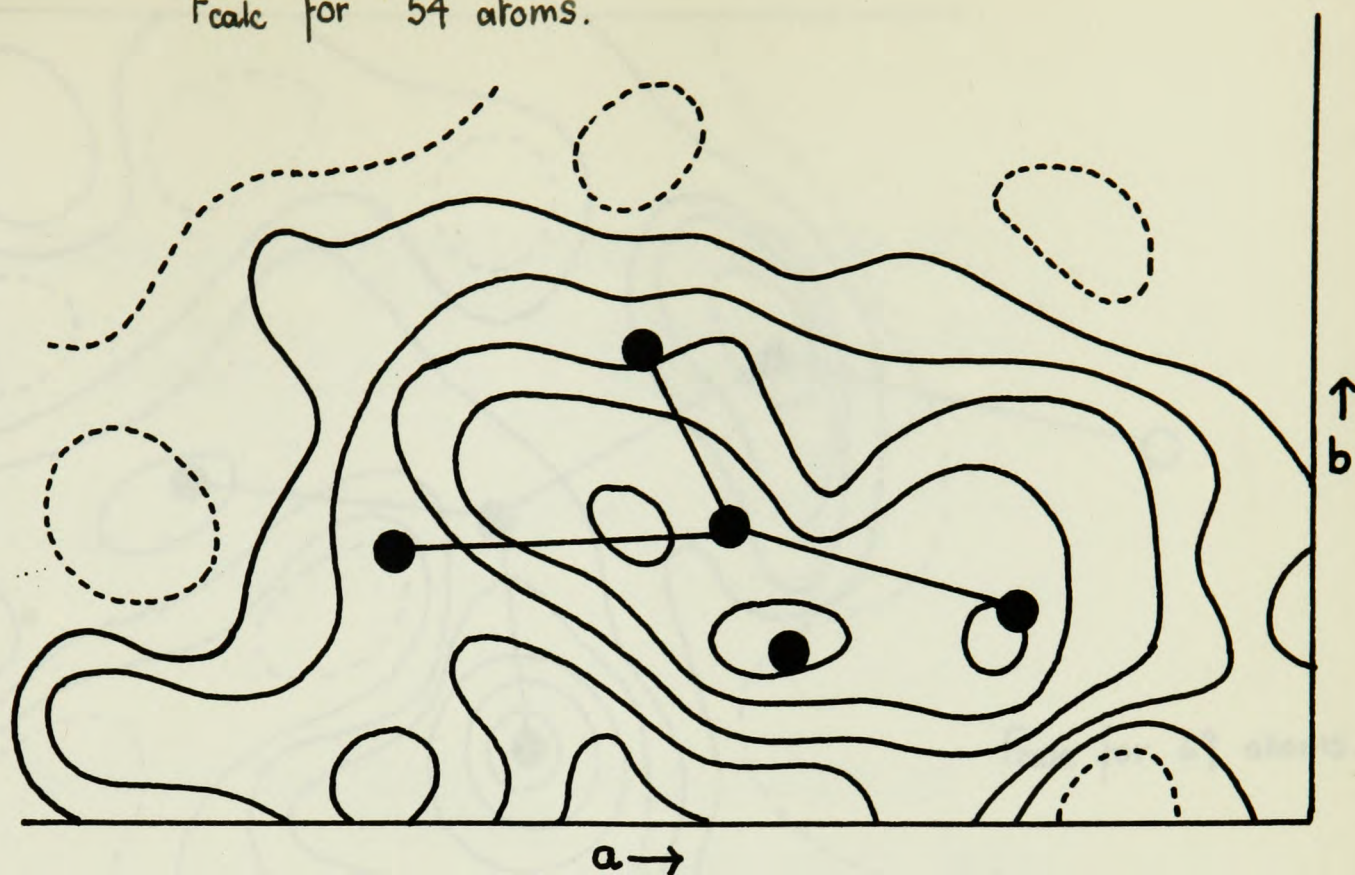
Such methods were used in an attempt to locate atoms in the last side chain in the degradation product of vitamin B₁₂. In earlier stages of the refinement atoms of a molecule of acetone and water of crystallisation were indicated by difference Fourier, as shown in figure 17. Peaks appeared in the three-dimensional maps in positions with these parameters. In all cases only reflections with observed intensities were used. The appearances of difference syntheses in the region of the last side chain are shown in figure 18. The x and y parameters of the atoms were indicated clearly but the z parameters initially chosen were shown, by a three-dimensional electron density calculation on phases of 72 atoms, to be incorrect. Revised co-ordinates, differing only in z parameters, were chosen from these three-dimensional maps, but the agreement between calculated and observed structure factors was no better in the resulting calculation. It seems that there is definitely some disorder here, possibly caused by the swinging of the $-CH_2-COOH$ part of the group in the space available.

(Contour interval

1x/2

Shaded atoms not indicated in figure)

F_{calc} for 54 atoms.



F_{calc} for 68 atoms.

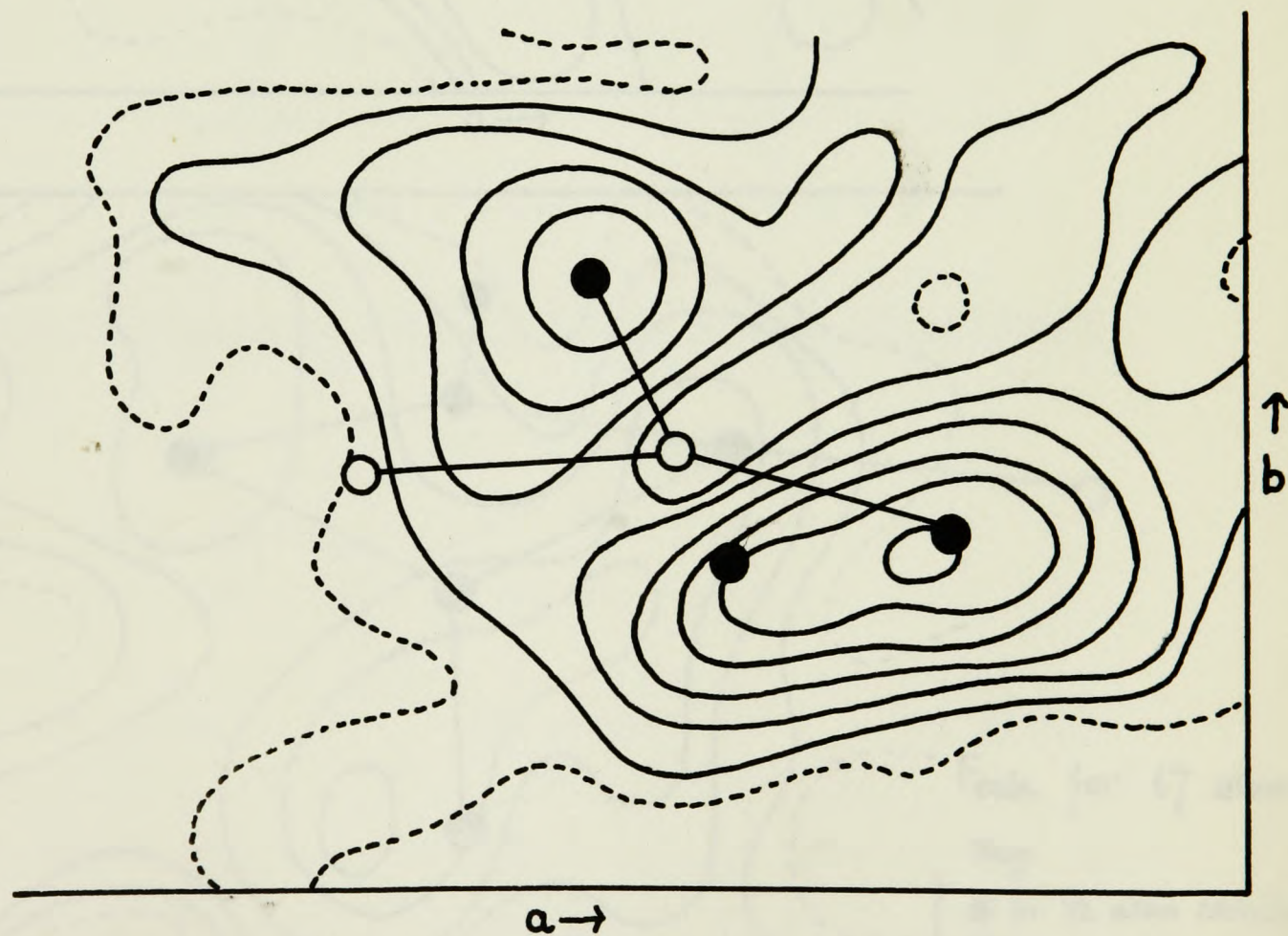


Figure 17. Difference Fouriers showing acetone and water molecules.

(Contour interval $1e/A^2$. Shaded atoms not included in F_{calc})

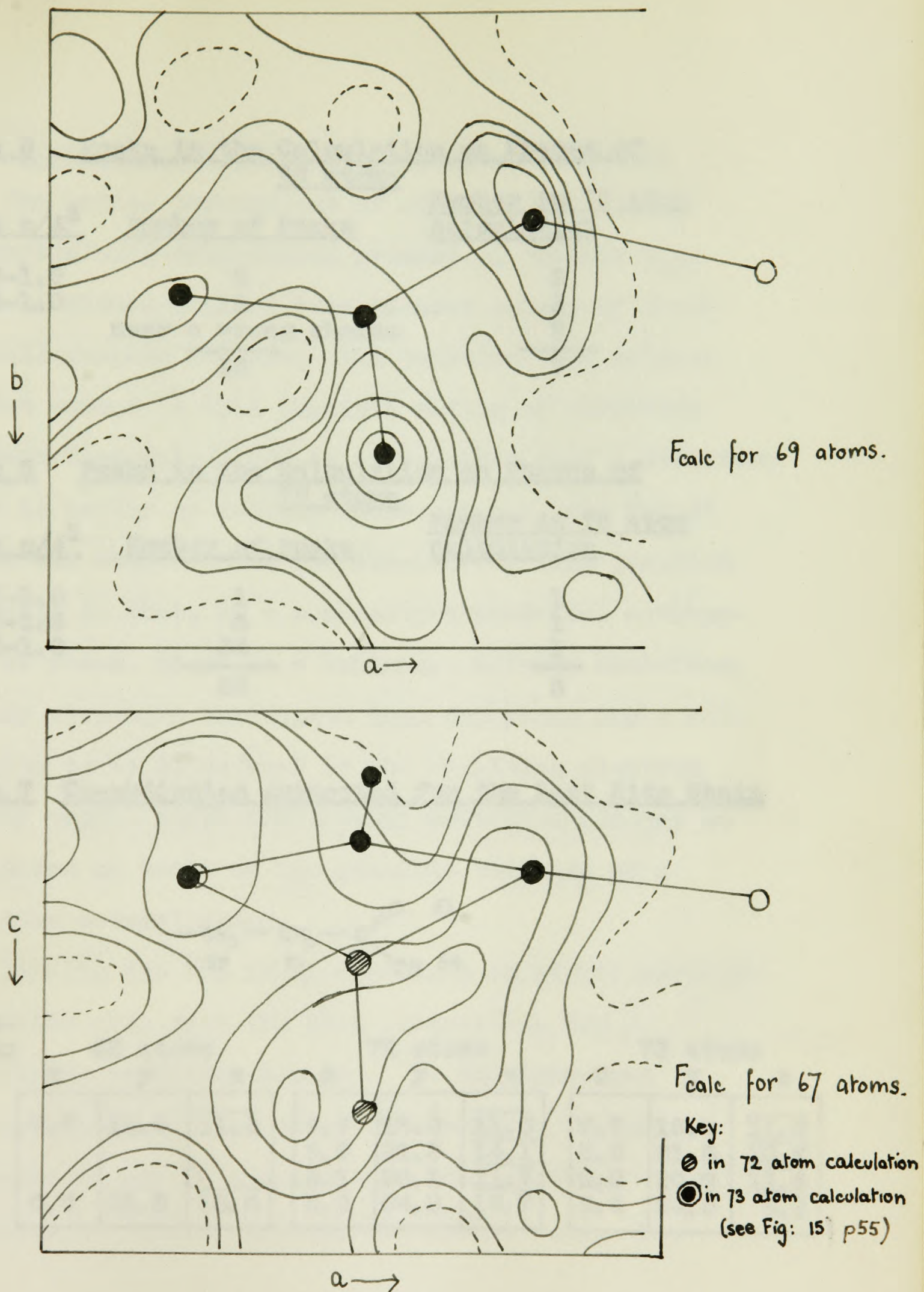


Figure 18. Difference Fouriers : Last side chain.

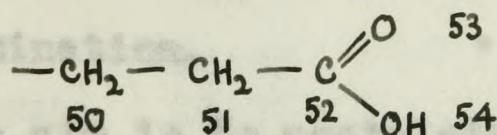
Table 5 Peaks in the Calculation on Phases of
68 atoms

<u>Range e/A^3</u>	<u>Number of Peaks</u>	<u>Number in 73 atom calculation</u>
2.0-1.6	2	2
1.6-1.3	12	3
	near a wrong choice	2
	<u>14</u>	<u>7</u>

Table 6 Peaks in the Calculation on Phases of
72 atoms

<u>Range e/A^3</u>	<u>Number of Peaks</u>	<u>Number in 73 atom calculation</u>
1.7-1.5	1	1
1.5-1.3	3	1
1.3-1.0	34	1
	<u>38</u>	<u>3</u>

Table 7 Co-ordinates suggested for the Last Side Chain



Calculation will give the same intensities for reflections as follows:

	68 atoms			72 atoms			73 atoms		
	x	y	z	x	y	z	x	y	z

51	7.7	18.5	11.5	7.7	18.9	11.8	7.7	18.9	11.8
52				5.0	21.3	14.1	5.0	21.5	10.7
53				2.1	20.1	11.7	2.2	20.0	11.6
54	6.0	26.8	13.0	5.0	24.3	18.7	5.4	24.5	8.9

Discussion of Three-Dimensional Refinements

The entire arrangement of atoms in the hexacarboxylic acid degradation product of vitamin B₁₂, shown in figure 13, has been deduced solely by X-ray crystallographic studies. The question then arises: To what extent is this physical method of structure determination valid, and is it possible that a structure which is partly or totally incorrect has been found? One of the main reasons for considering this question carefully is that, in a non-centrosymmetrical arrangement of atoms, there is a tendency, already described, for any structure introduced into the phase angle calculation to be reproduced in the resulting electron density maps. There are several methods which may be considered as tests of the possible validity of a structure determination.

Ideally the aim is to postulate an atomic arrangement which will give the same intensities for reflections as those observed. As the agreement between calculated and observed structure factors increases it may be assumed that the structure suggested is tending towards the correct one. The

disagreement is usually expressed by the ratio: comparison of calculated and observed structure

$$R = \frac{\sum \{ |F_{obs}| - |F_{calc}| \}}{\sum |F_{obs}|}$$

the summation extending over all reflections. In obtained. In this work intensities were visually and no corrections for absorption were made. Table 8 some values of R are listed. R is shown to increase if the structure is wrong and to decrease slowly as more atoms are used in the calculation. Furthermore the data from each layer line were roughly scaled and these do not extend equally well unless several of these are incorrectly placed. R three directions in reciprocal space. No hydrogen might be expected to decrease somewhat, however, if atom positions were used in the calculation of spurious peaks which appear in the electron density structure factors but, in the calculation for 73 atoms, maps are included in the structure factor calculation the hydrogen atoms were considered, for purposes of and so its significance is limited.

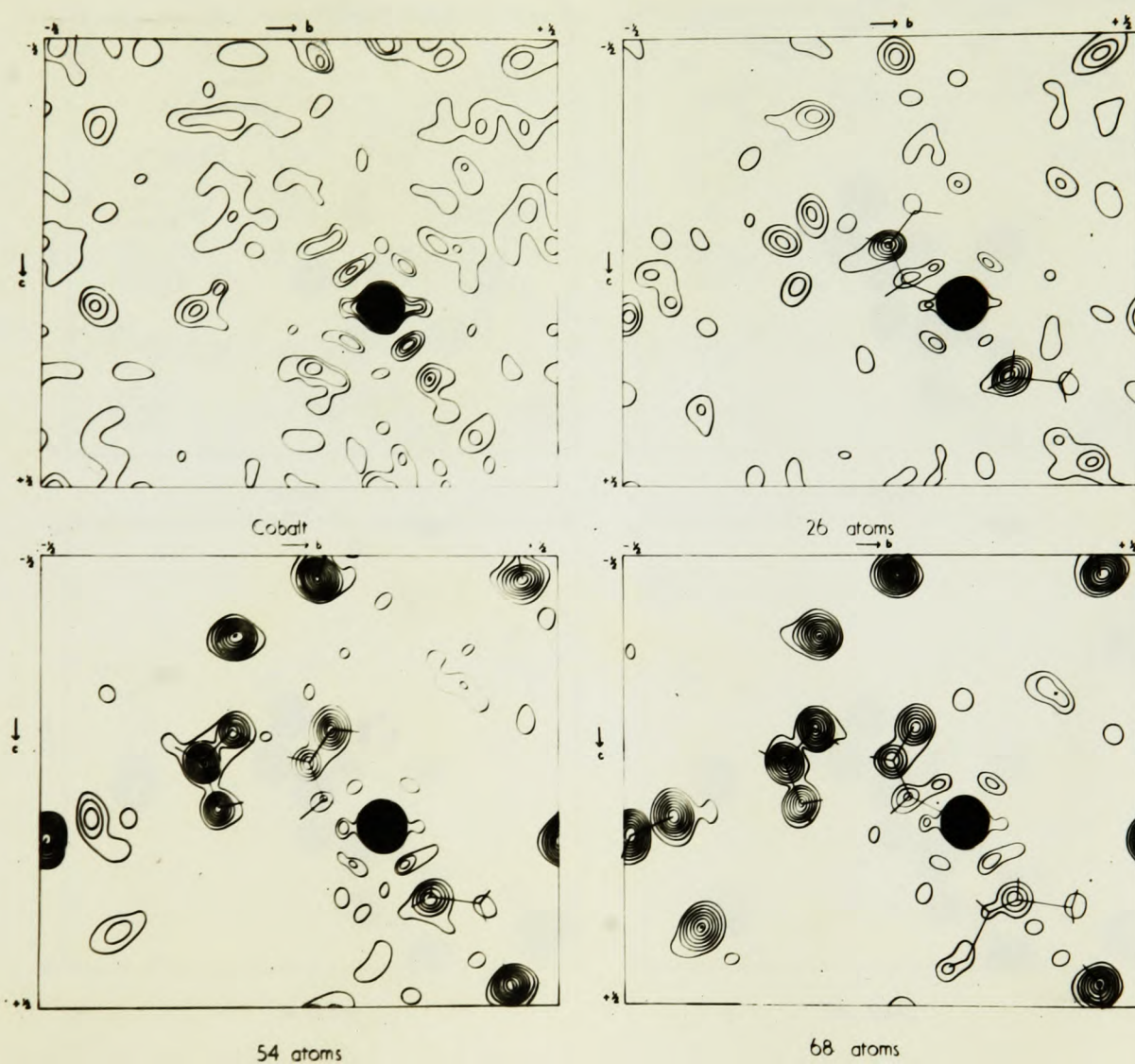
Table 8 Values of R at stages in the Three-dimensional Refinement for 3351 Observed Reflections

<u>Fourier</u>	<u>R</u>	<u>Remarks</u>
54 atoms	31.8%	
54 atoms (one wrong)	33.8%	One wrongly placed
68 atoms	27.6%	Two wrongly placed
72 atoms	23.7%	{ Two wrongly placed. Some moved incorrectly.
73 atoms	28.7%	

The last values listed for R may be compared with selection of high peaks in each calculation is values of 22% and 24% which were quoted for sodium and followed in tables 4-6. (41) (43) potassium benzylpenicillin respectively. A

comparison of calculated and observed structure factors is given in table 24 on pages 138 to 152. The accuracy of the experimental data will, necessarily, affect R and also details of the structure finally obtained. In this work intensities were estimated visually and no corrections for absorption were made. Furthermore the data from each layer line were only roughly scaled and these do not extend equally in all three directions in reciprocal space. No hydrogen atom positions were used in the calculation of structure factors but, in the calculation for 72 atoms, the hydrogen atoms were considered, for purposes of calculation, to be concentrated at the nuclei of the atoms to which they were attached.

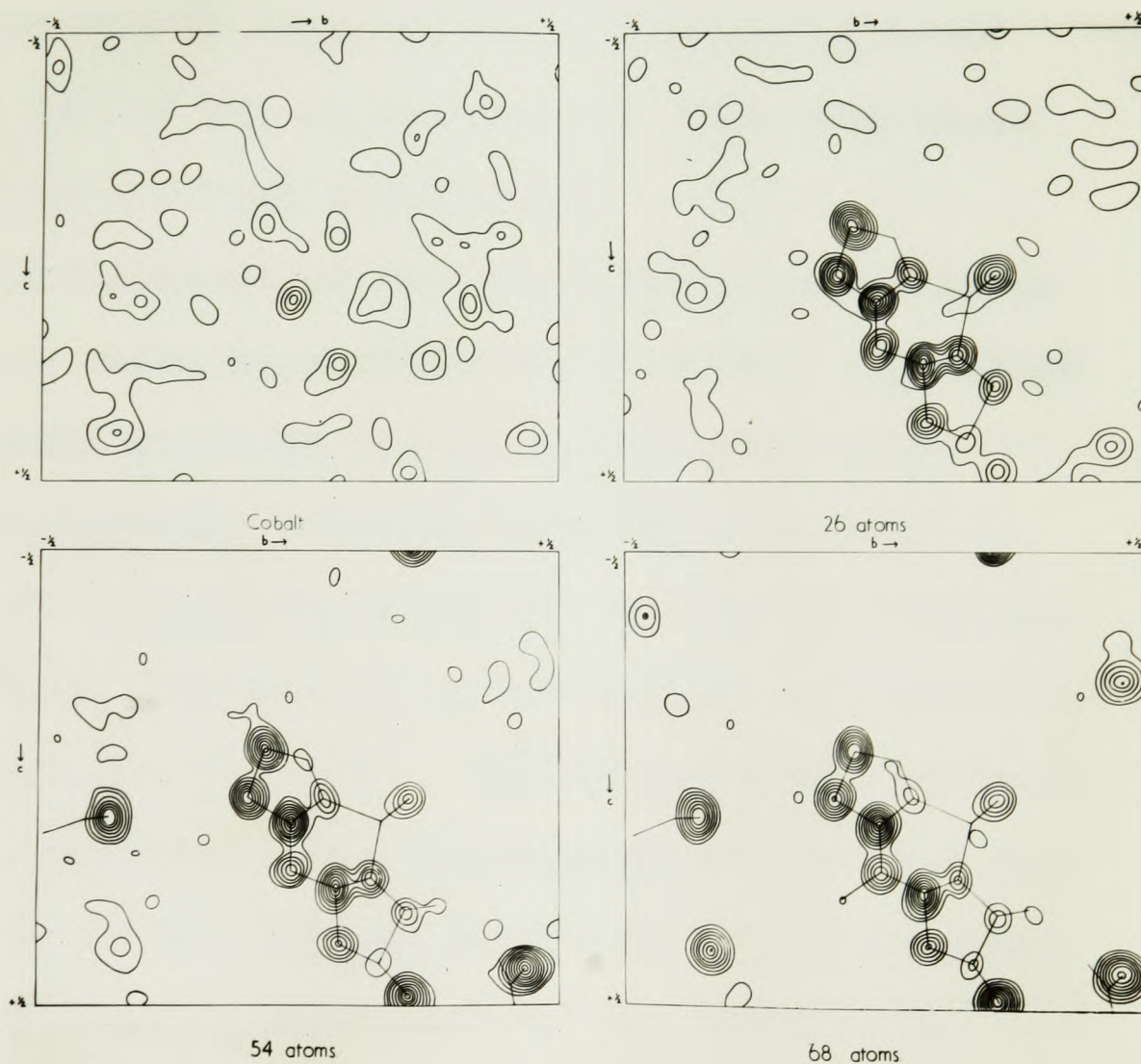
In figures 19 and 20 the appearances of two three-dimensional sections at various stages in the refinement are shown. There were no unexplained peaks over $1\frac{1}{2} e/A^3$ in height in the calculation on phases of 72 atoms (see table 6, page 61.). The course of the selection of high peaks in each calculation is followed in tables 2-6. Perhaps, however, if the appearances of electron density calculations are



Vitamin B₁₂ Fragment Sections at $x = \frac{2}{5}$ for 4 successive three-dimensional Fourier syntheses

Figure 19. One section in four successive calculations.

$$x = \frac{2}{5}$$



Vitamin B₁₂ Fragment Sections at $x = \frac{6}{60}$ for 4 successive three-dimensional Fourier syntheses

Figure 20. One section in four successive calculations.
 $x = 6$.

considered, the fact that the positions suggested for atoms fit the centrosymmetric projections is the best proof of their validity. The situation for the projection down the shortest axis is shown in figure 16. The heights of peaks in each calculation were used as a test of the correctness of their positions but this method is not infallible.

The announcement of the isolation of succinimide derivatives, which came after the tentative suggestion of the formula given in figure 12 (see page 46), provided independent evidence that the suggested structure is essentially correct. During the last few months Fourier refinements on wet and dry crystals of vitamin B₁₂ have been made. The method which has been found to be successful for the degradation product was used. Atoms of the "planar group" and the nucleotide part of the molecule were used in the phase angle calculations. Additional peaks which appeared in the electron density calculations were consistent, in most cases, with the arrangement found by work on the degradation product, but there were also other unexplained peaks in the series. This work was done

by Mrs. M.F. Mackay and Miss M.J. Harrison.

Incorrect Atomic Positions

At several stages in this work electron density calculations have been made with some atomic positions incorrectly chosen. As a result, it is possible to review the effect of the introduction of atoms in wrong positions into the phase angle calculation. There are two possible situations: the position may be totally incorrect, or else it may lie near an actual atomic position. These are shown, in part, in figure 21.

It has already been mentioned that an atom was accidentally introduced in an incorrect position into the phase angle calculation for 54 atoms. This provides a good example of the first situation listed above. The R values, listed in table 8, show an increase from 31.8 to 33.8%. The two three-dimensional calculations showed differences only in the regions directly affected. The atom should have been put at $x = 4.3$ (A; figure 22) but was put, instead, at $x = 13.0$ (B; figure 22). The peak heights are given in table 9.

Figure 21.

Atom in wrong position.

One-dimensional calculation

F data calculated from positions A, B, C.

— calculation using phases of atoms A, B, C.

— calculation using phases of atoms A, B, D.

(centre of symmetry introduced at X so that E and F are equivalent positions)

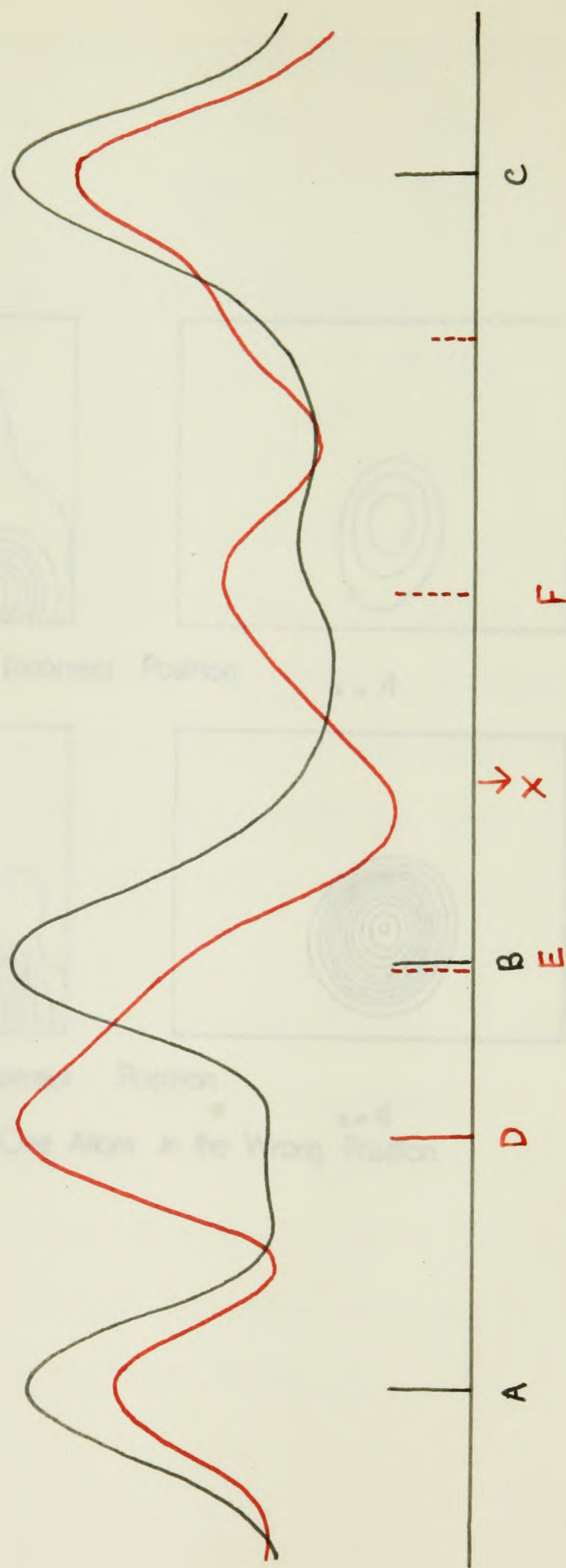


Figure 21.

Atom in wrong position.

One-dimensional calculation.

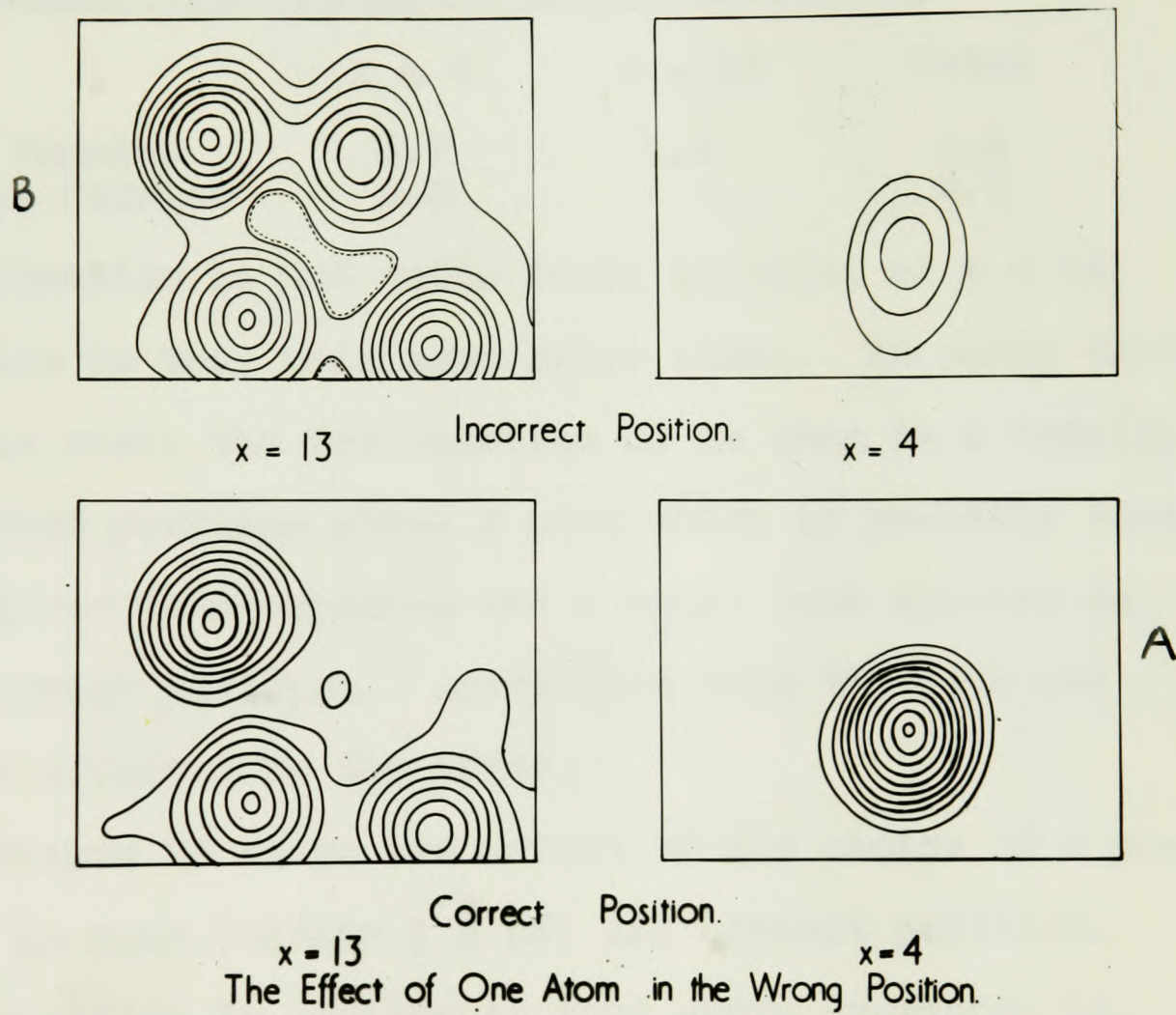


Figure 22.

Table 9 Correct and Incorrect Positions for Peaks

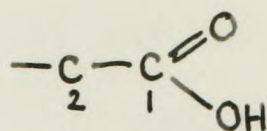
<u>Peak Heights</u> $e/\text{\AA}^3$	$x = 4$	$x = 13$	Total
Wrong Fourier	3.6	6.0	9.6
Correct Fourier	9.7	-	9.7

Approximate total electron count (electrons)

	$x = 4$	$x = 13$	Total
Wrong Fourier	4.7	1.8	6.5
Correct Fourier	5.5		5.5

The situation is not quite ideal because, at $x = 13$, the atom is very near some other atoms.²⁶ It seems that, in this case, the introduction of an atom in a totally incorrect position gives a peak which is probably somewhat lower than expected and a small peak appears in the correct position. Apart from this the maps are hardly affected by the error.

Figure 23 shows the effect of the choice of a peak which is near (within 1 Å of) the correct position. The situation is similar to that shown in figure 15. There was an ambiguity in the choice of the position of one atom in the 26 atom Fourier calculation, as shown. As the two atoms shown are carbon atoms of a group



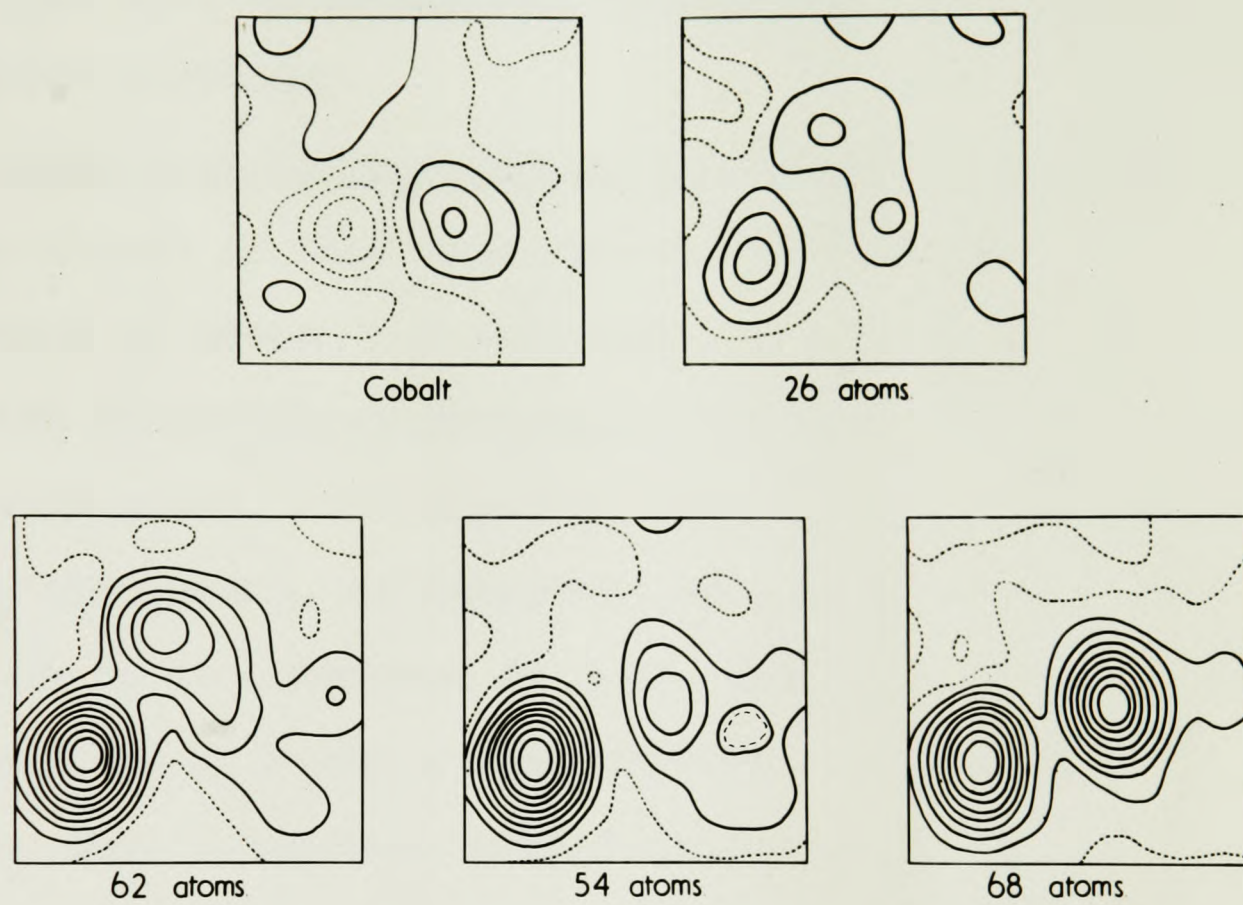


Figure 23. Atom near correct position.
(Three-dimensional section at $x = 9$)

Table 10(A)

Co-ordinates of Atoms at each stage

of which positions for C_2 and the oxygen atoms are known, the position of C_1 is stereochemically rigidly defined. The wrong position was chosen in the 62 atom calculation and, dramatically, a bulge is shown towards the correct position.

Three-dimensional Refinements of Co-ordinates of Atoms

The object of this research was to find the arrangement of atoms in a molecule, but the accuracy with which internuclear distances have been determined is not very great. The electron density was evaluated, in three dimensions, at intervals of $1/60$ each unit cell edge, that is at intervals of 0.42 , 0.26 and $0.22 \text{ \AA}$ in the directions x , y and z respectively. All peak positions were determined graphically. In table 10, page 74, the positions of atoms at each stage are listed, and final parameters for each peak are given to within ± 0.1 to 0.2 of $1/60$ th in each direction.

These values are equivalent to movements of ± 0.05 to $0.10 \text{ \AA}$, but this is probably much less than the accuracy of the determination of atomic positions. The data are extensive, but are cut off at the limit of $\sin \theta$ for copper $K\alpha$ radiation, and this will affect

Table 10(A) Co-ordinates of Atoms at each stage

	INPUT			OUTPUT			OUTPUT			INPUT			OUTPUT			INPUT			OUTPUT			FINAL CO-ORDINATES		
	26 ATOM FOURIER			26 ATOM FOURIER			54 ATOM FOURIER			68 ATOM FOURIER			68 ATOM FOURIER			72 ATOM FOURIER			72 ATOM FOURIER					
	60x	60y	60z	60x	60y	60z	60x	60y	60z	60x	60y	60z	60x	60y	60z	60x	60y	60z	60x	60y	60z	60x	60y	60z
1	9.1	9.6	5.8	9.1	9.6	5.8	9.1	9.6	5.8	9.1	9.6	5.8	9.0	9.6	5.9	9.0	9.6	5.9	9.1	9.6	5.9	9.1	9.6	5.9
2	13.7	5.3	9.7	13.7	5.3	9.7	13.7	5.3	9.7	13.7	5.3	9.7	13.8	5.3	9.7	13.9	5.2	9.7	13.8	5.3	9.6	13.8	5.3	9.6
3	5.0	13.0	2.8	5.1	12.9	2.7	5.1	12.8	2.8	5.2	12.6	2.7	5.2	12.8	2.9	5.2	12.9	3.0	5.2	13.0	3.1	5.2	13.0	3.1
4	2.7	14.0	1.5	2.8	14.2	1.5	2.8	14.4	1.5	2.9	14.6	1.5	2.9	14.6	1.3	2.9	14.6	1.2	3.0	14.6	1.1	3.0	14.6	1.1
5	7.1	12.4	17.3	7.1	12.4	17.4	7.2	12.4	17.6	7.3	12.4	17.8	7.3	12.4	17.8	7.3	12.4	17.8	7.3	12.4	17.8	7.3	12.4	17.8
6	7.0	8.5	13.3	7.1	8.4	13.3	7.1	8.3	13.2	7.2	8.1	13.1	6.9	8.2	13.0	6.8	8.2	13.0	7.0	8.2	13.0	7.0	8.2	13.0
7	6.4	4.5	14.5	6.3	4.4	14.6	6.2	4.3	14.7	6.0	4.2	14.8	6.1	4.2	15.2	6.1	4.2	15.5	6.1	4.3	15.3	6.1	4.3	15.2
8	5.2	1.0	12.1	5.2	0.8	12.1	5.2	0.8	12.1	5.2	0.6	12.1	5.2	0.6	12.1	5.2	0.6	12.1	5.3	0.6	12.1	5.3	0.6	12.1
9	6.1	1.0	6.0	6.0	1.0	6.2	6.0	0.8	6.1	5.9	0.7	6.3	5.9	0.7	6.3	5.9	0.7	6.3	5.9	0.7	6.3	5.9	0.7	6.3
10	7.2	3.0	3.0	7.3	3.1	2.9	7.4	3.1	2.9	7.6	3.2	2.8	7.6	3.1	2.7	7.6	3.2	2.8	7.6	3.1	2.8	7.6	3.1	2.8
11	8.0	1.1	2.4	8.0	1.1	2.4	8.1	1.3	2.4	8.2	1.4	2.4	8.1	1.3	2.3	8.2	1.3	2.4	8.0	1.3	2.3	8.0	1.3	2.3
13	11.3	9.0	6.0	11.3	8.6	6.3	11.3	8.7	6.2	11.3	8.3	6.5	11.2	8.3	6.4	11.1	8.3	6.4	11.2	8.4	6.2	11.2	8.4	6.2
14	11.2	11.2	1.1	11.1	11.4	1.0	11.2	11.4	1.1	11.1	11.6	1.0	11.1	11.5	1.0	11.1	11.5	1.1	11.1	11.5	1.1	11.1	11.5	1.1
15	13.1	16.0	2.5	12.9	16.0	2.7	12.8	15.9	2.6	12.6	15.8	2.8	12.6	16.0	2.7	12.6	16.1	2.7	12.6	16.2	2.7	12.6	16.2	2.7
16	13.1	20.5	1.2	13.2	20.5	1.3	13.2	20.4	1.3	13.3	20.3	1.4	13.3	20.3	1.4	13.3	20.3	1.4	13.3	20.2	1.3	13.3	20.2	1.3
17	12.0	19.5	6.5	11.9	19.6	7.0	12.0	19.6	7.2	11.9	19.7	7.7	12.2	19.6	7.5*	12.5	20.2	7.5	12.4	19.9	7.5	12.4	19.6	7.5
18	10.8	15.7	9.5	10.7	15.7	9.5	10.7	15.7	9.6	10.6	15.7	9.7	10.6	15.6	9.6	10.7	15.6	9.6	10.6	15.6	9.6	10.6	15.6	9.6
19	9.5	15.2	15.5	9.7	15.4	15.7	9.8	15.4	15.8	10.0	15.6	16.0	10.2	15.6	16.0*	10.3	15.7	16.1	10.3	15.7	16.0	10.3	15.7	16.0
20	7.3	9.5	24.5	7.3	9.5	24.4	7.3	9.6	24.2	7.3	9.7	24.0	7.3	9.7	24.1*	7.3	9.6	23.8	7.3	9.6	23.9	7.3	9.6	24.0
21	5.1	4.9	22.0	5.1	4.8	22.2	5.2	4.9	21.9	5.3	4.8	22.0	5.3	4.8	21.8	5.3	4.8	21.8	5.3	4.8	21.8	5.3	4.8	21.8
22	5.4	5.7	2.8	5.4	5.7	2.7	5.4	5.7	2.8	5.4	5.7	2.7	5.4	5.8	2.7	5.4	5.7	2.7	5.5	5.7	2.7	5.5	5.7	2.7
23	6.4	4.0	4.0	6.3	3.8	4.0	6.3	3.8	3.8	6.2	3.6	3.6	6.3	3.6	3.6	6.2	3.6	3.6	6.2	3.6	3.6	6.2	3.6	3.6
24	13.3	10.0	10.5	13.3	10.3	10.7	13.4	10.6	10.8	13.5	10.9	11.0	13.5	10.9	10.9	13.5	10.9	10.9	13.5	10.9	11.0	13.5	10.9	11.0
25	13.3	16.1	9.8	13.3	16.2	9.7	13.4	16.2	9.7	13.5	16.3	9.6	13.6	16.4	9.3	13.6	16.4	9.0	13.6	16.4	9.2	13.6	16.4	9.2
26	13.4	23.5	13.0	13.2	23.3	12.8	13.3	23.3	12.8	13.1	23.1	12.6	12.9	23.2	12.7*	12.9	23.6	12.7	12.9	23.3	12.7	12.9	23.2	12.7
27	10.3	20.8	16.5	10.4	21.3	16.6	10.4	21.3	16.8	10.5	21.8	17.0	10.4	21.8	16.9*	10.4	21.5	16.9	10.4	21.7	17.0	10.4	21.8	17.0

For numbering of atoms see figures 25 and 26, pages 89 and 90.

For explanation of * see page 77.

Table 10(B)

Co-ordinates of Atoms at each stage

	INPUT			OUTPUT			INPUT			OUTPUT			INPUT			OUTPUT			FINAL		
	54 ATOM FOURIER			54 ATOM FOURIER			68 ATOM FOURIER			68 ATOM FOURIER			72 ATOM FOURIER			72 ATOM FOURIER			CO-ORDINATES.		
	60x	60y	60z	60x	60y	60z	60x	60y	60z	60x	60y	60z	60x	60y	60z	60x	60y	60z	60x	60y	60z
12	9.8	3.8	6.8	9.8	3.8	6.9	9.8	3.8	7.0	9.8	3.8	6.8	9.8	3.8	6.8	9.8	3.8	6.7	9.8	3.8	6.6
28	4.3	15.9	16.8	4.3	15.9	16.8	4.3	15.9	16.8	4.3	15.9	16.8	4.3	15.9	16.8	4.3	15.9	16.8	4.3	15.9	16.8
29	4.1	5.5	15.7	3.9	5.4	15.7	3.7	5.3	15.7	3.8	5.2	15.7	3.9	5.1	15.7	3.9	5.2	15.7	3.9	5.2	15.7
30	15.1	25.0	0.4	15.0	24.9	0.4	14.9	24.8	0.4	14.8	24.7	0.4	14.7	24.6	0.4	14.7	24.8	0.5	14.7	24.8	0.5
31	10.9	7.6	24.7	10.8	7.6	24.8	10.7	7.6	24.9	11.1	7.6	24.7	11.1	7.6	24.8	10.9	7.6	24.9	10.9	7.6	24.9
32	11.3	3.2	29.8	11.4	3.4	29.9	11.5	3.6	30.0	11.6	3.5	29.8	11.6	3.5	29.6	11.6	3.5	29.7	11.6	3.5	29.7
33	14.3	3.0	30.5	14.3	2.8	30.6	14.3	2.6	30.7	14.4	2.6	30.6	14.5	2.6	30.5	14.4	2.6	30.4	14.4	2.6	30.4
34	9.3	2.0	32.8	9.2	2.0	32.9	9.1	2.0	33.0	9.2	1.9	32.9	9.3	1.8	32.8	9.3	1.8	32.9	9.3	1.8	32.9
35	1.8	4.8	24.0	1.7	4.8	23.8	1.7	4.8	23.6	1.6	4.7	23.6	1.6	4.7	23.6	1.6	4.7	23.6	1.6	4.7	23.6
40	8.3	9.4	3.4	8.2	9.4	3.4	8.1	9.4	3.4	8.3	9.3	3.4	8.5	9.2	3.4	8.4	9.3	3.3	8.4	9.3	3.3
41	9.4	11.2	2.5	9.4	11.2	2.3	9.4	11.2	2.1	9.4	11.1	2.3	9.4	11.2	2.1	9.4	11.1	2.3	9.4	11.1	2.4
42	11.1	14.5	4.5	11.3	14.4	4.2	11.5	14.3	3.9	11.5	14.3	3.9	11.5	14.3	3.9	11.5	14.3	3.8	11.5	14.3	3.8
43	8.3	7.9	6.8	8.3	7.8	6.8	8.3	7.7	6.8	8.3	7.7	6.8	8.3	7.7	6.8	8.2	7.6	6.8	8.2	7.6	6.8
47	0.9	4.9	14.7	0.8	4.9	14.7	0.7	4.9	14.7	0.7	5.1	14.5	0.7	5.3	14.3	0.7	5.4	14.2	0.7	5.4	14.2
49	17.1	8.3	9.2	17.1	8.2	9.2	17.1	8.1	9.2	17.1	8.0	9.5	16.8	7.9	9.5	16.9	7.9	9.6	17.0	7.9	9.7
55	12.3	29.3	11.4	12.4	29.4	11.3	12.5	29.5	11.2	12.4	29.4	11.3	12.3	29.3	11.4	12.4	29.2	11.4	12.4	29.2	11.4
56	8.8	30.7	7.5	8.8	30.7	7.6	8.8	30.7	7.7	8.8	30.7	7.6	8.9	30.7	8.3	8.9	30.7	7.9	8.9	30.7	7.6
58	11.7	37.7	4.2	11.7	37.6	4.0	11.7	37.5	3.8	11.7	37.6	3.6	11.7	37.7	3.4	11.6	37.6	3.5	11.6	37.6	3.5
59	6.3	38.2	5.5	6.4	38.3	5.4	6.5	38.4	5.3	6.5	38.4	5.1	6.5	38.4	5.0	6.5	38.3	4.9	6.5	38.3	4.9
50	11.5	23.1	23.8	11.4	22.8	23.7	11.3	22.5	23.6	11.3	22.7	23.6	11.3	22.8	23.6	11.4	22.9	23.5	11.4	22.9	23.5
61	8.7	24.8	27.2	8.7	24.8	27.2	8.7	24.8	27.2	8.6	24.9	27.2	8.6	25.2	27.8	8.6	25.1	27.4	8.6	25.0	27.2
62	6.2	26.0	25.3	6.2	26.4	25.3	6.2	26.8	25.3	6.3	26.6	25.4	6.4	26.4	25.3	6.3	26.4	25.4	6.3	26.4	25.4
63	9.7	25.7	33.3	9.6	25.5	33.2	9.5	25.3	33.1	9.5	25.5	33.1	9.5	25.7	33.1	9.5	25.7	33.0	9.5	25.7	33.0
64	6.1	12.7	28.6	6.2	12.6	28.8	6.3	12.5	29.0	6.3	12.5	29.1	6.3	12.5	29.2	6.3	12.5	29.1	6.3	12.5	29.1
65	2.3	8.6	3.6	2.3	8.6	3.7	2.3	8.6	3.8	2.3	8.6	3.6	2.3	8.6	3.4	2.3	8.7	3.3	2.3	8.6	3.3
66	13.3	9.3	17.2	13.3	9.4	17.2	13.3	9.5	17.2	13.2	9.4	17.2	13.2	9.3	17.2	13.1	9.3	17.2	13.1	9.3	17.2
67	16.3	22.4	14.3	16.3	22.3	14.3	16.3	22.2	14.3	16.4	22.3	14.3	16.4	22.3	14.3	16.4	22.3	14.3	16.4	22.3	14.3
68	8.7	7.7	19.3	8.8	7.4	19.1	8.9	7.1	18.9	8.7	7.1	19.0	8.6	7.1	19.0	8.6	7.2	19.1	8.6	7.2	19.1

Table 10(C) Co-ordinates of Atoms at each stage

	INPUT			OUTPUT				INPUT			OUTPUT			FINAL CO-ORDINATES		
	68 ATOM FOURIER			68 ATOM FOURIER				72 ATOM FOURIER			72 ATOM FOURIER			FINAL CO-ORDINATES		
	60x	60y	60z	60x	60y	60z		60x	60y	60z	60x	60y	60z	60x	60y	60z
36	0.6	2.5	30.4	0.6	2.6	30.5	*	0.6	3.0	29.8	0.7	2.9	29.9	0.7	2.8	30.0
37	0.7	2.8	30.6	0.7	2.7	30.7		0.7	2.7	30.7	0.7	2.6	30.6	0.7	2.6	30.6
38	1.8	4.7	36.0	1.8	4.8	35.7		1.8	4.8	35.4	1.8	4.8	35.2	1.8	4.8	35.1
39	1.4	5.2	27.5	1.3	5.2	27.3		1.3	5.2	27.1	1.3	5.2	27.0	1.3	5.2	27.0
44	3.2	2.5	7.1	3.2	2.5	7.2		3.2	2.5	7.2	3.2	2.5	7.2	3.2	2.5	7.2
45	0.6	1.5	4.8	0.6	1.5	4.8		0.6	1.5	4.8	0.6	1.6	4.9	0.6	1.6	4.9
46	2.0	2.6	9.0	1.9	2.5	9.0	*	2.2	3.7	9.8	2.0	3.2	9.5	1.9	2.6	9.1
48	4.7	2.3	8.8	4.7	2.2	8.7	*	4.9	3.8	8.1				4.7	2.2	8.7
57	8.9	35.8	5.2	8.9	35.8	5.2		8.9	35.8	5.2	8.8	35.8	5.1	8.8	35.8	5.1
70	5.8	39.9	22.5	5.9	39.9	22.5		5.9	39.9	22.5	5.9	39.9	22.6	5.9	39.9	22.6
71	9.3	39.2	19.8	9.2	39.1	19.9		9.2	39.1	19.9	9.1	39.1	19.9	9.1	39.1	19.9
50	11.1	20.3	11.8	11.3	20.2	12.0		11.5	20.0	12.3	11.4	20.1	12.4	11.3	20.2	12.4
51	7.7	18.5	11.5					7.7	18.9	11.8	7.7	18.9	11.8	7.7	18.9	11.8
54	6.0	26.8	13.0					5.0	24.3	18.7				5.4	24.5	8.9
52								5.0	21.3	14.1				5.0	21.5	10.7
53								2.1	20.1	11.7	2.2	20.0	11.6	2.2	20.0	11.6
72								3.1	38.5	20.2	3.2	38.4	20.2	3.2	38.4	20.2
73								6.7	42.6	28.4	6.7	42.7	28.3	6.7	42.7	28.3
69														4.8	38.7	9.8

positions of peaks⁴⁴⁾. The experimental inaccuracies in the initial data will, together with these other factors, give peaks which are probably within $\pm 0.2 \text{ \AA}$ of the actual atomic positions.

A three-dimensional refinement of the structure of l-threonine was described by Schoemaker, Donohue, Schomaker and Corey⁴⁵⁾. These workers found that if, in a non-centrosymmetric refinement, an atomic co-ordinate moves a small distance in space from the position in which it was put into the calculation, the atomic peak should be chosen twice this distance from its original position. This "double shift rule" has some theoretical justification⁴⁶⁾. The method was used to refine co-ordinates for the degradation product and seemed to be valid. In table 10 asterisked atoms were moved into positions which were considered stereochemically more probable. Most of them moved back towards the original positions. Table 11 shows movements for two atoms.

Table 11 Some Movements of Peaks

<u>Calculation</u>	<u>Atom 56</u>			<u>Atom 46</u>		
	<u>x</u>	<u>y</u>	<u>z</u>	<u>x</u>	<u>y</u>	<u>z</u>
Appeared in 68	8.8	30.7	7.6	1.9	2.5	9.0
Put in 72	8.9	30.7	8.3	2.2	3.7	9.8
Appeared in 72	8.9	30.7	7.9	2.0	3.2	9.5
Final co-ordinates	8.9	30.7	7.6	1.9	2.6	9.1

In both these cases co-ordinates were wrongly altered for the 72 atom calculation. The movement for atom 46 was extremely large as this was moved so that it would lie a reasonable distance from positions tentatively chosen for atoms in the last side chain. These positions were incorrect and the atom 46 moved back towards its original position.

Heights of Peaks in the Electron Density Calculations

The height of a peak in an electron density calculation will depend on the form of the scattering factor curve of the atom it represents. This means it depends on the atomic number and temperature factor of the atom. In table 12 the approximate heights of peaks in each calculation are listed. These heights are sensitive to any error in the scaling factor; and, as this is not known accurately, the relative values of peak heights may have more significance than the absolute values. Apart from this there is a random background fluctuation in electron density which, in the 72 atom calculation, seems to be of the order of $\pm 1.3 \text{ e}/\text{\AA}^3$. It is also difficult to find the true height of a peak when none of its parameters are integral numbers of 1/60th. In spite

Table 12(A) Approximate Peak Heights in $e/\text{\AA}^3$

Atoms in phase angle calculation:

		<u>Cobalt</u>	<u>26</u>	<u>54</u>	<u>62</u>	<u>68</u>		<u>72</u>
1	Co	76.4	61.7	58.8	48.6	49.9	Co	50.3
2	Cl	7.0	31.2	28.5	26.3	26.2	Cl	27.3
4	N	1.5	10.9	9.6	8.4	9.2	N	8.3
6		3.3	12.3	11.9	9.9	10.5		9.1
10		3.5	12.7	11.6	10.0	10.5		9.8
14		3.3	12.5	10.8	9.8	10.5		10.5
18		2.5	9.8	10.3	8.7	10.5		10.0
3	C	2.2	9.2	8.4	7.5	6.8	C	7.1
5		2.5	9.4	9.1	8.1	8.2		8.8
7		3.1	8.8	8.1	6.1	8.2		8.4
8		2.0	8.6	8.5	7.7	7.7		7.3
9		4.0	7.9	8.6	7.3	8.6		8.5
11		3.8	10.3	8.7	7.9	7.7		7.5
13		2.7	9.0	8.5	7.4	8.2		7.8
15		3.2	8.3	7.2	7.3	7.6		6.9
16		3.0	8.3	9.4	7.0	8.3		7.3
20		4.1	8.3	9.8	7.8	9.0		8.3*
22		1.8	9.0	8.8	7.3	8.2		8.2
23		2.4	9.0	8.3	7.1	7.7		7.3
24		2.7	7.6	7.4	5.7	7.7		7.4
26		2.6	8.0	8.6	7.7	7.8		7.8*
17		3.9	8.0	7.9	6.9	7.9	CH	7.9*
19		4.0	8.6	8.6	7.2	8.2		9.3*
21		2.5	8.6	8.6	7.7	7.4		7.7
25		2.7	8.3	7.8	6.9	7.1		8.3
27		2.5	8.6	8.5	6.0	8.0		7.8*

* see page 77

Table 12(B) Approximate Peak Heights in e/A^3

Atoms in phase angle calculation:

		<u>26</u>	<u>54</u>	<u>62</u>		<u>68</u>		<u>72</u>
32	C put in	3.3	8.2	7.9	N	8.7	C	6.8
41	as N	3.1	10.5	7.3		8.6		7.3
61		3.0	9.5	7.7		7.2		5.8*
12		2.8	9.2	(3.9)		8.6	CH	7.3*
30		2.7	8.1	7.9		8.2	CH ₂	7.8
31		4.1	10.1	9.4		9.5		9.3
35		3.3	10.3	(2.8)		8.6		8.2
40		3.1	9.4	6.8		8.6		9.0
55		2.5	10.1	(2.5)		8.9		7.9
56		3.1	10.0	8.4		8.2		8.3*
60		2.9	8.6	6.7		8.2		8.4
28		3.2	9.7	(3.4)		9.5	CH ₃	9.3
29		3.3	9.3	8.2		8.2		9.0
49		2.7	9.4	7.8		8.2		8.7*
64		3.1	9.5	8.4		9.5		8.8
65		3.0	8.9	7.3		7.8		7.8
66		3.0	7.8	7.5		7.3		7.8
67		2.8	8.7	6.9		7.8		8.6
33	O put in	4.0	10.5	9.2	O	10.9	O	10.5
34	as N	3.8	11.6	(4.5)		10.9		11.4
42		4.4	10.2	8.0		10.5		10.0
43		4.2	9.7	8.5		10.9		10.3
47		2.8	7.9	7.4		8.2		7.9
58		2.5	10.0	8.5		9.6		10.7
59		2.6	7.7	6.3		8.6		8.8
62		3.0	9.6	8.2		8.4		9.0
63		3.0	9.2	7.3		9.4		9.8
68		2.9	8.6	7.5		9.5	H ₂ O	10.8

Table 12(C) Approximate Peak Heights in e/A^3

Atoms in phase angle calculation:

		<u>26</u>	<u>54</u>	<u>62</u>		<u>68</u>		<u>72</u>
37	N	1.7	2.6	1.9	N	7.4	C	5.4
46		1.5	1.8	1.5		7.2		4.3*
57		2.0	3.7			8.2		6.8
70		1.7	2.0	1.7		6.9		5.6
71		1.8	1.2			1.5		4.7 wrong
36		2.0	2.8	2.2		6.8	CH ₂	7.3*
44		2.1	2.9	2.6		8.1		7.5
45		1.7	1.8	1.2		6.9		7.0
50		2.1	3.2	7.4		7.7		7.3
51		2.3	2.7	5.2		5.6		5.9
71		1.9	2.6	6.3		6.8	CH ₃	6.9
73		1.8	1.4	1.8		2.0		7.3
38		2.0	2.1	7.0	O	7.3	O	7.8
39		2.0	2.8	6.7		9.1		9.8
48		2.0	1.8	6.8		8.2		6.8*
54		1.7	1.6			5.6		6.6 wrong
53		1.5	1.7	1.4		1.4		6.3
72		1.5	1.2	6.5		1.5		6.7
69		1.5	1.7	1.4		1.6	H ₂ O	1.7

The rest goes into small peaks in atomic positions and into spurious peaks. The average in table 13 which gives average peak heights for atoms in the "phase angle".

Table 13 also shows the heights of peaks which are in atomic positions but were not included in the phase angle calculation. These are generally much less than half the expected peak heights. The heights of

of all this it is possible to make some relevant remarks on peak heights.

The temperature factor for this compound is much lower than for the vitamin, so that the data are more extensive and atomic peaks much higher. The position of the first diffraction ring is about $1.4 \text{ \AA}$ from the centre of the peak so that this will enhance any peak due to a bonded neighbouring atom. This effect may also have helped the structure determination greatly.

As more atoms are included in the phase angle calculation the heights of peaks due to these atoms decreases. In the initial stages too much electron density is pushed into peaks on known atomic positions. The rest goes into small peaks in atomic positions and into spurious peaks. The change in heights is indicated in table 13 which gives average peak heights for atoms in the "planar group".

Table 12 also shows the heights of peaks which are in atomic positions but were not included in the phase angle calculation. These are generally much less than half the expected peak heights. The heights of

Table 13 Average Approximate Heights for Atomic Peaks in the Planar Group (e/A³)

Atoms in phase angle calculation:		<u>Co</u>	<u>26</u>	<u>54</u>	<u>68</u>	<u>72</u>
Atom	Co	76.4	61.7	58.8	49.9	50.3
	Cl		31.2	28.5	26.2	27.3
	N		11.6	10.8	10.2	9.5
	C		8.6	8.5	7.9	7.9

Table 14 Average Approximate Heights for Atomic Peaks in the Side Chains (e/A³)

Atoms in phase angle calculation:		<u>54</u>	<u>68</u>	<u>72</u>
Atom	C	9.4 N	7.8 N	-
	CH ₂	9.5	8.2	8.0 CH ₂
	CH ₃	9.0	8.2	8.2 CH ₃
	O	9.5	9.7 O	9.7 O

C	1.5-3.0	1.2-3.7
CH ₂	1.7-4.1	1.5-3.9
CH ₃	1.8-3.5	1.4-2.8
O	1.5-4.4	1.7-3.8

Table 15 Ranges of Heights of Peaks (e/A^3)

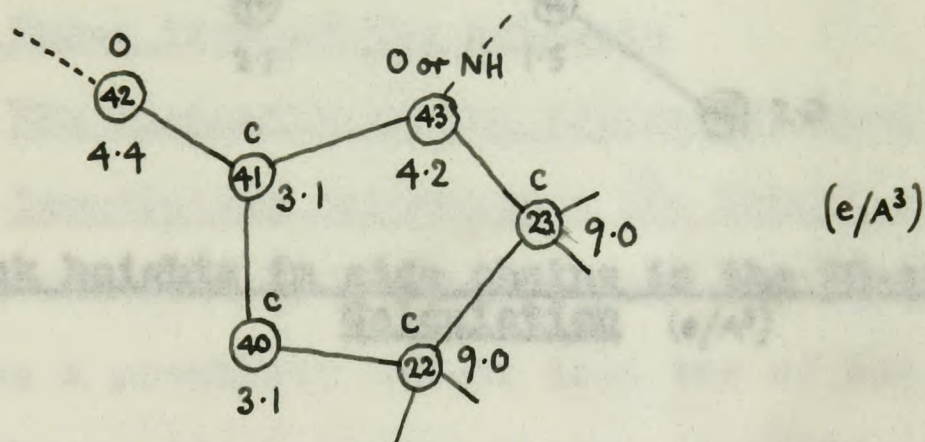
A. Atoms included in the phase angle calculation

	<u>26</u>	<u>54</u>	<u>68</u>	<u>72</u>
N	9.8-12.5	9.6-11.9	9.2-10.5	8.3-10.0
C	7.6-10.3	7.2-10.5	6.8- 9.0	5.4- 8.8
CH ₂		8.1-10.3	6.8- 9.5	5.9- 9.3
CH ₃		7.8- 9.7	6.8- 9.5	6.9- 9.3
O		7.9-11.6	7.3-10.9	6.3-11.4

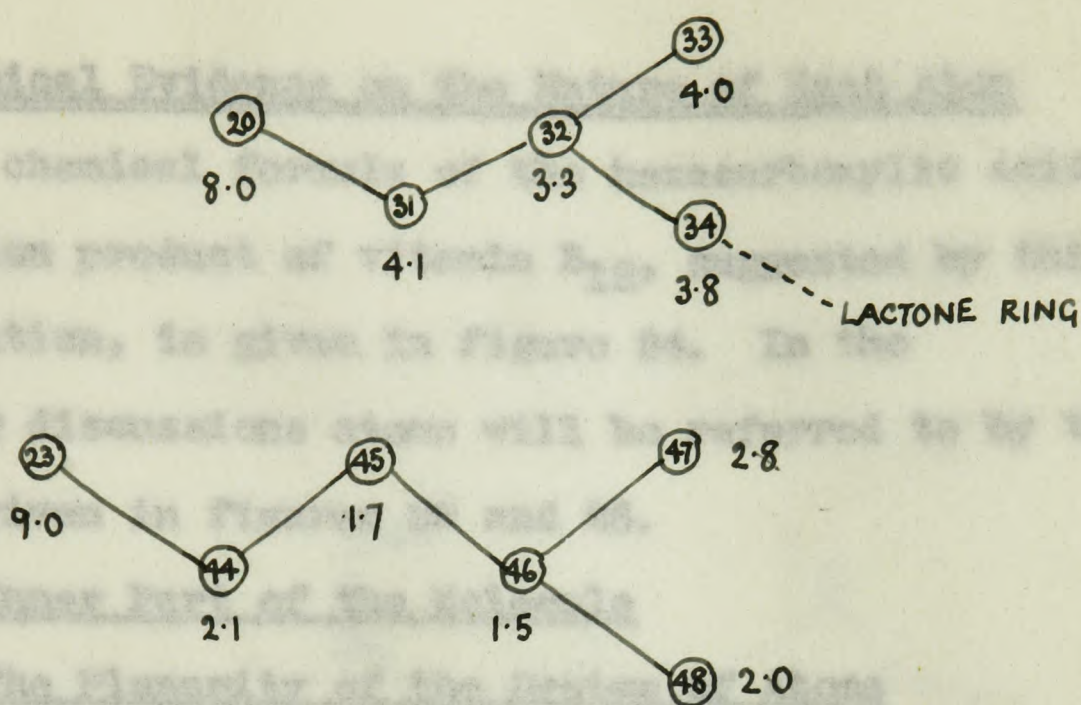
B. Atoms not included in the phase angle calculation

	<u>26</u>	<u>54</u>
C	1.5-3.0	1.2-3.7
CH ₂	1.7-4.1	1.8-3.2
CH ₃	1.8-3.3	1.4-2.6
O	1.5-4.4	1.7-2.8

such peaks depend on the atomic number of the atoms they represent and, apparently, the rigidity of the structure in which they lie. The γ -"lactone" ring provides a very rigid structure and exemplifies this point. In the 26 atom calculation heights of peaks in this are:



The structure analysis must give a considerable but other side groups are less rigid. The positions of atoms in the propionic acid side chains were the last in the molecule to be found. The peaks are very low and diffuse presumably because the group is not very rigidly held in the crystal. This fact complicated the structure determination. If, however, the carboxylic group of the side chain is hydrogen bonded to a rigid part of the structure, then peak heights are higher.



Some peak heights in side chains in the 26-atom
Calculation (e/A^3)

The structure analysis must owe a considerable part of its success to the low temperature factor found for the crystal. Peaks due to atoms not included in the calculation are higher than the general fluctuations in electron density and so their selection has been, with a few exceptions (which are no doubt due to the non-rigidity of the group considered), comparatively simple.

Chemical Evidence on the Nature of Each Atom

The chemical formula of the hexacarboxylic acid degradation product of vitamin B₁₂, suggested by this investigation, is given in figure 24. In the following discussions atoms will be referred to by the numbers given in figures 25 and 26.

A. The Inner Part of the Molecule

1. The Planarity of the System of Atoms

Immediately Surrounding the Cobalt Atom

The molecule is a tetrapyrrole derivative and resembles a porphyrin except that two of the five-membered rings (A and D) are directly linked. Approximate internuclear distances and interbond angles are shown in figure 27. Within the experimental accuracy of $\pm 0.2 \text{ \AA}$ the system of atoms immediately surrounding the cobalt atom, with due exception of the two atoms, (5) and (19), at the direct link, is planar (i.e. atoms (6) to (18) are coplanar).

The ultraviolet spectrum of the vitamin and the degradation product shows a strong absorption band near 360 mμ. This must surely mean that there is a conjugated system of double bonds in these compounds,

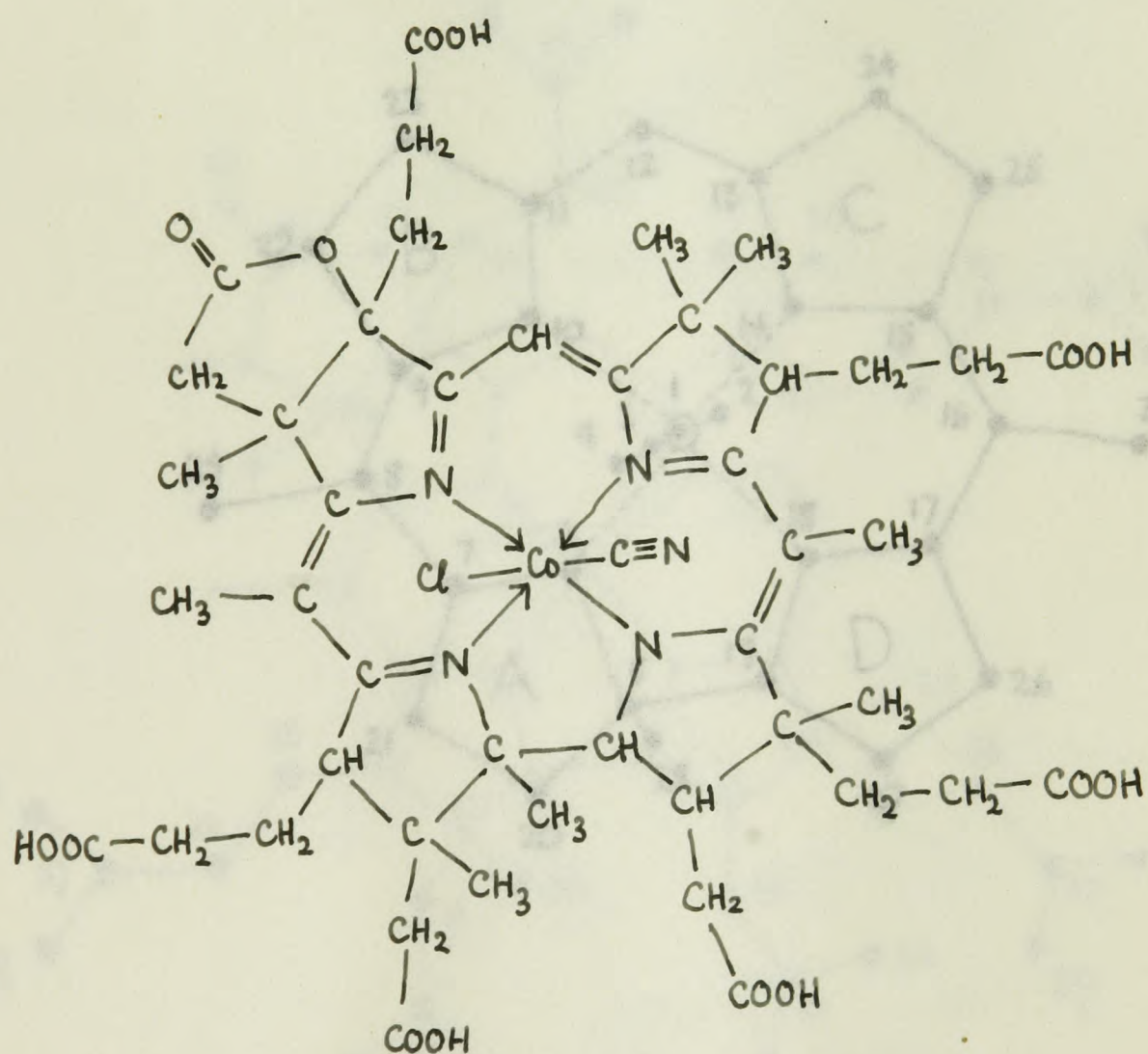


Figure 25 Numbering of atoms in the inner nucleus.

Figure 24 Formula suggested for the hexacarboxylic acid.

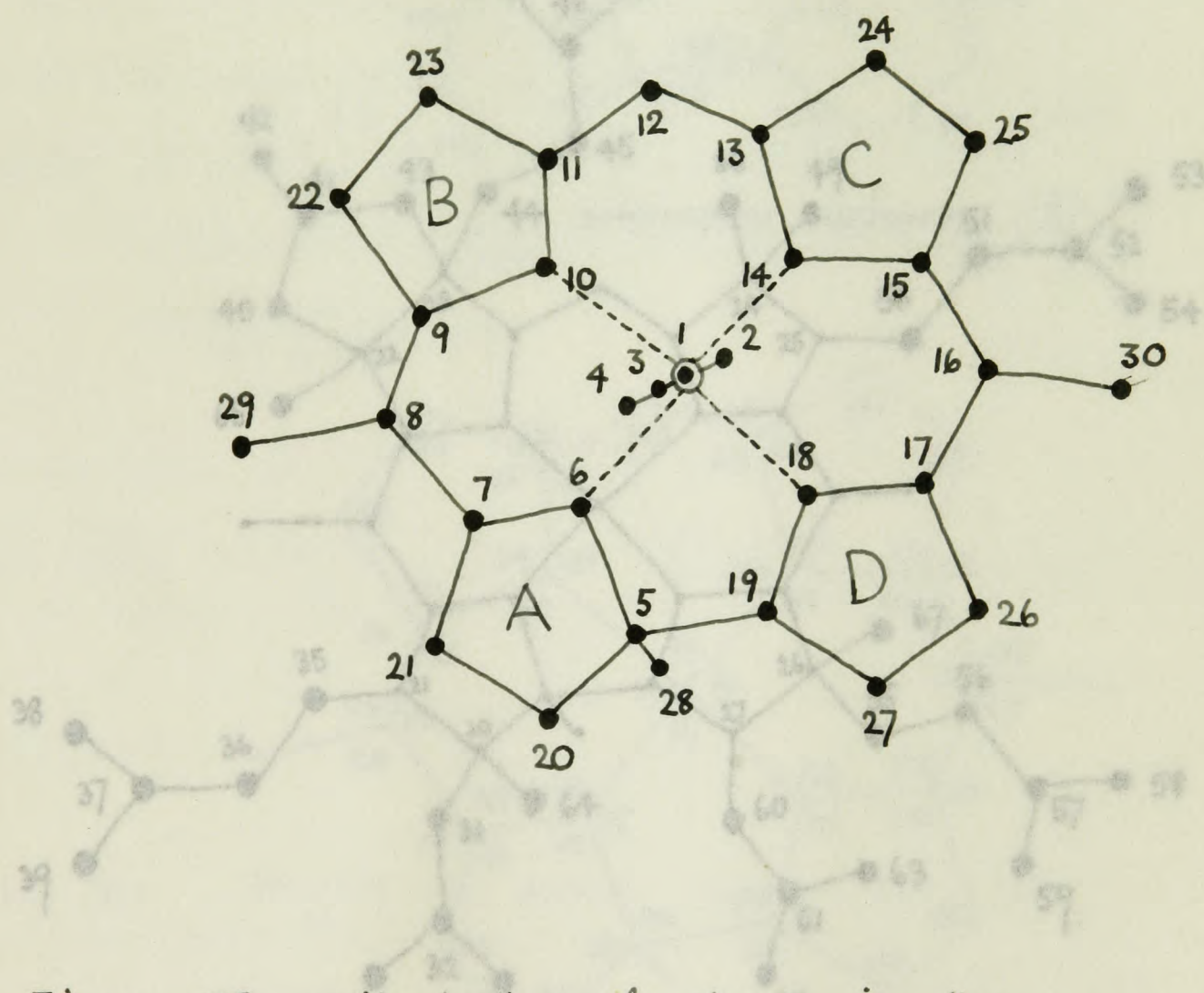


Figure 25. Numbering of atoms in the
inner nucleus.

Figure 26. Numbering of atoms in the side chains.

Figure 27. Dimensions of the inner nucleus.

(From final manuscript of paper 1954)

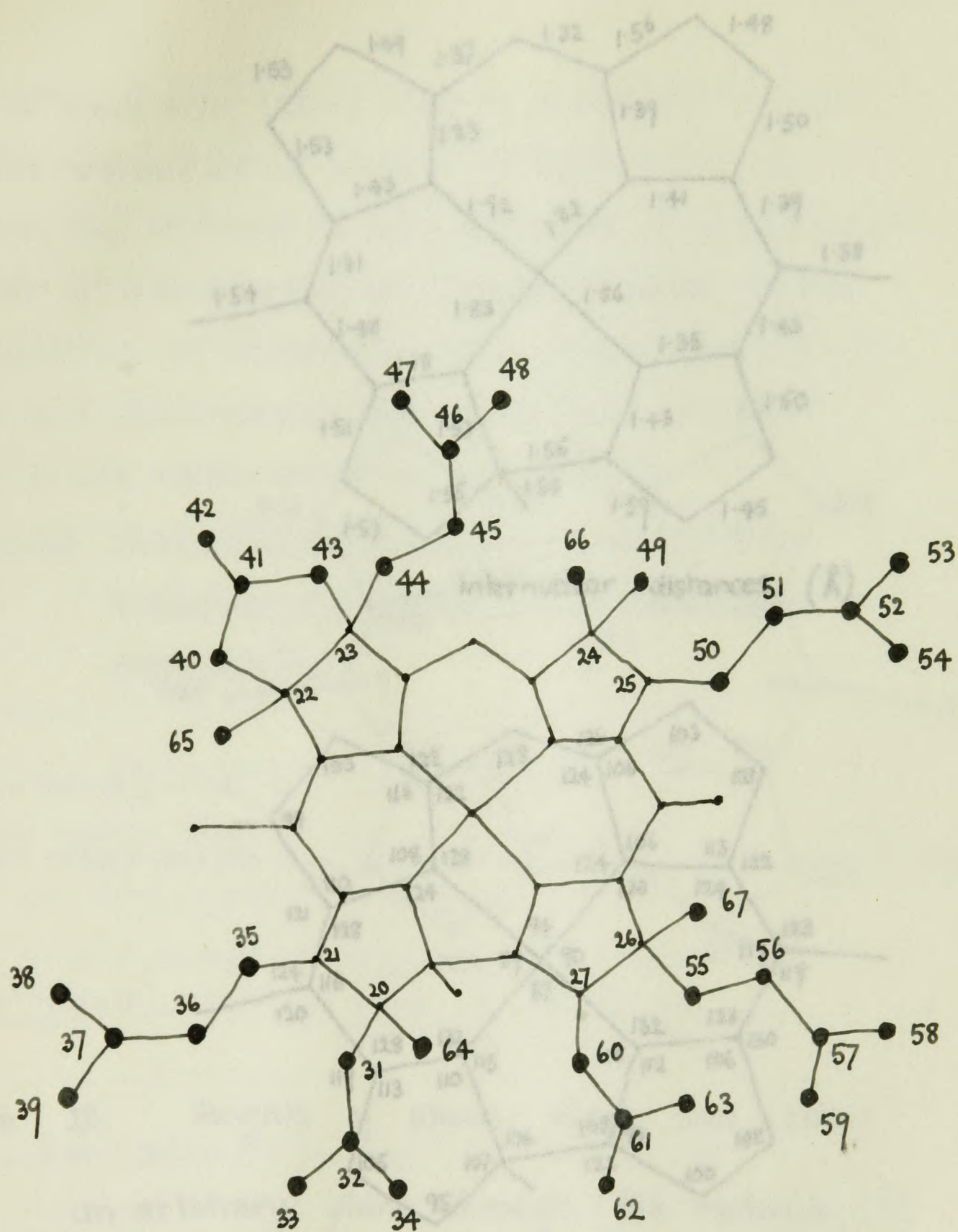


Figure 26. Numbering of atoms in the side chains.

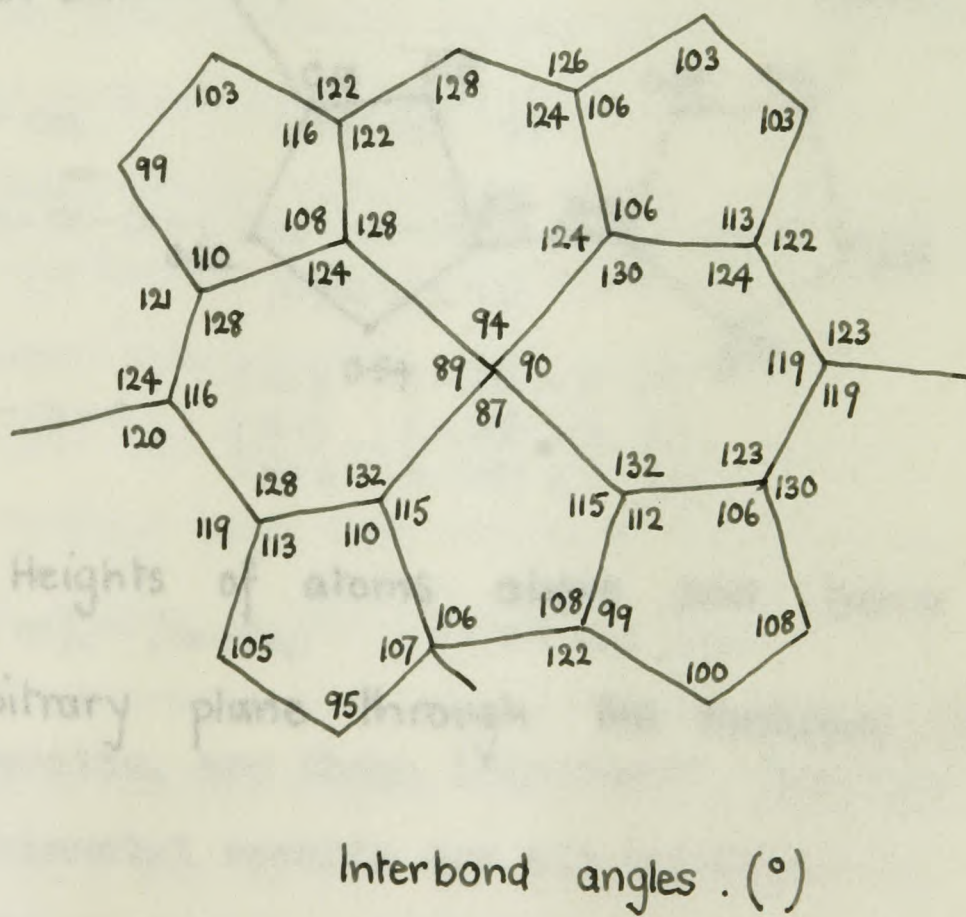
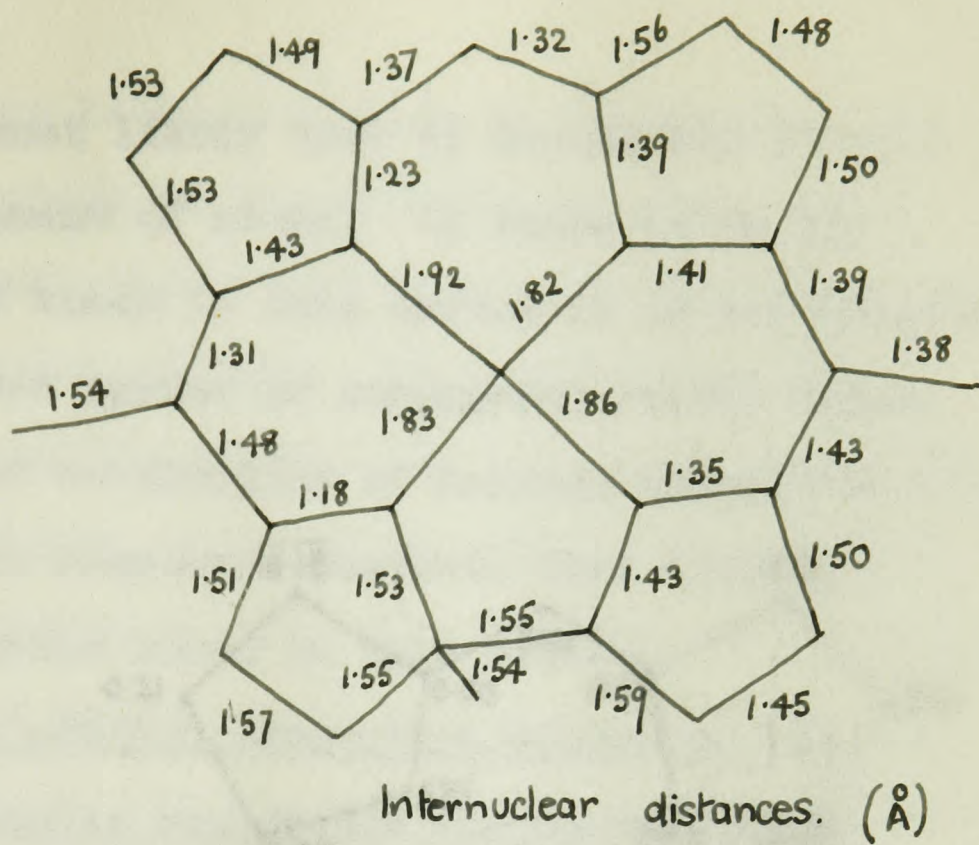


Figure 27. Dimensions of the inner nucleus.
(From final co-ordinates in Table 10)

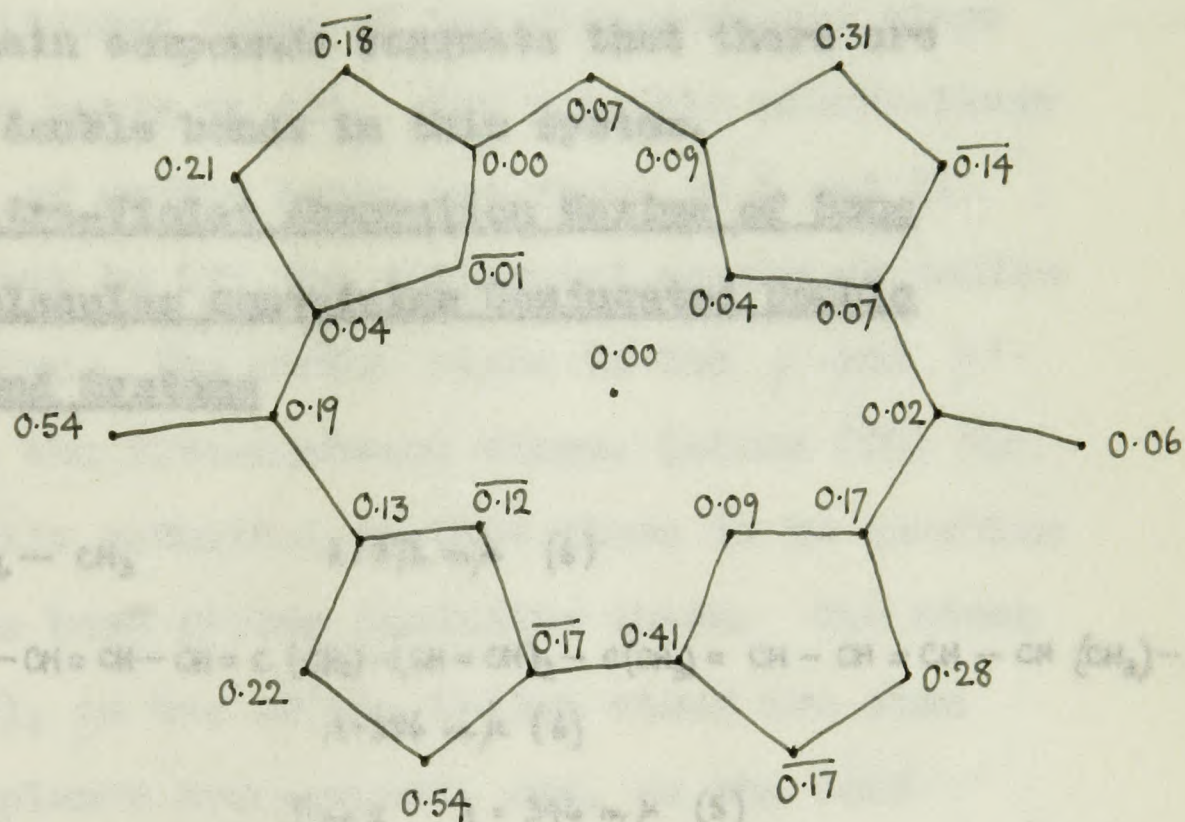
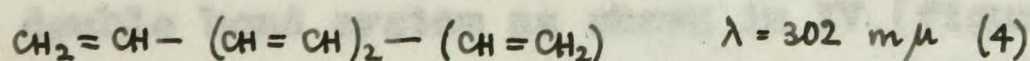
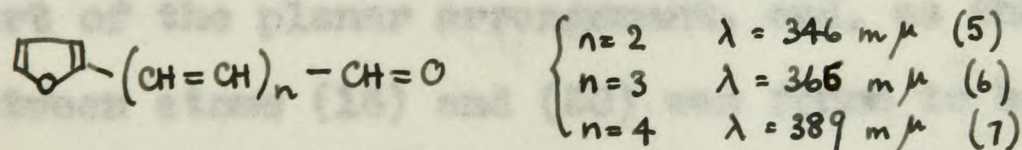
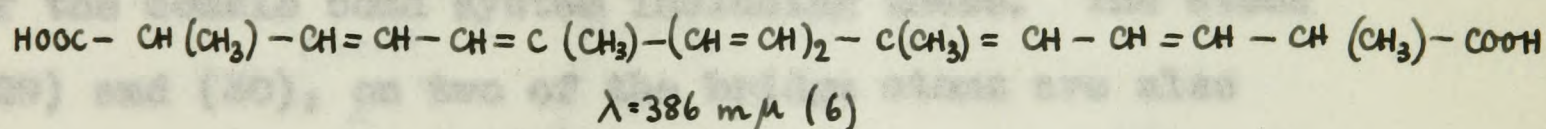
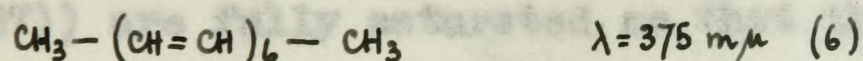


Figure 28. Heights of atoms above and below an arbitrary plane through the molecule. (Å)

and it seems most likely that it should lie in this planar arrangement of atoms. As there is an odd number, 15, of bonds in this system it is not possible to have a closed system of conjugated double bonds. A comparison of wavelengths of maximum absorption for some open chain compounds suggests that there are five or six double bonds in this system.

Table 16 Ultra-Violet Absorption Maxima of Some Molecules containing Conjugated Double Bond Systems



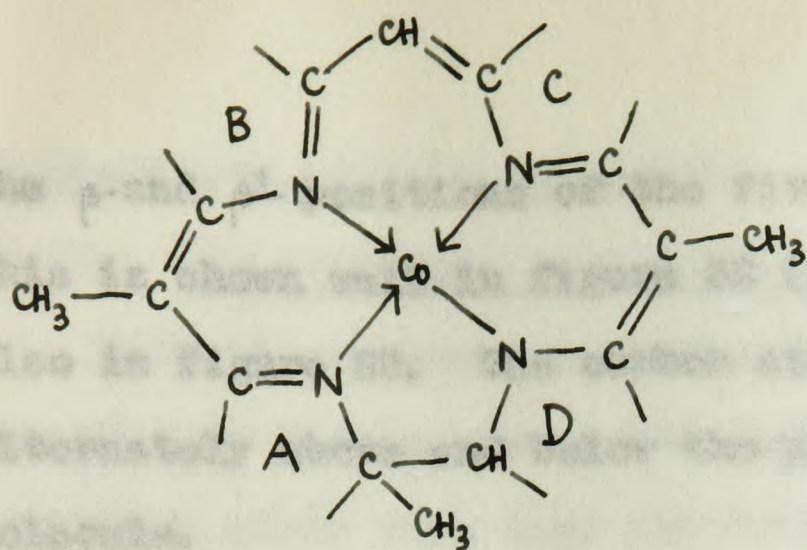
The question remains, are these localised? Unfortunately the experimental results are not sufficiently accurate to give an answer. Possible arrangements of

localised double bonds are shown in figure 29.

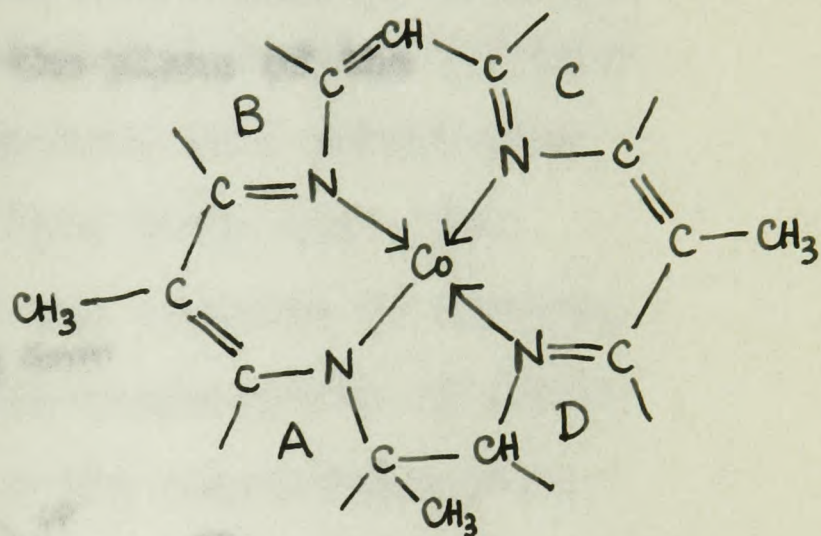
Cobalt is trivalent in the vitamin and there is no reason to suppose that its valency state has changed in the degradation product. It is bonded to the chloride and cyanide groups, and so must be bonded to one of the nitrogen atoms of the five-membered rings to make three bonds in all. Two possible mirror-image arrangements of double bonds are shown in I and II (figure 29) but in III the conjugated system of double bonds is broken. The carbon atoms in the β - and β' -positions in the five-membered rings, (atoms (20) to (27)) are fully saturated so that there is no question of the double bond system including these. The atoms (29) and (30), on two of the bridge atoms are also part of the planar arrangement, and, as the bond between atoms (16) and (30) was found to be rather short, it was thought that this might be included in the double bond system as shown in IV (figure 29). From the bond distances found, it seems that, if there is localisation of double bonds, then the arrangements of these shown in I and IV are the most likely.

The planar part is strained by the puckering of

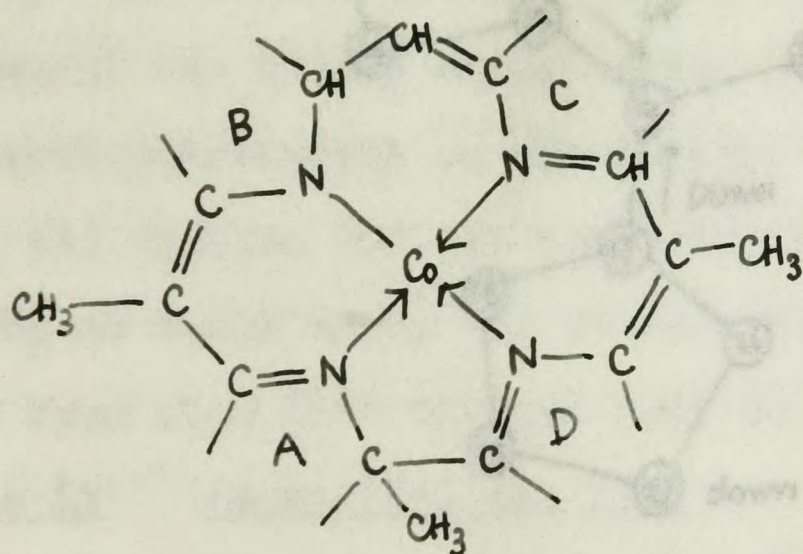
Figure 29. Some possible arrangements of double bonds in the planar group.



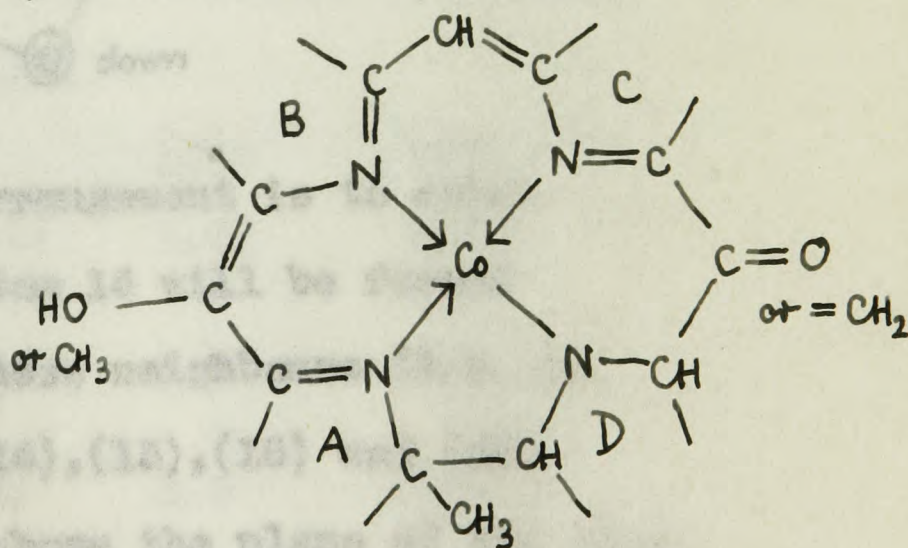
I



II



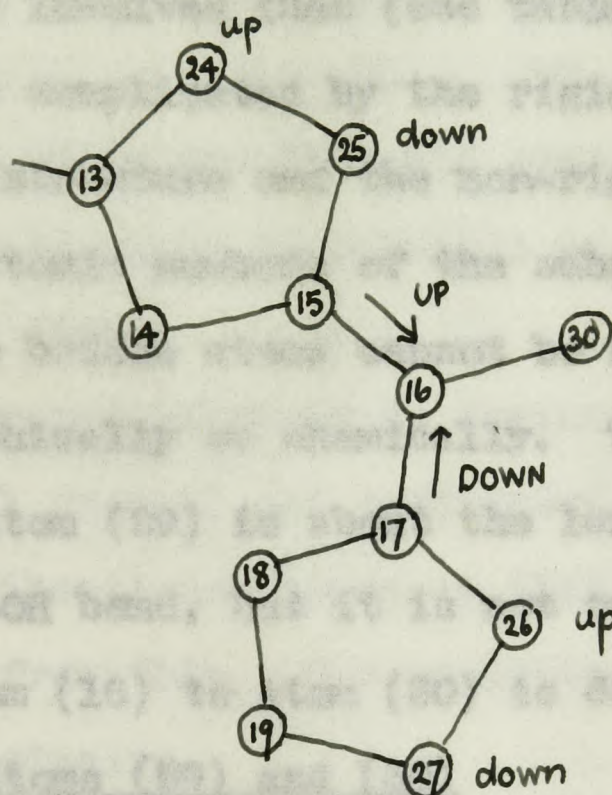
III



IV

Figure 29. Some possible arrangements of double bonds in the planar group.

the β - and β' -positions of the five-membered rings. This is shown well in figure 32 (see page 108) and also in figure 28. The carbon atoms (20) to (27) lie alternately above and below the plane of the molecule.



If, for example, a planar arrangement is to exist around atoms 15 and 17, atom 16 will be forced in opposite directions by these neighbours (i.e. to keep the system of atoms, (14), (15), (16) and (25) planar, atom (16) must lie above the plane of the ring, but if the system (16), (17), (18) and (26) is planar (16)

should lie below this plane).

2. Substituents on Two of the Bridge Atoms

In this structure analysis it was not generally possible to distinguish peaks due to carbon, nitrogen or oxygen atoms when they appeared in a calculation which had not involved them (see table 15). The situation was complicated by the rigidity of certain parts of the structure and the non-rigidity of other parts. The atomic numbers of the substituent atoms on two of the bridge atoms cannot be found either crystallographically or chemically. The bond from atom (8) to atom (29) is about the length of a C—CH₃ or C—OH bond, but it is not certain if the bond from atom (16) to atom (30) is double or single.

Table 17 Atoms (29) and (30)

	<u>(29)</u>	<u>(30)</u>
Bond length to bridge atom (Å) (in 68 atom calculation)	1.57	1.38
Peak height in 26 atom calculation (e/Å ³)	3.3	2.7
Peak height in 68 atom calculation (e/Å ³) put in atomic number 7	8.2	8.2

It is rather long for the group to be a carbonyl group. The possibilities are:-

<u>Group at Atom 29</u>	<u>Group at Atom 30</u>
-------------------------	-------------------------

a) $-\text{CH}_3$

c) $-\text{CH}_3$

b) $-\text{OH}$

d) $=\text{CH}_2$

e) $-\text{OH}$

f) $=\text{O}$

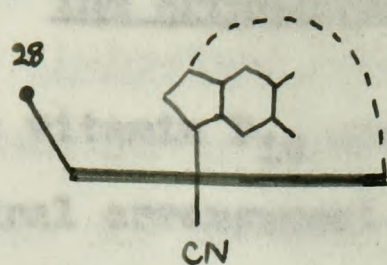
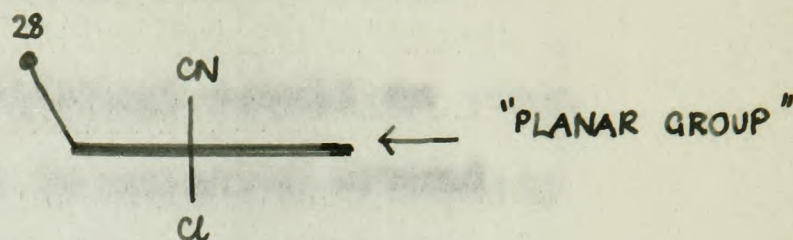
A combination of b) and f) would give a keto-enol system and so it might be possible to obtain two non-identical forms.

The infra-red spectrum is dominated by carbonyl (from carboxyl groups) and conjugated ($\text{C}=\text{C}$ and $\text{C}=\text{N}$) double bond vibrational bands so that no conclusions may be drawn from this. The analytical figures are equally inconclusive.

Table 18 Analytical Figures for the Hexacarboxylic Acid

Atoms (29) and (30)	C%	H%	N%
20	52.7	5.33	6.99
O, C	54.0	5.54	7.00
2C	55.3	5.76	7.02
Found	52.8	6.28	7.85

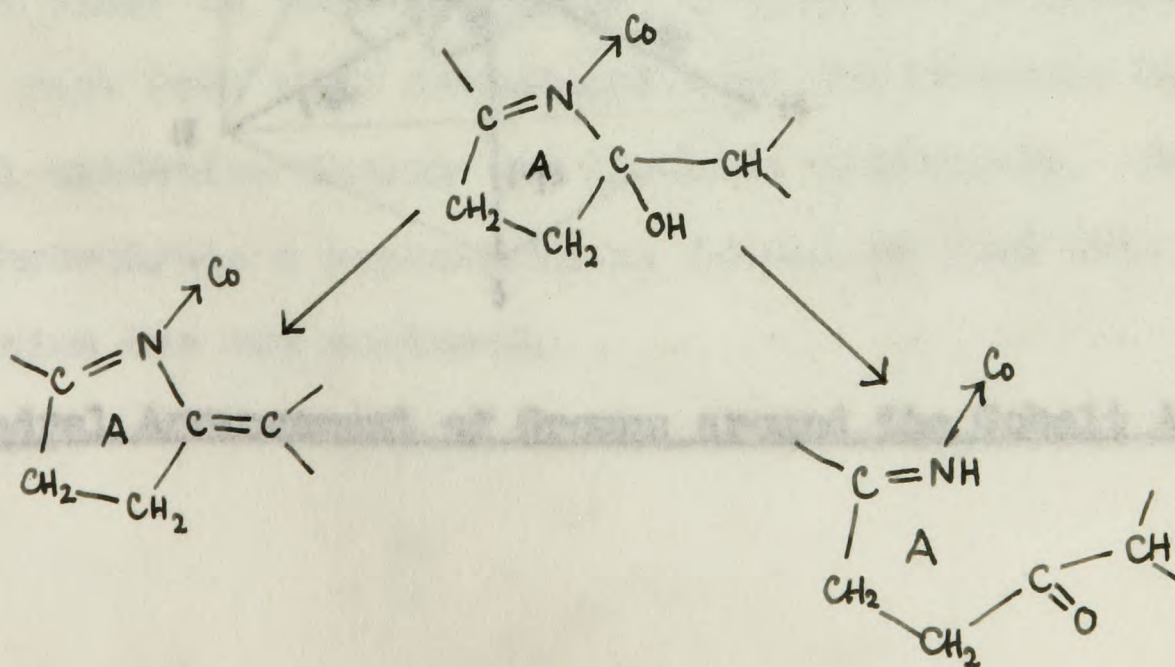
Neither of these atoms, (29) or (30), is within a hydrogen bonded distance (about $2.7 \text{ \AA}$) of another

VITAMIN B₁₂

DEGRADATION PRODUCT

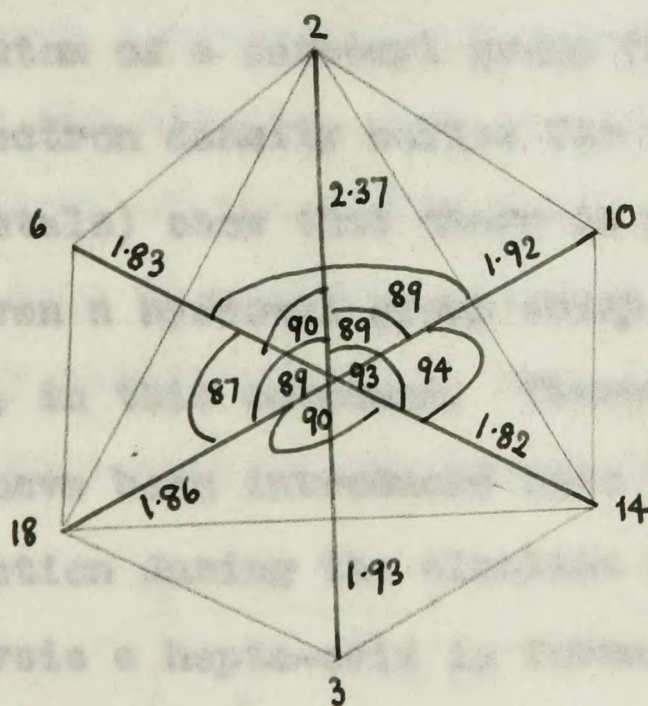
During the alkaline hydrolysis the cyanide group has been replaced by a group, presumably a hydroxyl group, and the nucleotide by a cyanide group. The hydroxyl is later replaced by chloride during the working-up of the hydrolysis mixture.

The atom (28) must be the carbon atom of a methyl group. It is then effectively the missing bridge atom. If it were a hydroxyl group the ring to which it is attached should open readily. Also the tertiary alcohol it would represent should dehydrate easily.



4. The Arrangement of Atoms Around the Cobalt Atom

As vitamin B₁₂ contains trivalent cobalt an octahedral arrangement of atoms is expected around this. In the degradation product the four nitrogen atoms of the "planar group", and the chloride and cyanide groups surround the cobalt but the configuration is not that of a regular octahedron, for the Cl-Co-C≡N line is not quite perpendicular to the planar group. Also in the "planar group" the N-Co-N angles are not all 90° owing to the dissymmetry of the arrangement due to the lack of one bridge atom.



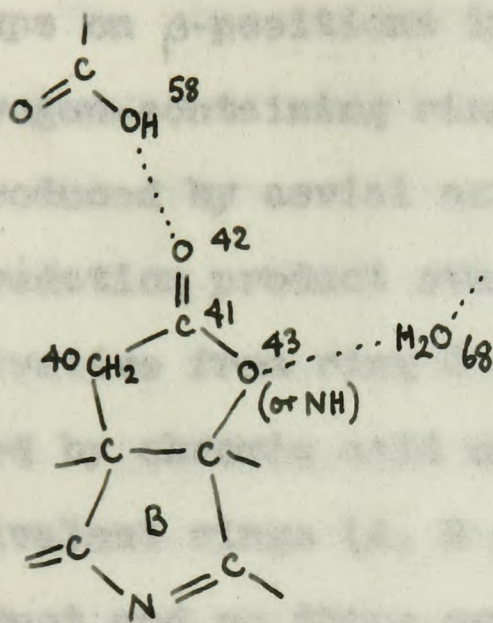
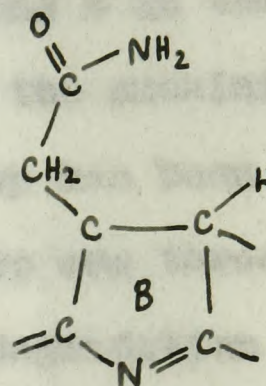
Octahedral Arrangement of Groups around the Cobalt Atom

B. Side Chains

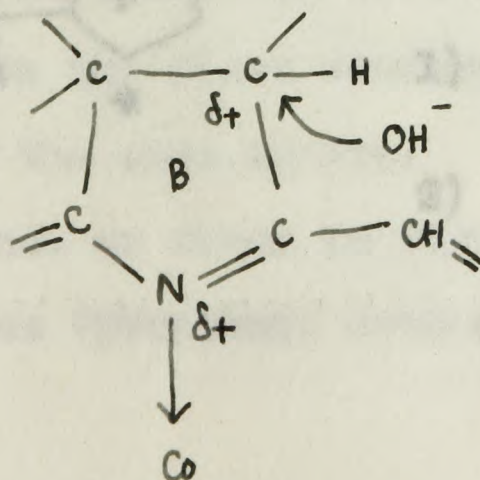
1. γ -Lactone Ring

Attached to ring B there is a five-membered ring. The peaks due to this in the 26 atom electron density maps were high, especially for atoms (42) and (43) (see figure 11), possibly because this ring represents a rigid structure. As atom (43) is about $2.7 \text{ \AA}$ from an isolated peak, (68), a water molecule, it was supposed to be an oxygen (or nitrogen) atom. The other high peak, (42), is about $1.2 \text{ \AA}$ from another peak (41). These must then be in a carbonyl group. The oxygen atom, (42), of this is hydrogen bonded to the oxygen atom of a carboxyl group (58).

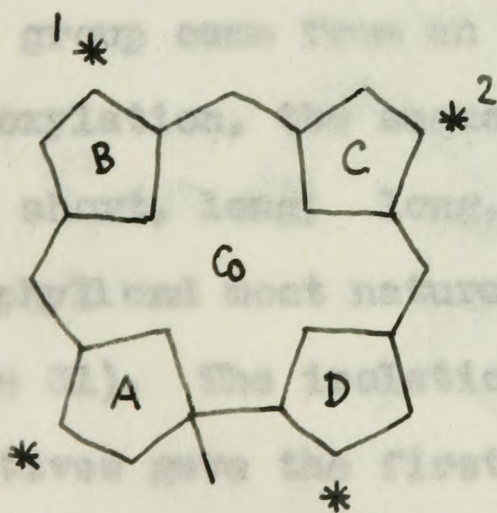
The electron density series for vitamin B₁₂ (wet and dry crystals) show that there is no equivalent ring, not even a hydroxyl group which could have formed such a ring, in this compound. Therefore a hydroxyl group must have been introduced into the molecule by aerial oxidation during the alkaline hydrolysis. On acid hydrolysis a hepta-acid is formed so that this oxidation has not occurred.

degradation productvitamin B₁₂

The mechanism of the introduction of a hydroxyl group into such a system is not clear. A deficit of electrons on the nitrogen atom is implied, regardless of the positions of the double bonds in the conjugated system, and this is possibly aided by the cobalt atom. Alternatively peroxidation may have occurred. These suggestions were made by Sir Robert Robinson.



Two compounds have been isolated with hydroxyl groups on β -positions in the five-membered nitrogen-containing rings. A hydroxyl group has been introduced by aerial oxidation into ring B in the degradation product studied here. In the succinimide derivative from ring C a hydroxyl group has been introduced by chromic acid oxidation. There are three equivalent rings (A, B and C) in the degradation product and so three equivalent positions which could be hydroxylated. Therefore mixtures due to degradation products differing in the positions of hydroxyl groups (or lactone rings) may result.



* hydrogen atom

1) hydroxyl group in hexacarboxylic acid

2) hydroxyl group in succinimide derivative

The ring could be either a lactone or lactam ring. The nitrogen analysis figures give 5.5 atoms per molecule of which 5 have been accounted for. Again the evidence from the infra-red spectrum is inconclusive. The present studies cannot differentiate between the two possibilities.

2. Acetic and Propionic Acid Side Chains

Attached to the β - and β' positions of the five-membered rings are groups which, from their stereochemistry and the original chemical evidence, were taken to be acetic acid ($-\text{CH}_2-\text{COOH}$) and propionic acid ($-\text{CH}_2-\text{CH}_2-\text{COOH}$) side chains. The sequence of these was not regular but, assuming one methyl group came from an acetic acid residue by decarboxylation, the sequence was short, long; short, long; short, long; long, short: as in haematin, chlorophyll and most naturally occurring porphyrins (figure 31). The isolation of the succinimide derivatives gave the first chemical evidence of the full nature of the side chains.

It is found, as shown in figure 32, that all the long side chains (propionic acid groups) lie on the

(from final co-ordinates in table 2)

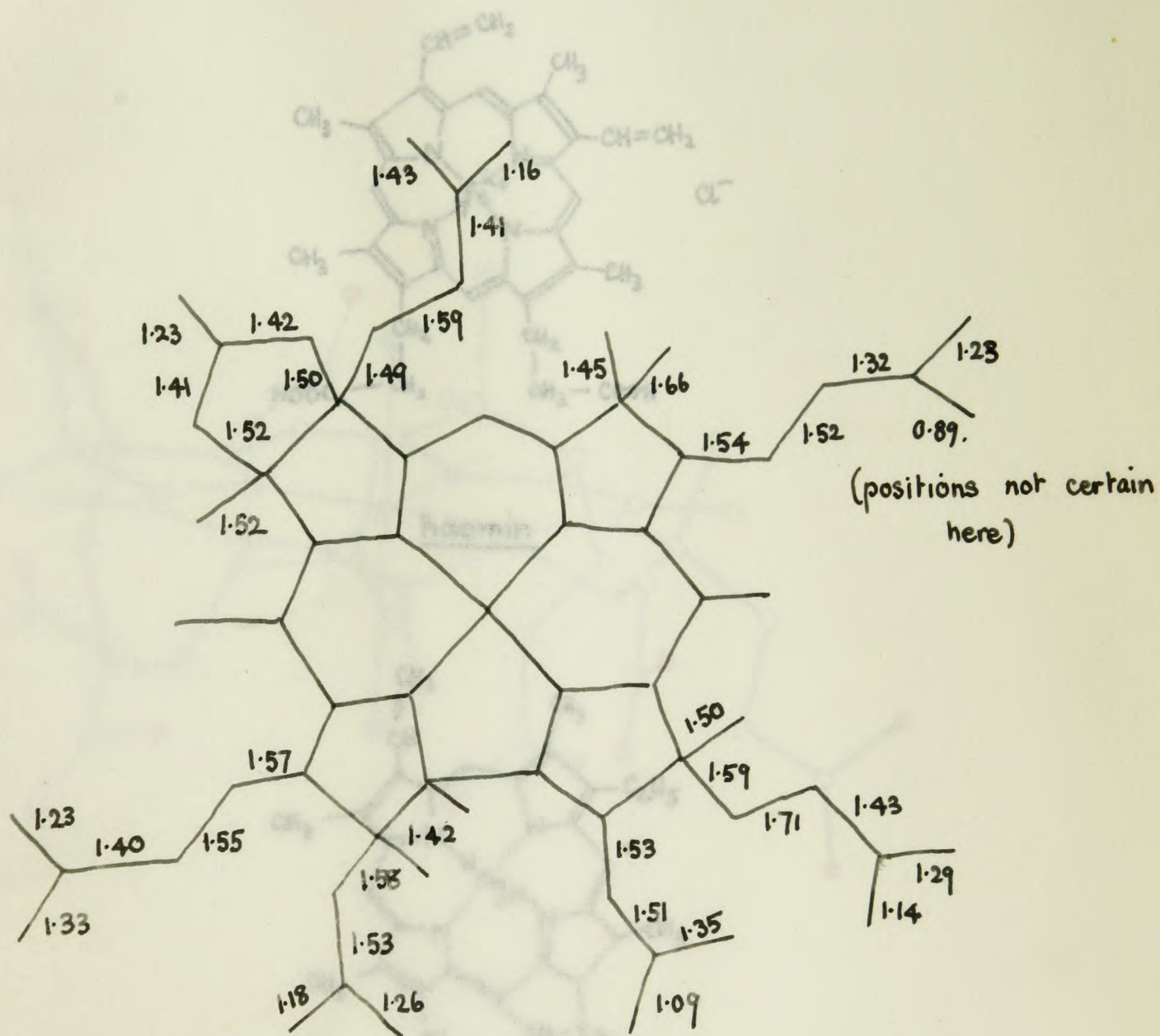
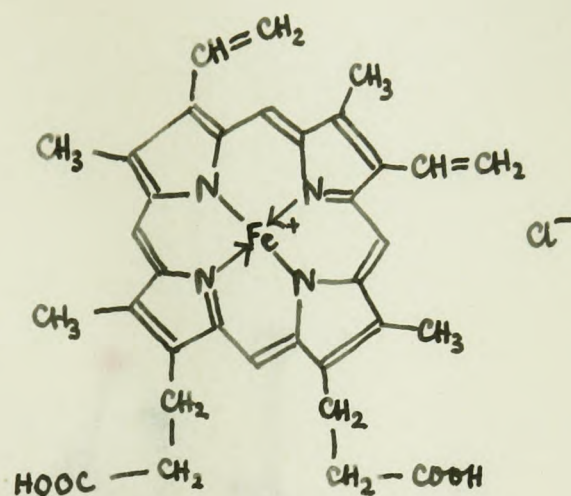
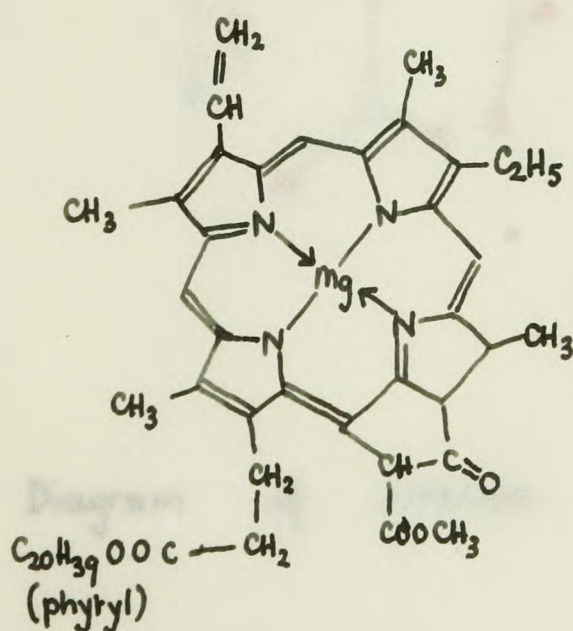


Figure 30. Internuclear distances in side chains.
(from final co-ordinates in table 10).

Figure 31. Formulae of some porphyrins.



haemin



chlorophyll a.

Figure 31.

Formulae of some porphyrins.

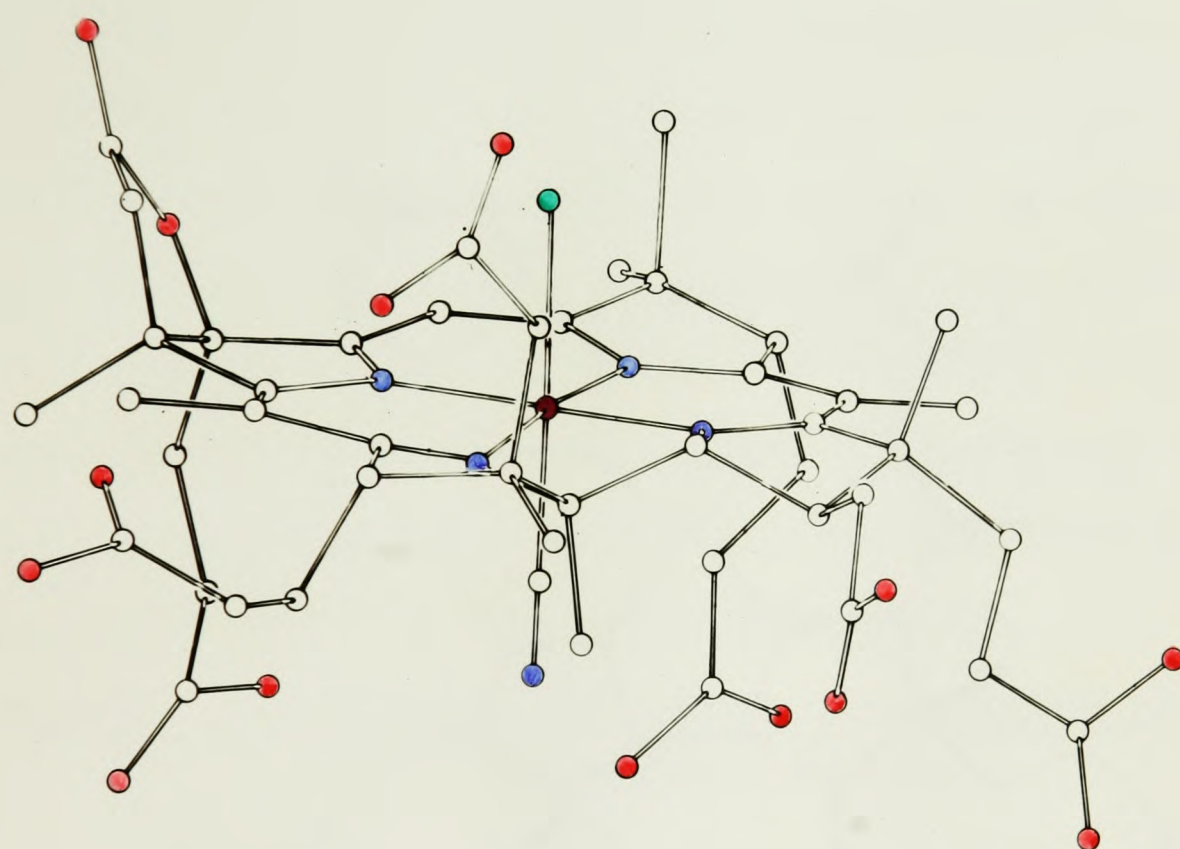


Figure 32. Diagram of molecule.

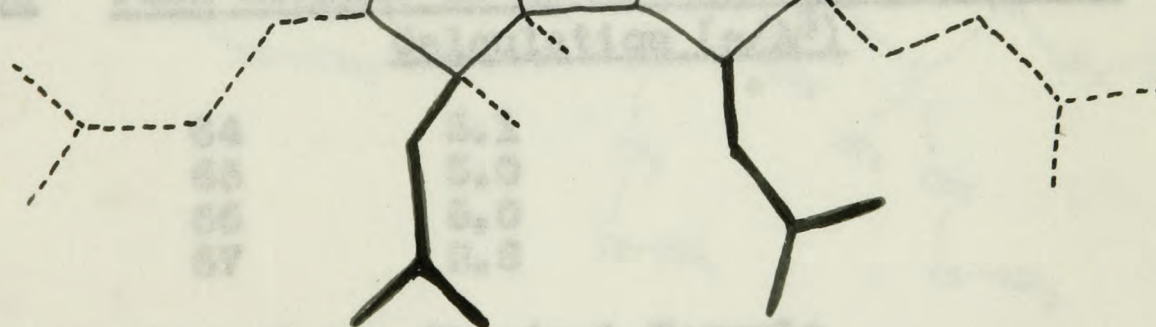
cobalt — purple
chlorine — green.
nitrogen — blue
carbon — white
oxygen — red.

same side of the "planar group" as the "planar group".
 and the shorter chains (acetic acid) and the shorter chains (acetic acid) lie on the same side of the
 "planar group" as the "planar group". In the vitamin the
 benzene ring is held in by the longer chains.

3. Stereochemistry

There were single bonds attached to one
 β -position in each ring. These could have been due
 to either methyl or ethyl groups, but the first
 suggestion is probably, otherwise the carbon count
 in the molecule would be wrong, and lactones could be
 formed or hydrolyzed to form
 lie in a regular manner. The ring B is
 quite different from the other rings.

Table 22 Peak Heights of Methyl Groups in 30 MHz



C. Discussion of the Chemical Formula

The chemical formulae of vitamin B_{12} and its hom-
 carboxylic acid degradation product are given in

Figure 33. Stereochemistry of side chains

same side of the "planar group" as the cyanide group, and the shorter chains (acetic acid groups and the γ -"lactone" ring) lie on the same side of the "planar group" as the chloride. In the vitamin the benziminazole is walled in by the longer chains.

3. Methyl Groups

There were single atomic peaks attached to one β -position in each ring. These could have been due to either methyl or hydroxyl groups, but the first suggestion is preferable, otherwise the carbon count in the molecule would be wrong, and lactones could be formed or dehydration could occur. Surprisingly these lie in a regular sequence which means that ring D is quite different from the other three rings.

Table 20 Peak Heights of Methyl Groups in 26 atom
Calculation (e/A^3)

64	3.1
65	3.0
66	3.0
67	2.8

C. Discussion of the Chemical Formula

The chemical formulae of vitamin B₁₂ and its hexacarboxylic acid degradation product are given in figures 34 and 24 respectively. The arrangement of

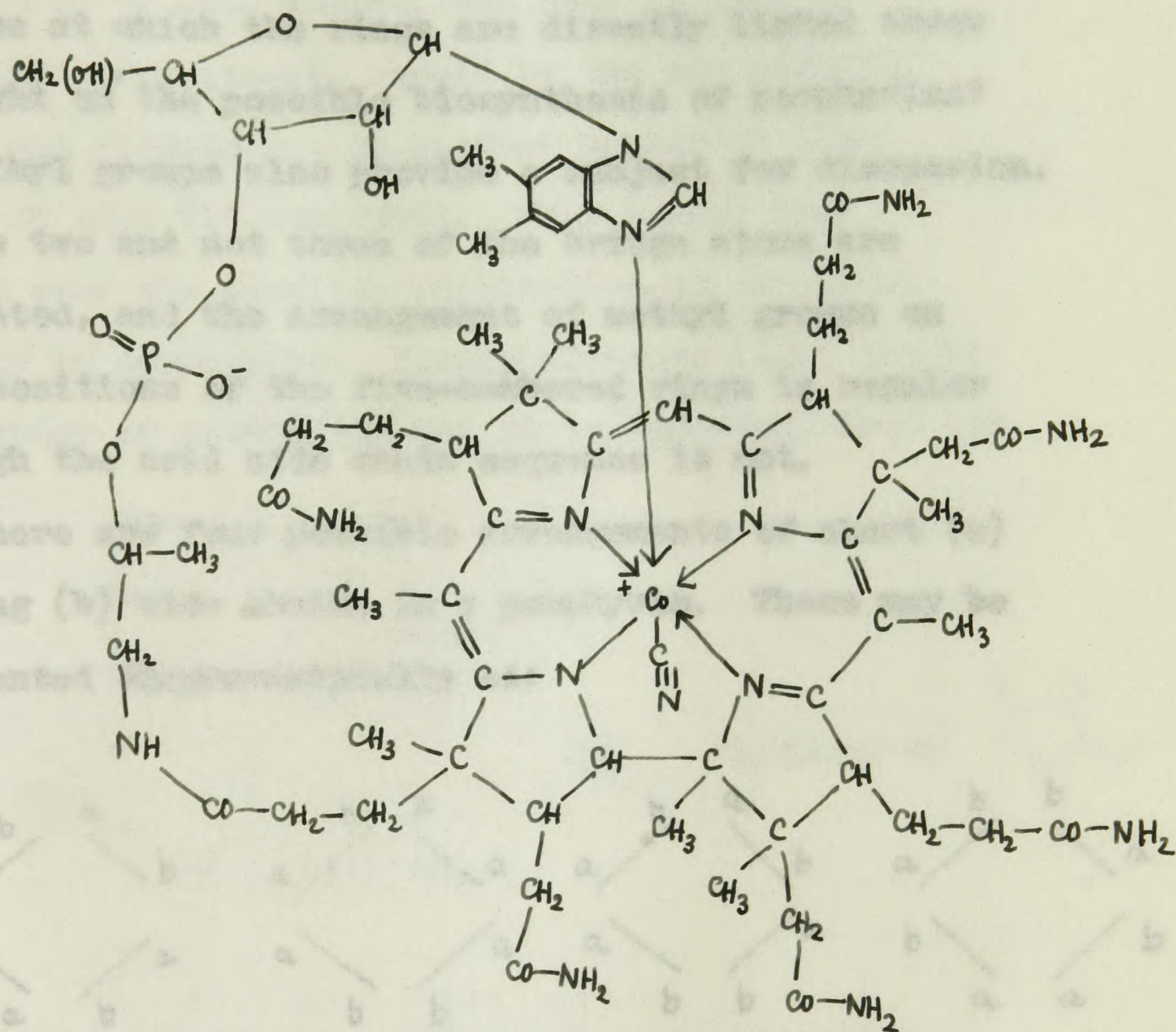
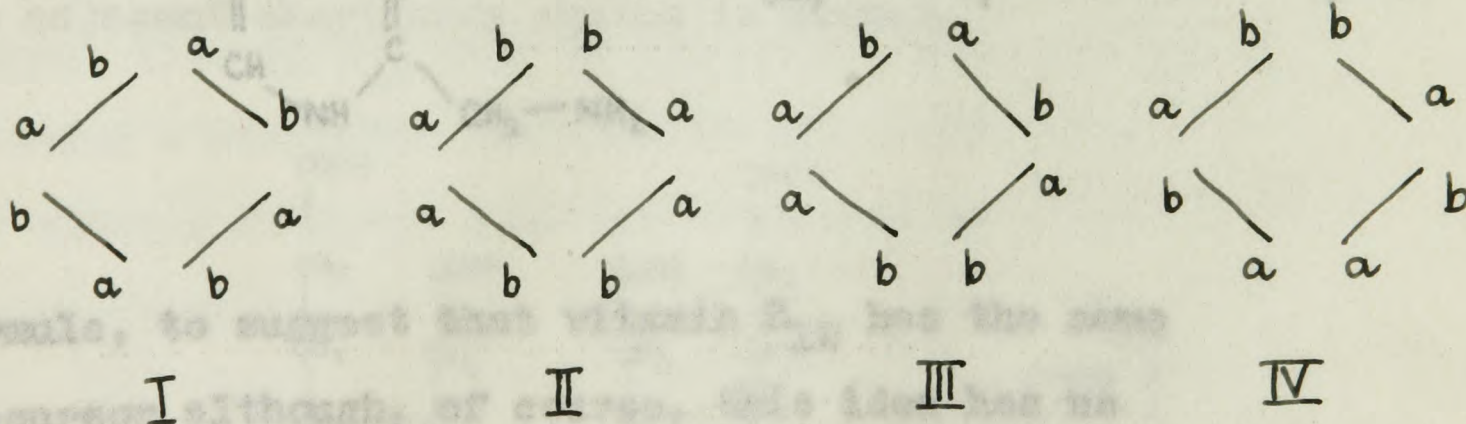


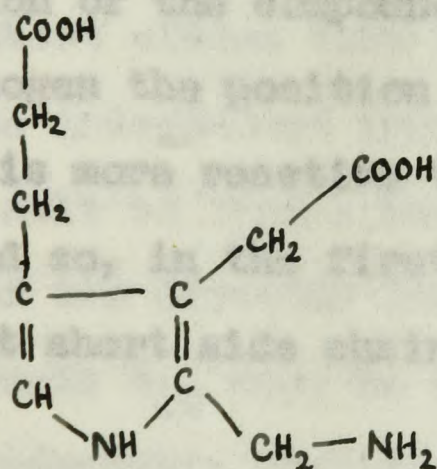
Figure 34. Formula tentatively suggested for vitamin B₁₂.

atoms in the hexacarboxylic acid and in the analogous part of the vitamin gives rise to many questions, for instance why is one bridge atom excluded from a porphyrin, and does the point in the side chain sequence at which the rings are directly linked throw any light on the possible biosynthesis of porphyrins? The methyl groups also provide a subject for discussion, because two and not three of the bridge atoms are methylated, and the arrangement of methyl groups on the β -positions of the five-membered rings is regular although the acid side chain sequence is not.

There are four possible arrangements of short (a) and long (b) side chains in a porphyrin. These may be represented diagrammatically as:

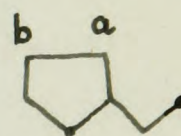


The dissymmetry must be produced first to give a compound in which the reversal is no longer possible. Type III compounds are generally found in nature although, rarely, type I compounds may be found. It is therefore exceedingly interesting that only one arrangement of side chains, and what is more an asymmetric arrangement, should be the one normally found, and vitamin B₁₂ is another member of this series of type III compounds. Many people have puzzled over the possible explanation of this dissymmetry in porphyrins. Porphobilinogen ⁴⁸⁾ (XII) can be a precursor of porphyrins and it is tempting, from its



(XII)

represented as

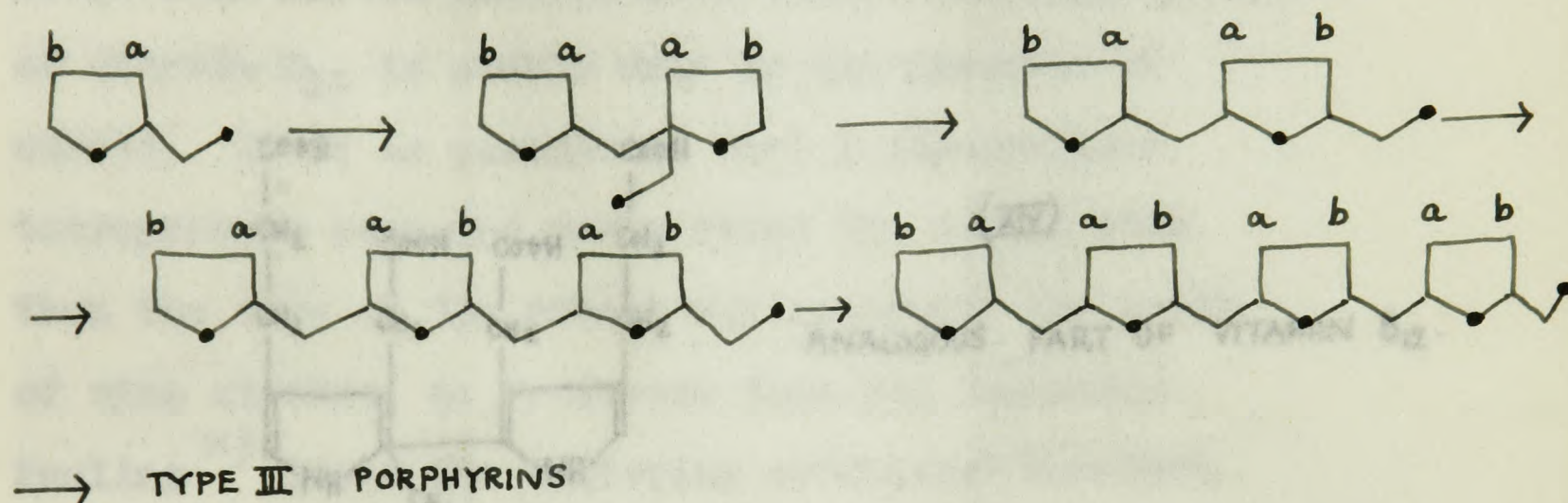


formula, to suggest that vitamin B₁₂ has the same precursor although, of course, this idea has no experimental basis. Robinson has a theory of porphyrin biosynthesis which it is interesting to discuss here. ⁴⁹⁾

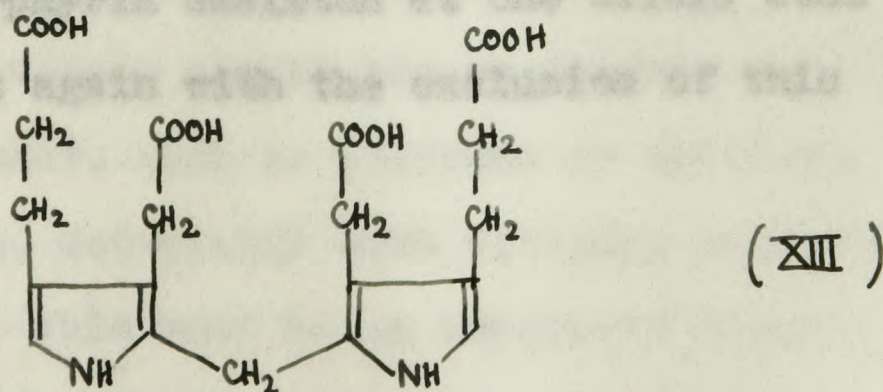
SUGGESTED FIRST STAGE IN PORPHYRIN SYNTHESIS

The dissymmetry must be produced first to give a compound in which the reversal is no longer possible.

He postulates the following series of reactions:

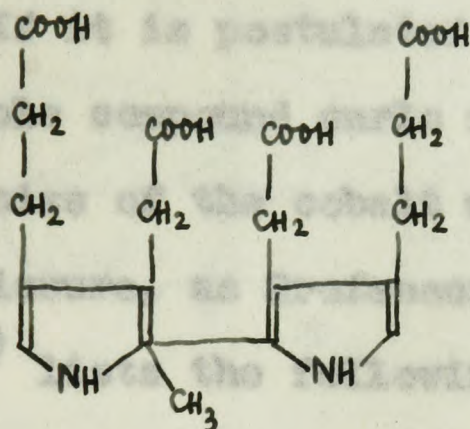


This theory is quoted because the initial stage is the formation of the compound (XIII). The idea is that, in porphobilinogen the position ortho to the acetic acid side chain is more reactive than the other ortho position and so, in the first stage, a compound with two adjacent short side chains is formed.



SUGGESTED FIRST STAGE IN PORPHYRIN SYNTHESIS

Yet in vitamin B₁₂ the two adjacent acetic acid residues are on either side of the direct link (XIV).



(XIV)

ANALOGOUS PART OF VITAMIN B₁₂.

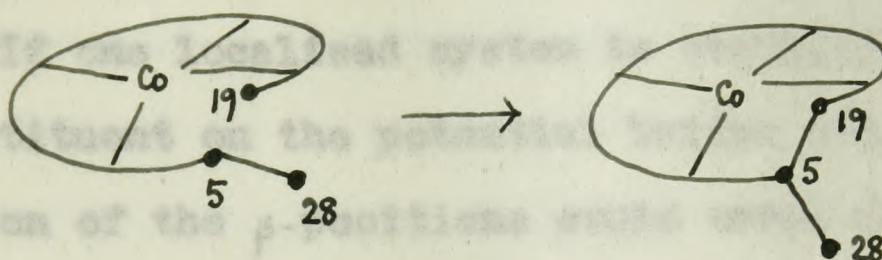
Sir Robert claims that his theory cannot be used for the independent synthesis of vitamin B₁₂. It is difficult to find a mechanism for the initial formation of two directly linked five-membered rings. Vitamin B₁₂ may, he suggests, be formed by breaking a porphyrin skeleton at one bridge atom and closing it again with the exclusion of this bridge atom.

But why is this compound formed in the first place? Cobalt porphyrins are stable⁵⁰⁾ but the metal atom may be removed from them. The ring system of vitamin B₁₂ is stable only in the presence of cobalt. If it is postulated that a non-cyclised tetrapyrrole compound curls round the cobalt atom then the size of the cobalt may determine the point of ring closure, as Professor Todd has suggested. Pauling⁵¹⁾ lists the following octahedral covalent radii:

	Fe ^{III}	1.23 Å
	Fe ^{IV}	1.20 Å
	Co ^{III}	1.22 Å

so that it seems that trivalent cobalt has about the same covalent radius as iron. Therefore, if the size of the metal atom were the sole determining factor, an analogous compound with, for example, iron might be expected. Trivalent cobalt has as strong a tendency as any other element, such as platinum or chromium, to form complexes, especially with nitrogen containing compounds⁵²⁾. This must be an important feature in the formation of vitamin B₁₂. Also, as mentioned on page 96,

the puckering of the five-membered rings in vitamin B_{12} causes a strain in the conjugated double bond system. The effect of this will be to cause atom (19) to lie above, and not at the same level as, atom (5).



If, owing to the stereochemistry about atom (5), the last bridge atom, (28), lies in the opposite direction to or too far away from atom (19) and a bond between these cannot be formed, then a direct link from atom (5) to atom (19) would form if this were chemically possible. It has been assumed that, whereas porphyrins are preformed and the metal atom enters the skeleton if it can do so without distorting it too much, the precursor of vitamin B_{12} undergoes ring closure after the introduction of the metal atom and it is the strength of the Co—N link which causes the steric situation

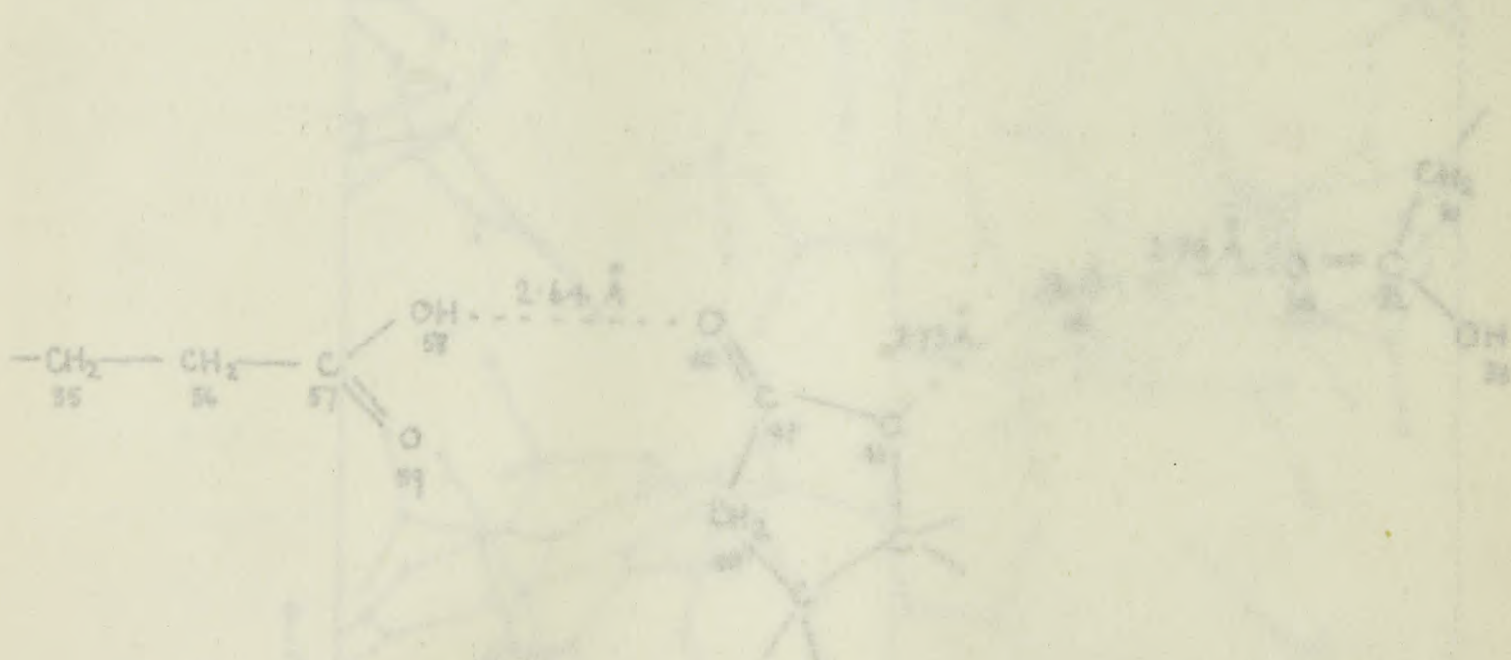
described above.

The methyl groups are arranged at the β -positions of the five-membered rings in a regular sequence. This indicates, as Professor Todd pointed out, that methylation must have occurred after the formation of a tetrapyrrole compound, rather than when porphobilinogen was formed. If one localised system is stabilised, perhaps by a substituent on the potential bridge atom, the methylation of the β -positions could occur as expected. The strange fact is not that the sequence of these methyl groups is regular, but that only two of the three bridge atoms are methylated. These could be methylated if they were the carbon atoms of methylene groups α to —C=N— groups and it is difficult to see why, if this is the case at some stage, it should not be so for all three bridge atoms.

Now that the formula of this compound is known, many such questions will be asked. The oxidative degradation products are consistent with such a formula, but chemical verification for some parts of the structure is lacking. At Cambridge, experiments are in progress in which attempts are being made to isolate all possible

degradation products. When these are completed the road will be clear for biogenetic studies on vitamin B₁₂ to proceed.

The molecule, which is rather an awkward shape, packs in the unit cell making contacts where it can. It seems that it is rarely possible for direct hydrogen bond contacts to be made. There appears to be one well-defined and rigid hydrogen bond system, which includes the lactone ring, one water molecule and two carboxyl groups.

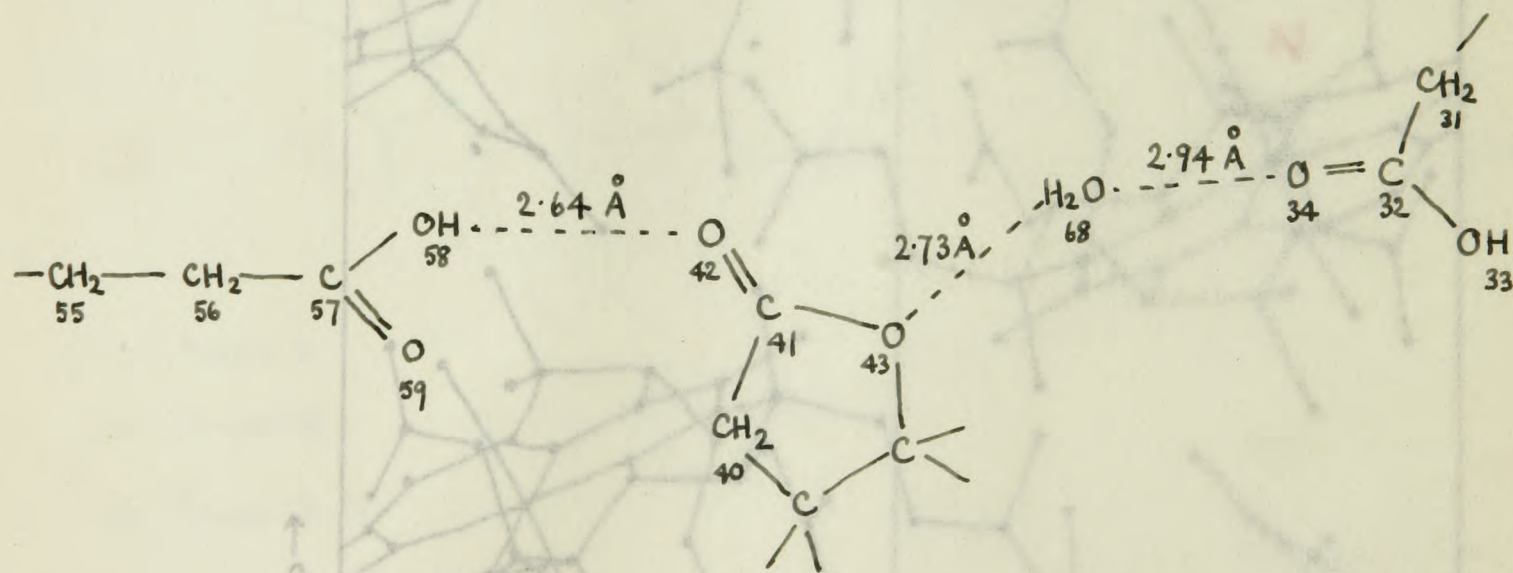


Approximate hydrogen bond distances from structure
FIGURE 36

The Arrangement of Atoms in the Crystal

A diagram of the molecule is given in figure 32 and the manner in which it packs in the unit cell is shown in figures 35 and 36.

The molecule, which is rather an awkward shape, packs in the unit cell making contacts where it can. It seems that it is rarely possible for short hydrogen bond contacts to be made. There appears to be one well-defined and rigid hydrogen bond system, which includes the lactone ring, one water molecule and two carboxyl groups.



Approximate hydrogen bond distances near the lactone ring

Figure 35. Arrangement of four molecules in the unit cell.

(N = near side of planar group)

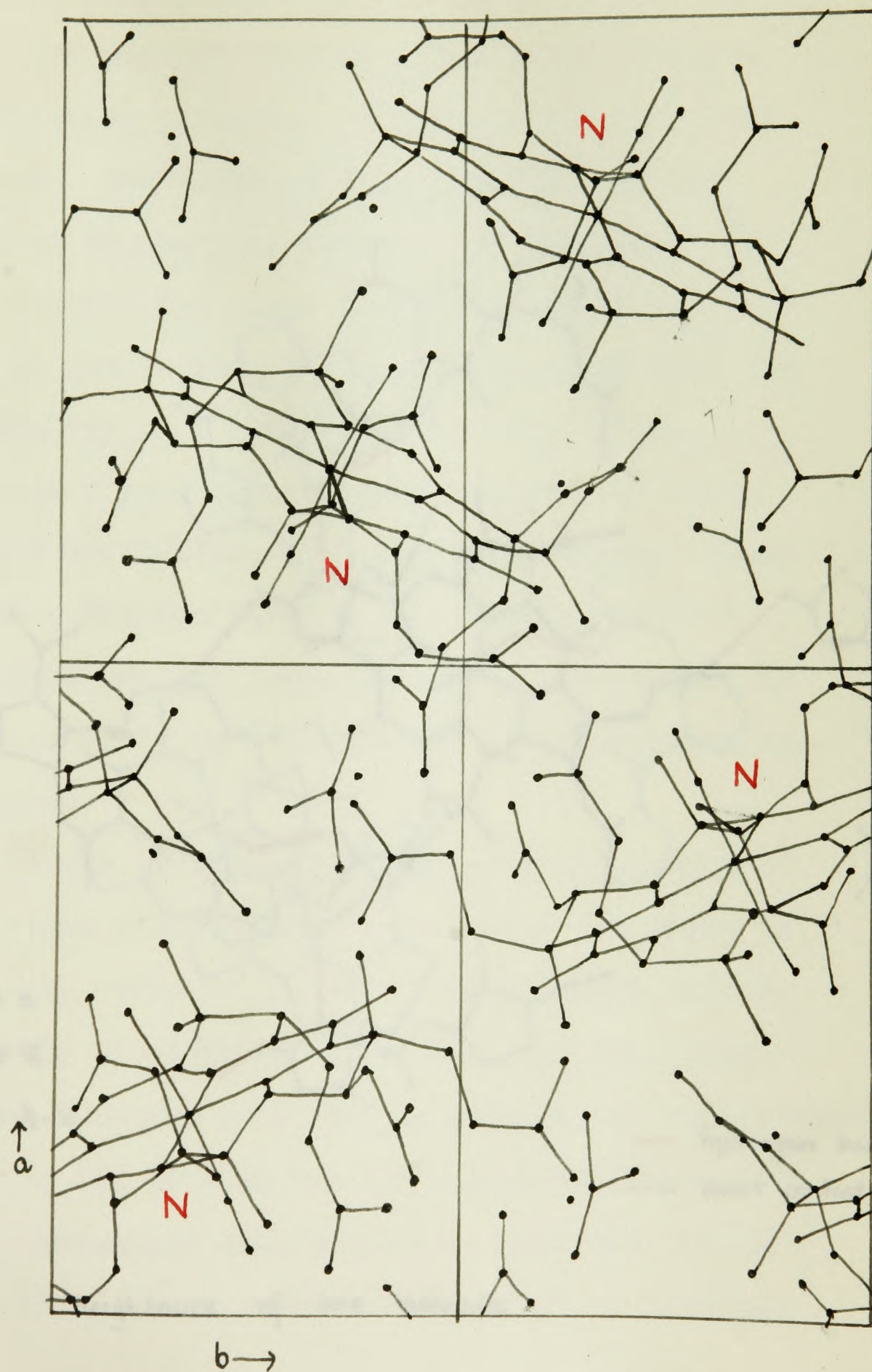


Figure 35. Arrangement of four molecules in the unit cell.
 (N = near side of planar group)

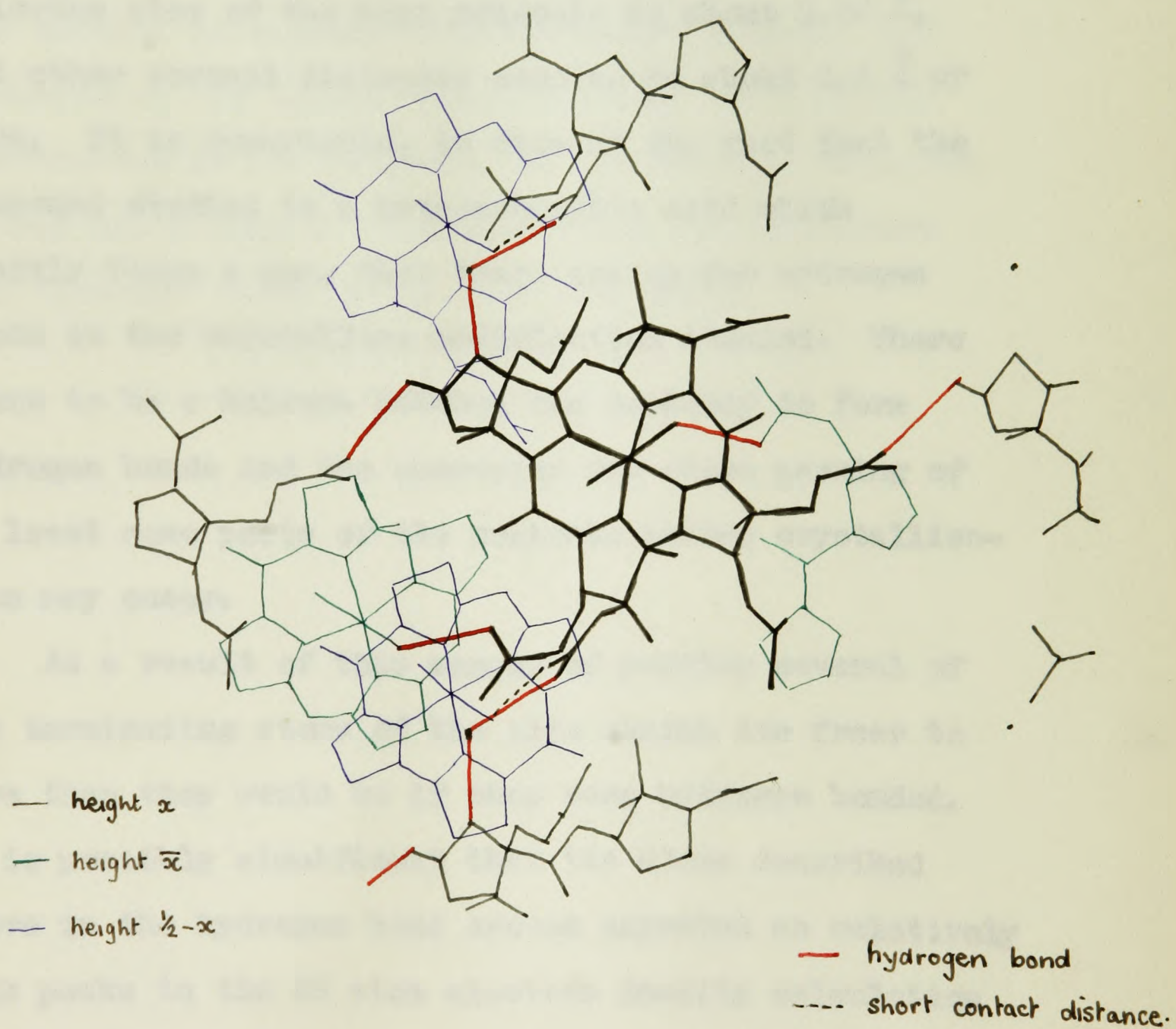


Figure 36. Neighbours of one molecule

There is also a hydrogen bond between atom (39) of a carboxyl group and the nitrogen atom of the cyanide group of the next molecule, as these atoms are about 2.76 Å apart. The distance from atom (33) to the chlorine atom of the next molecule is about 3.05 Å. All other contact distances seem to be about 3.4 Å or more. It is remarkable, in view of the fact that the compound studied is a hexacarboxylic acid which readily forms a gum, that there are so few hydrogen bonds in the crystalline modification studied. There seems to be a balance between the tendency to form hydrogen bonds and the necessity for close packing of at least some parts of the molecule before crystallisation may occur.

As a result of this manner of packing several of the terminating atoms of the side chains are freer to move than they would be if they were hydrogen bonded. It is possibly significant that the atoms described above in the hydrogen bond system appeared as relatively high peaks in the 26 atom electron density calculation and in all subsequent calculations. When the end of a side chain is not held firmly in position by hydrogen

bonds then it may be free to move in the space available or, alternatively, disorder may arise—and this is possibly the case for the last side chain—when a water molecule is lost or moves as the crystal is removed from the mother liquor. The presence of the second water molecule, (76), is uncertain and is not indicated by the chemical analyses. It may exist only in some unit cells and so produce the disorder found in the last side chain.

Appendix

A Comparison of Electron Density and Phase

Distribution Calculations

This work represents the analysis, by means of successive three-dimensional electron density calculations, of the structure of a very large molecule. Although the method has been successful in this case, it is not suggested that it could be used for all such complicated molecules. Each structure determination presents its own problems and suitable methods of overcoming these must be found.

Patterson function is set down with $1/r$ instead of $1/r^2$ in terms of the equivalent positions (four in the case of the space group $P2_12_12_1$) of each heavy atom in the unit cell. Peaks in the resulting distribution then indicate the positions of other atoms. This calculation may be made directly, simply by adding the heights of the Patterson function for each superposition, but the method is tedious and, for the calculation of a three-dimensional distribution, mechanical computing is preferable. The terms which are to be used in such a calculation may be derived as follows:

Appendix

A Comparison of Electron Density and Vector Convergence

Distribution Calculations

In the early stages of the structure determination it was clear that phase angle calculations could only involve a partial molecular structure. It was not certain which Fourier method of calculation would best indicate the structure of the remainder of the molecule. Beevers and Robertson⁵³⁾ suggested the use of a vector convergence distribution (V.C.D.) in the determination of structures in which there is a heavy atom. The Patterson function is set down with its origin in turn on the equivalent positions (four in the case of the space group $P2_12_12_1$) of each heavy atom in the unit cell. Peaks in the resulting distribution then indicate the positions of other atoms. This calculation may be made directly, simply by adding the heights of the Patterson function for each superposition, but the method is tedious and, for the calculation of a three-dimensional distribution, mechanical computing is preferable. The terms which are to be used in such a calculation may be derived as follows⁵⁴⁾.

Suppose there are m heavy atoms in the unit cell with parameters $x_1, y_1, z_1; x_2, y_2, z_2; \dots \dots \dots x_m, y_m, z_m$, then the vector convergence distribution (V.C.D.) corresponds to m Patterson functions (P) with their origins on each of these positions in turn.

$$\begin{aligned} \text{V.C.D.}(x, y, z) &= \frac{1}{V} \sum_{j=1}^m P(x-x_j, y-y_j, z-z_j) \\ &= \frac{1}{V} \sum_h \sum_k \sum_l \left\{ |F_{hkl}|^2 \sum_{j=1}^m \exp \left[-2\pi i \{ h(x-x_j) + k(y-y_j) + l(z-z_j) \} \right] \right\} \\ &= \frac{1}{V} \sum_h \sum_k \sum_l \left\{ |F_{hkl}|^2 \sum_{j=1}^m \exp \left[2\pi i (hx_j + ky_j + lz_j) \right] \right\} \exp \left[-2\pi i (hx + ky + lz) \right] \end{aligned}$$

For all the heavy atoms in the unit cell:-

$$\sum_{j=1}^m f_j \exp \left[2\pi i (hx_j + ky_j + lz_j) \right] = F_{hkl_{HA}} = A_{hkl_{HA}} + iB_{hkl_{HA}}$$

If f_j , the scattering factor, is the same for all these heavy atoms, i.e. $f_j = f_{HA}$

$$\begin{aligned} \sum_{j=1}^m \exp \left[2\pi i (hx_j + ky_j + lz_j) \right] &= \frac{1}{f_{HA}} \left[A_{hkl_{HA}} + iB_{hkl_{HA}} \right] \\ &= A'_{hkl_{HA}} + iB'_{hkl_{HA}} \end{aligned}$$

where A' and B' are the geometric values of the structure factors.

Therefore:

$$V.C.D(x,y,z) = \frac{1}{V} \sum_h \sum_k \sum_l \left\{ |F_{hkl}|^2 [A'_{hkl_{HA}} + iB'_{hkl_{HA}}] \exp [-2\pi i(hx + ky + lz)] \right\}$$

This result may be compared with the corresponding equation for the electron density

$$\rho(x,y,z) = \frac{1}{V} \sum_h \sum_k \sum_l [A_{hkl} + iB_{hkl}] \exp [-2\pi i(hx + ky + lz)]$$

The calculation of a superposed Patterson function is then equivalent to a Fourier calculation with

$$|F_{hkl}|^2 A'_{hkl_{HA}} \quad \text{and} \quad |F_{hkl}|^2 B'_{hkl_{HA}} \quad \text{as coefficients.}$$

These take the place of A_{hkl} and B_{hkl} which are used in electron density calculations. The values of $|F_{hkl}|^2$ are sharpened, as for a simple Patterson summation.

Dr. Brink²⁸⁾ investigated the relative merits of three Fourier methods in the determination of a structure in which there is a heavy atom. She calculated an electron density distribution, a vector convergence distribution and a distribution by Rogers' heavy atom method⁵⁵⁾. $\left(\frac{|F|^2}{F_{HA}} \right)$ as coefficients (see table 21) using data for a non-centrosymmetric

projection of sodium benzylenediacetate. Rogers'

heavy atom method, in which the contributions of the

Table 21.

lighter atoms in the structure amplitudes are weighted.

Coefficients used in the Different Calculations

was found to give too many false maxima in the

density map. He suggested that the vector convergence

distribution method of calculation should be used with

Electron density distri-

bution (heavy atom phases)

$$|F_{obs}| \cos \alpha_{HA}$$

$$|F_{obs}| \sin \alpha_{HA}$$

investigations on the structure of vitamin B₁₂

She worked with experimental data but it seemed

better to review the situation using calculated data.

V.C.D.

$$|F_{obs}|_s^2 F_{HAg} \cos \alpha_{HA}$$

$$|F_{obs}|_s^2 F_{HAg} \sin \alpha_{HA}$$

A hypothetical structure in which the heavy atom is

group Pb, was chosen. An arrangement of six atoms, one

doubly weighted atom, the heavy atom, was chosen.

Rogers' heavy atom

$$\frac{|F_{obs}|^2}{F_{HA}} \cos \alpha_{HA}$$

$$\frac{|F_{obs}|^2}{F_{HA}} \sin \alpha_{HA}$$

density and vector convergence distribution method

method

calculated using phase from the "heavy" (doubly

weighted) atom, from a partial structure of three atoms

and from a total correct structure.

The appearance of these electron density and

s = sharpened data

vector convergence distribution method was shown in

g = geometric part

Figure 37. When only one atom is used in the phase

angle calculation a false center of symmetry is

introduced into the density map. The calculations

projection of sodium benzylpenicillin. Rogers' heavy atom method, in which the contributions of the lighter atoms to the structure amplitudes are weighted, was found to give too many false maxima in the density map. She suggested that the vector convergence distribution method of calculation should be used with the three-dimensional Patterson series for further investigations on the structure of vitamin B₁₂.

She worked with experimental data but it seemed better to review the situation using calculated data. A hypothetical structure in a non-centrosymmetric plane group P6, was chosen. An arrangement of six atoms, one doubly weighted made the asymmetric unit. Electron density and vector convergence distributions were calculated using phases from the "heavy" (doubly weighted) atom, from a partial structure of three atoms and from a total correct structure.

The appearances of these electron density and vector convergence distribution maps are shown in figure 37. When only one atom is used in the phase angle calculation a false centre of symmetry is introduced into the density maps. The calculations

Figure 37. Comparison of two Fourier methods.

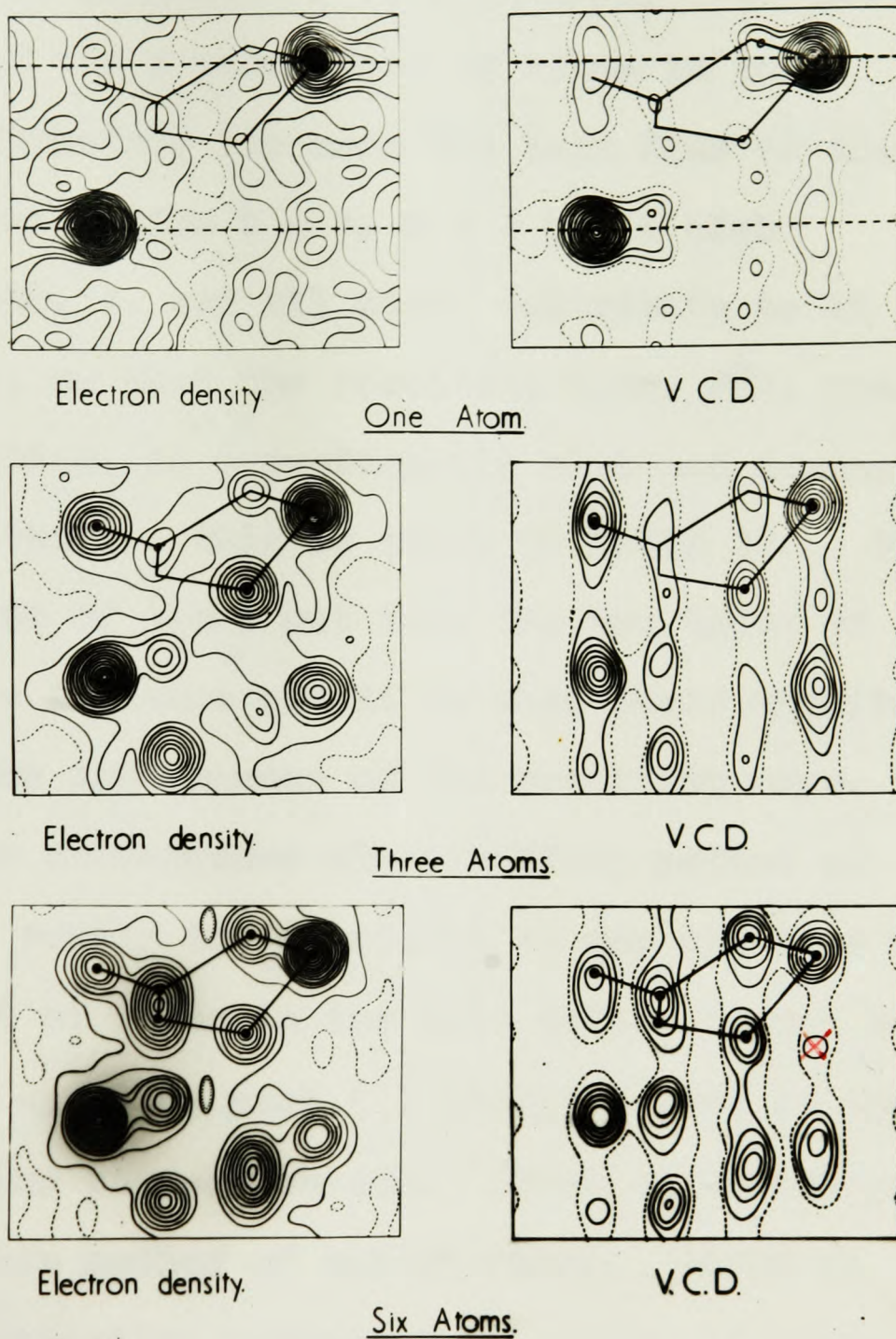
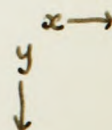


Figure 37. Comparison of two Fourier methods.



using heavy atom phases indicated atomic positions but showed several spurious peaks. In the other vector convergence distribution calculations there are high density lines at $x = 0, \frac{1}{5}, \frac{2}{5}, \frac{3}{5}, \frac{4}{5}$ of the unit cell. All the x parameters of atoms in the hypothetical structure lie near the last four of these values. The reflection $h = 5, k = 0$ has a high structure factor, A , and all atoms contribute to it in the same sense, so that the resulting term, F^2A , used in the calculation, is exceptionally high and dominates all the other terms. This is shown in table 22. Of course the signs of terms put into the two types of calculation are the same and it is only their magnitudes which affect the appearances of the resulting maps.

The vector convergence distributions method of calculation is really too sensitive to any symmetry in the arrangement of atoms in the unit cell. It may be noted that one spurious peak (X) persists even in the calculation using correct phases. Other disadvantages are found in this method of calculation:- There is very little distinction between the heights of peaks due to atoms of different atomic numbers and peaks are

S = sharpened

Table 22

not as round as in electron density calculation.
Contributions of Each Atom to Three Terms in the
Structure Factor Calculation and the Resulting
Coefficients used in the Electron Density and
V.C.D. Calculations

is used, $k=0$ atom (0), is not indicated in this calculation. It is a broad maximum in the $h=6$ vector convergence.

Atom	$h=4$	$h=5$	$h=6$
1	.59	.93	.13
2	1.00	.69	0
3	.95	.93	.48
4 (twice)	.81 x 2	.69 x 2	.59 x 2
5	1.00	.73	0
6	0.81	1.00	.81
2 x Total = A_g	3.10	11.32	5.20
$ F $	2.00	1.40	1.03
$ F ^2$	6.2	15.9	5.4
$ F ^2_s$	38.4	252.8	29.2
$ F ^2_s A_g$	63.0	556.2	87.9
$ F ^2_s A_g$	195.3	6296.2	457.1
Relative Values			
$ F $	1	2.6	0.9
$ F ^2_s A_g$	1	32.4	2.4

S = sharpened

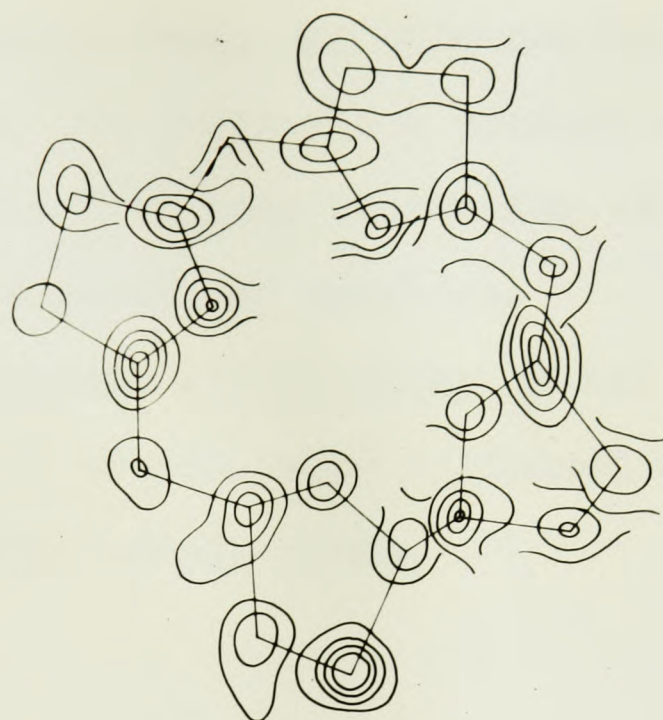
not as round as in electron density calculation. It is easier to select atomic positions from the electron density calculation when a partial structure is used, but one atom (O), is not indicated in this calculation. It lies on a broad maximum in the vector convergence distribution calculation. A comparison of the density maps from these two Fourier methods might indicate more of the structure than the distributions separately, but the extra calculations that this would involve would not make it worth while.

A three-dimensional vector convergence distribution map was calculated in Oxford for the degradation product using cobalt atom phases. It provided a test of the validity of these remarks. Figure 38 compares the appearance of atoms in the "planar" group in the two methods of calculation. There is very little difference. Several high, spurious peaks were found which had no equivalent in the electron density calculation. Most of these were related to the position of the cobalt atom. A comparison of the numbers of high peaks and the proportions of these which indicate atomic

VCD

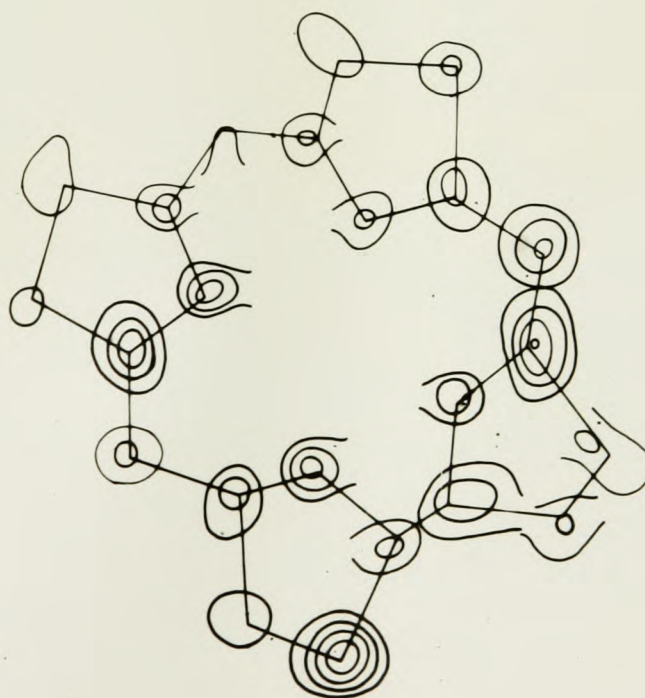
Figure 38. Comparison of appearance of planar group

using cobalt atom phases but different methods of calculation.



+

Electron Density



+

V.C.D.

Figure 38. Comparison of appearance of planar group
using cobalt atom phases but different methods of
calculation.

positions in the two calculations is given in table 23.

If this method had been the only one used for either vitamin B₁₂ or the degradation product many wrong

positions would have been chosen for atoms as several of the highest peaks are spurious.

All subsequent calculations on the degradation product and the vitamin were performed as Fourier electron density calculations.

2. Vector Convergence Distribution Calculation

Arbitrary Peak Heights	Total For Peaks	Not atomic positions indicated
200-300	34	17
300-400	11	6
400-500	6	3
500-600	1	1
600-700	3	-
700-800	-	-
800-900	3	(17)
	58	27

Table 23.

Peaks in the Electron Density and V.C.D. Calculations
for the Degradation Product

1. Electron Density Calculations

<u>Peak Heights $e/\text{\AA}^3$</u>	<u>Total No: Peaks</u>	<u>No: atomic positions indicated</u>
2-3	84	34
3-4	17	12
4-5	-	-
5-6	-	-
6-7	1	1
	102	47

2. Vector Convergence Distribution Calculation

<u>Arbitrary Peak Heights</u>	<u>Total No: Peaks</u>	<u>No: atomic positions indicated</u>
200-300	34	17
300-400	11	6
400-500	5	2
500-600	1	1
600-700	2	-
700-800	-	-
800-900	2	(1?)
	55	27

Table 24 Observed and Calculated Structure Amplitudes

For each reflection the upper value is that observed, the lower value that calculated. Observed structure amplitudes have been roughly scaled to the calculated ones. The calculated ones are those at the 72 atom stage.

Temperature factor: 2.77 A⁻²

Mean value of F obs: 34.7

Mean value of

Fobs-Fcalc: 10.0

Mean value of Fcalc

when Fobs not

measured: 12.6

h k 0

n	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
0			135		233		66		62		52		28		21		91		28		40		25		48		12				17	
			186		217		90		62		10		3		28		109		34		41		27		70		48		4		30	
1		108	180	98	190	13	90	32	33	105	13	43	17	52	35	14	30		37			21	18	28				14		14		
		130	258	102	183	12	103	46	58	131	7	37	3	3	55	17	23	2	34	5	8	10	24	48	3	10	3	7	16	13	5	1
2	75	61	142	98	55		54	49	152	74			66	48		43	48	38	35		26	33		58				24	25	40		
	85	31	172	108	94	14	9	43	130	81	15	18	85	35	25	41	26	60	31	31	25	37	11	52	3	16	17	23	20	38	13	1
3		26	11	13	144	148	53	42		92	39	52	37	66	45	40			17	32	24				24	36				21	16	
		13	30	28	179	176	56	2	32	79	25	58	43	71	60	54	22	16	25	35	8	2	26	4	37	45	18	8	2	28	14	17
4	250	80	98	35	19	174	82	18	34		43	31	45	32	24		42	26	29	52	13		21		38	23	25	31				10
	339	49	99	45	15	170	37	18	43	14	59	19	42	30	32	17	59	17	30	71	2	0	22	39	44	31	28	21	10	11	17	17
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	12	11	8	12	42	25	21	13	20	12	29	14	1	30	11	11	11	10	12	5	24	

h k 14

$k \backslash h$	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
0	25				28		21			30	21								
1	22	3	0	19	32	5	28	7	4	42	35	0	7	7	16	4	21		
2		17				17			21			22							
3	18	19	15	20	11	24	7	10	15	17	16	43	10	10	3	5	12		
4			18										33		23				
5	1	6	20	12	5	21	5	15	15	7	15	11	37	10	38	15	4		
6					14					11									
7	10	21	10	13	11	12	18	30	9	8	16	12	5	23	18	10	3		

h k 15

$k \backslash h$	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0			24	27	24											
1		17	0	37	7	15	26	5	9	9	7					
2	37	16	16	20	16	16	11		19							
3	37	15	9	31	20	26	6	16	5	8	13					
4		38		12		17										
5	10	36	3	15	7	5	5	3	14	14	2					
6						24				11						
7	24	18	1	25	15	5	11	12	3	20	12					

h k 16

$k \backslash h$	0	1	2	3	4	5	6	7	8	9	10	11	12
0	30												
1	17	8	19	1	8	6	11	8	5				
2			29		22								
3	4	18	23	16	25	11	4	14	7				
4		22	20	10	17								
5	23	21	3	17	10	10	8	16	12				
6	13				16		9						
7	9	15	6	1	13	5	9	1	5				

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ABSTRACT

Vitamin B₁₂ has been shown, by spectroscopic experiments, to contain 5:5-dimethylimidazole-4-carboxylic acid, 5-methyltetrahydrofuran, propionamide, five of which are in the form of a cyclic structure, and a cobalt atom which is coordinated to a cyanide group, and a cobalt atom which is coordinated to a cyanide group, and a cobalt atom which is coordinated to a cyanide group. The chemical composition of the vitamin is unknown. The vitamin was obtained from a bacterial culture of *Corynebacterium* and was purified by a series of extractions with a series of solvents. The vitamin is a crystalline solid and its melting point is 170°C. The analysis of these crystals is as follows:

Very little of the material was available for chemical analysis. The material was purified by a series of extractions with a series of solvents. The vitamin is a crystalline solid and its melting point is 170°C. The analysis of these crystals is as follows:

The space group is $P2_12_12_1$. The unit cell dimensions are $a = 24.58 \text{ \AA}$, $b = 18.58 \text{ \AA}$ and $c = 15.58 \text{ \AA}$. The volume of the asymmetric unit is about 1000. The figures suggest that the structure of the vitamin is as follows:

ABSTRACT

Vitamin B₁₂ has been shown, by hydrolysis experiments, to contain 5:6-dimethylbenziminazole, ribose, phosphate, propanolamine, five or six amide groups, a cyanide group, and a cobalt containing fragment of unknown chemical composition. Todd and his co-workers at Cambridge obtained a cobalt-containing fragment, a hexacarboxylic acid with a great tendency to form gums, in a crystalline form and in this thesis an X-ray analysis of these crystals is described.

Very little of the material was available, and chemical analyses were performed on only a polymorphic modification which was not suitable for X-ray studies. The space group is $P2_12_12_1$, non-centrosymmetric in three dimensions. There are four molecules in the unit cell. The unit cell dimensions of the compound are $a = 24.58 \text{ \AA}$, $b = 15.52 \text{ \AA}$ and $c = 13.32 \text{ \AA}$. The weight of the asymmetric unit is about 1072. The analytical figures suggest that the formula of the dried compound

is approximately $C_{44-45} H_{62} N_{5-6} O_{14-15} Co Cl$. The chromophoric group of the molecule was found, spectroscopically, to have remained unchanged during the hydrolysis.

Three dimensional intensity data were collected on Weissenberg photographs, some of which were found to have spots to the limit of $\sin \theta$ using copper radiation. The intensities, estimated visually, were corrected and scaled.

The position of the heavy atom, cobalt, was found by two dimensional Patterson syntheses. It is in a more general position in the unit cell than it is in vitamin B_{12} and its selenocyanide.

A three dimensional electron density calculation using observed structure amplitudes and phase angles determined by the positions of the cobalt atom was calculated. The resulting sections show many peaks, some of which must be spurious, and others which were considered to correspond to actual atomic peaks. There are high diffraction maxima and minima around the cobalt atom but peaks corresponding to a porphyrin-like group (I), with one bridge atom missing,

were clearly distinguished. This agreed with an atomic arrangement

different from that of the previous model.

and nitrogen atoms put into the calculated positions

with peak heights of $1/2$ and $1/4$, while another

round peak up to 4×10^{-4} was observed in position which

corresponded to a carbon atom.

While about the entire arrangement of atoms was

found at this stage there were not all yet known.

suggested tentatively by crystallographic studies on

the vitamin. There was a high peak at about $2.3 \text{ \AA}$

from the cobalt atom and it was suggested that this

was the chlorine atom.

From this stage onwards work continued in

collaboration with Dr. K.N. Trueblood and Dr. R.J. Prosen

in California. They calculated structure factors and

three dimensional electron density series on the

electronic computer SWAC.

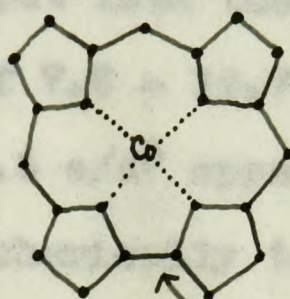
The co-ordinates of the 26 atoms around the cobalt

atom (leaving out one uncertain atom on the edge of a

diffraction maximum) and including chlorine and a

cyanide group were used in the next phase angle calcula-

(I)



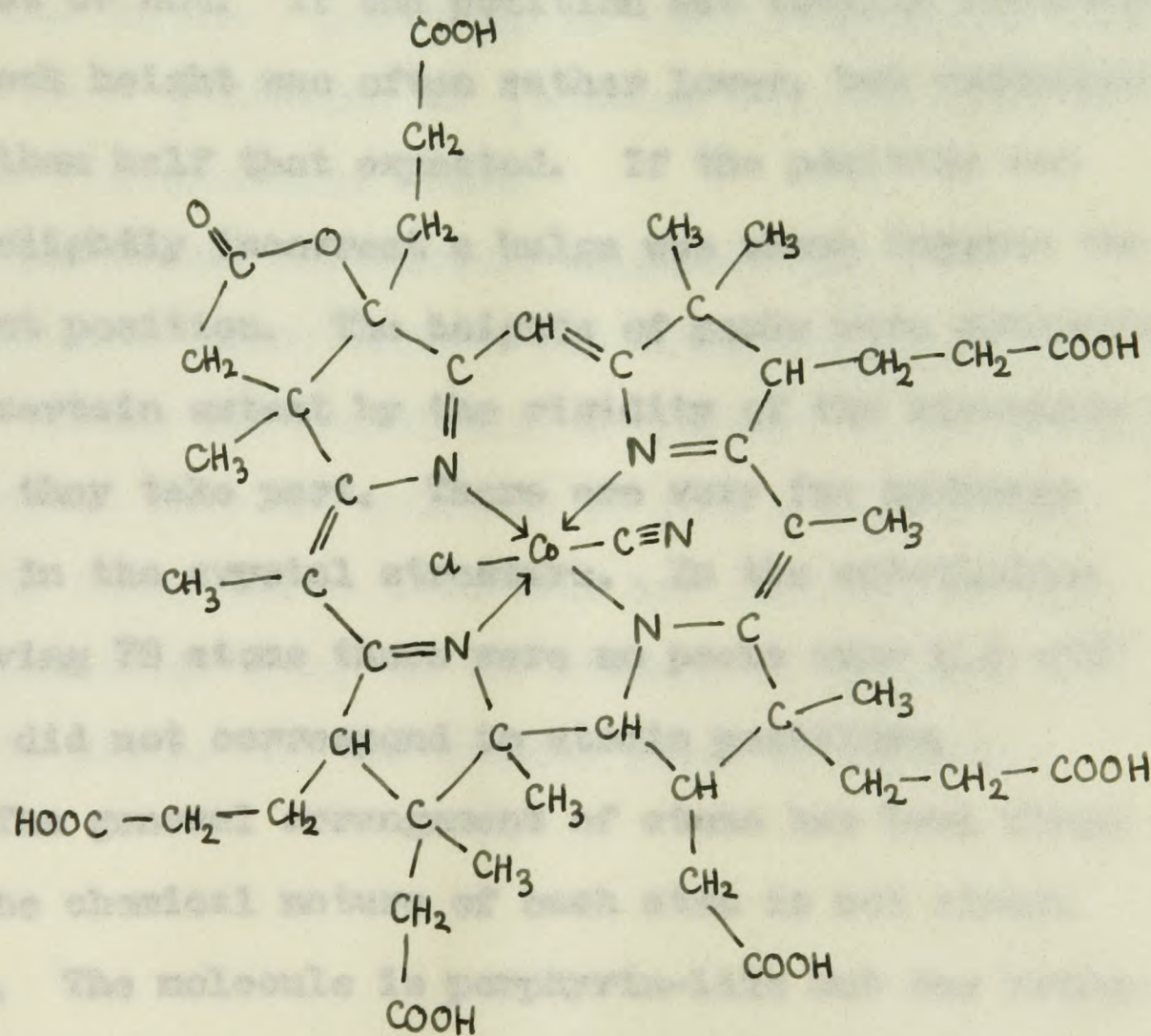
BRIDGE CARBON ATOM

MISSING

tion. The general appearance of the resulting three-dimensional electron density series was very different from that of the previous ones. Carbon and nitrogen atoms put into the calculation appeared with peak heights of $7.6 - 12.7 \text{ e}/\text{\AA}^3$, while smaller round peaks up to $4.5 \text{ e}/\text{\AA}^3$ appeared in positions which corresponded stereochemically to a reasonable formula.

While almost the entire arrangement of atoms was found at this stage these were not all put into the next calculation. The highest peaks, over $2.5 \text{ e}/\text{\AA}^3$ in height, were included in the next phase angle calculation involving 54 atoms. The method used in the structure analysis was to compute phase angles for gradually increasing groups of acceptable atomic positions and impose these on observed structure factors. The resulting terms were used to calculate the three dimensional electron density series. Several such series were calculated and only the positions for atoms in one side chain have not been found satisfactorily. There has recently been chemical evidence which establishes the nature of the last side chain and supports the formula (II) tentatively suggested for the

It was found that, in a non-planar structure of this kind, an atom occupies the position in which it was put in the planar calculation, regardless of whether that position was correct or not. If the position was taken as incorrect the peak height was only a rather lower, but more than half that of the correct one. If the position was only slightly incorrect the peak height was only a little lower than the correct position. This is a device which they have used to find out which bonds in the molecule are not planar, involving 79 atoms. There were a few atoms which did not correspond to the expected values but the chemical values for each atom is not clear. The molecule is a porphyrin-like one, but one atom is missing. The central ring system has a cobalt in planar except at the points where the nitrogen atom is missing. The four nitrogen atoms have their β and β' positions fully substituted, except for



(II) Formula suggested for the hexacarboxylic acid.

degradation product.

It was found that, in a non-centrosymmetric structure of this kind, an atom appeared in the position in which it was put in the phase angle calculation, regardless of whether that position was correct or not. If the position was totally incorrect the peak height was often rather lower, but certainly more than half that expected. If the position was only slightly incorrect a bulge was shown towards the correct position. The heights of peaks were determined to a certain extent by the rigidity of the structure in which they take part. There are very few hydrogen bonds in the crystal structure. In the calculation involving 72 atoms there were no peaks over $1.5 \text{ e}/\text{\AA}^3$ which did not correspond to atomic positions.

The general arrangement of atoms has been found but the chemical nature of each atom is not always clear. The molecule is porphyrin-like but one bridge atom is missing. The central ring system round the cobalt is planar except at the points where the bridge atom is missing. The four five-membered rings have their β and β' positions fully saturated. Acetic acid

($\text{—CH}_2\text{—COOH}$) and propionic acid ($\text{—CH}_2\text{—CH}_2\text{—COOH}$) groups were attached in the sequence found for most naturally occurring porphyrins. Peaks, probably due to methyl groups, are found attached to two of the bridge atoms, at the direct link, and in a regular sequence on the positions of the five-membered rings. There is a γ -lactone or γ -lactam ring attached to one of the five-membered rings.

The atomic arrangement suggested has been confirmed by other workers for vitamin B₁₂, except that, during the hydrolysis, a hydroxyl group has been introduced into the molecule to give a lactone (or lactam) ring, and an inversion has occurred so that the nucleotide group in the vitamin is replaced by a cyanide group, and the cyanide group by chloride.